World Journal of

Biological Chemistry

World Journal of Biological Chemistry

A peer-reviewed, online, open-access journal of biological chemistry

Editorial Board

2009-2013

The *World Journal of Biological Chemistry* Editorial Board consists of 529 members, representing a team of worldwide experts in biochemistry and molecular biology. They are from 40 countries, including Argentina (1), Australia (7), Austria (2), Belgium (6), Brazil (5), Bulgaria (1), Canada (18), Chile (1), China (36), Czech Republic (1), Denmark (1), Finland (3), France (14), Germany (17), Greece (4), India (9), Iran (2), Israel (5), Italy (26), Japan (43), Lithuania (1), Mauritius (1), Mexico (2), Netherlands (7), New Zealand (2), Norway (4), Portugal (4), Romania (1), Russia (2), Singapore (5), South Africa (1), South Korea (19), Spain (18), Sweden (4), Switzerland (2), Thailand (1), Turkey (1), Ukraine (1), United Kingdom (19), and United States (232).

WJBC

World Journal of
Biological Chemistry

Contents

Contents

Contents

ABOUT COVER   Editorial Board Member of *World Journal of Biological Chemistry*, Juan J Moreno, PhD, Professor, Department Physiology, Faculty Pharmacy, Institute Nutrition, University of Barcelona, Barcelona 08028, Spain

AIM AND SCOPE   *World Journal of Biological Chemistry* (*World J Biol Chem*, *WJBC*, online ISSN 1949-8454, DOI: 10.4331), is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJBC is to rapidly report the most recent developments in the research by the close collaboration of biologists and chemists in area of biochemistry and molecular biology, including: general biochemistry, pathobiochemistry, molecular and cellular biology, molecular medicine, experimental methodologies and the diagnosis, therapy, and monitoring of human disease.

We encourage authors to submit their manuscripts to *WJBC*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

INDEXING/ABSTRACTING   *World Journal of Biological Chemistry* is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

EDITORIAL

Can short-term fasting protect against doxorubicin-induced cardiotoxicity?

Amie J Dirks-Naylor, Samir A Kouzi, Sendra Yang, Ngan TK Tran, Joseph D Bero, Raean Mabolo, Diep T Phan, Stephanie D Whitt, Heather N Taylor

Amie J Dirks-Naylor, Samir A Kouzi, Sendra Yang, Ngan TK Tran, Joseph D Bero, Raean Mabolo, Diep T Phan, Stephanie D Whitt, Heather N Taylor, School of Pharmacy, Wingate University, Wingate, NC 28174, United States
Author contributions: Dirks-Naylor AJ and Kouzi SA wrote the mansucript and involved in data collection; Yang S, Tran NTK, Bero JD, Mabolo R, Phan DT, Whitt SD and Taylor HN involved in data collection and editing the manuscript.
Correspondence to: Amie J Dirks-Naylor, PhD, Associate Professor, School of Pharmacy, Wingate University, 515 N. Main Street, Wingate, NC 28174, United States. anaylor@wingate.edu
Telephone: +1-704-2338341 Fax: +1-704-2338332

Abstract

Doxorubicin (Dox) is one of the most effective chemotherapeutic agents used in the treatment of several types of cancer. However the use is limited by cardiotoxicity. Despite extensive investigation into the mechanisms of toxicity and preventative strategies, Dox-induced cardiotoxicity still remains a major cause of morbidity and mortality in cancer survivors. Thus, continued research into preventative strategies is vital. Short-term fasting has proven to be cardioprotective against a variety of insults. Despite the potential, only a few studies have been conducted investigating its ability to prevent Dox-induced cardiotoxicity. However, all show proof-of-principle that short-term fasting is cardioprotective against Dox. Fasting affects a plethora of cellular processes making it difficult to discern the mechanism(s) translating fasting to cardioprotection, but may involve suppression of insulin and insulin-like growth factor-1 signaling with stimulated autophagy. It is likely that additional mechanisms also contribute. Importantly, the literature suggests that fasting may enhance the antitumor activity of Dox. Thus, fasting is a regimen that warrants further investigation as a potential strategy to prevent Dox-induced cardiotoxicity. Future research should aim to determine the optimal regimen of fasting, confirmation that this regimen does not interfere with the antitumor properties of Dox, as well as the underlying mechanisms exerting the cardioprotective effects.

Core tip: Doxorubicin (Dox)-induced cardiotoxicity remains a significant cause of morbidity and mortality in cancer survivors, despite the intensive investigation of potential protective strategies. Studies have shown that short-term fasting induces cardioprotective effects against Dox-induced injury. Importantly, evidence suggests that fasting may enhance the antitumor effects of Dox. Thus, short-term fasting may be a feasible practice that can easily be incorporated into the treatment plans of cancer patients.

Dirks-Naylor AJ, Kouzi SA, Yang S, Tran NTK, Bero JD, Mabolo R, Phan DT, Whitt SD, Taylor HN. Can short-term fasting protect against doxorubicin-induced cardiotoxicity? *World J Biol Chem* 2014; 5(3): 269-274 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/269.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.269

INTRODUCTION

Doxorubicin (Dox) is one of the most effective chemotherapeutic agents currently used in the treatment of haematological malignancies and solid tumors such as breast cancer. It is a quinone-containing anthracycline antibiotic. Its mechanism of antitumor activity has been shown to

involve binding to topoisomerase-Ⅱ α, thereby resulting in DNA strand breaks and apoptosis[1]. Despite its effectiveness, its use is limited due to cardiotoxicity. In animal models, Dox has been shown to be hepatotoxic as well, but in humans it is the cardiotoxicity which primarily limits its use[2,3]. High cumulative doses of Dox are the most powerful predictor of Dox-induced congestive heart failure[4]. One early study reported that 4% of patients receiving a dose of 500-550 mg/m^2 and 18% and 36% of patients receiving 551 mg/m^2 or higher, respectively, experienced heart failure, which is often refractory to conventional therapy[4,5]. The clinical outcome for these patients is poor[4]. A variety of approaches to prevent cardiotoxicity have been tested, however, their efficacy has been limited[4]. Thus, continued investigation of viable strategies to protect the heart from Dox-induced toxicity is of vital importance.

MECHANISMS OF DOX-INDUCED CARDIOTOXICITY

Mechanisms of Dox-induced cardiotoxicity have not been clearly elucidated, but have been shown to involve oxidative stress, mitochondrial dysfunction, and apoptosis. For example, Dox treatment has been shown to increase mitochondrial depolarization, fission, and ROS production while decreasing the rate of ATP synthesis and content[6-10]. Lipid peroxidation, reduced aconitase activity (a marker of oxidative stress), and alterations in the expression and activity of antioxidant enzymes, such as superoxide dismutase (SOD), are also evident[11,12]. Oxidative stress and mitochondrial dysfunction can induce apoptosis which leads to loss of post-mitotic myocytes and altered cardiac function[8,13,14]. It has long been known that Dox can induce oxidative stress *via* semiquinone redox cycling, however it is unclear if this is the specific mechanism of cardiotoxicity since ROS scavengers failed to prevent cardiac toxicity in several studies[15,16]. Recently, topoisomerase-Ⅱ β has been shown to be a molecular target of Dox in cardiomyocytes[17]. Cardiac myocytes do not express topoisomerase-Ⅱ α, the molecular target in tumor cells[18]. Zhang *et al*[17] demonstrated that cardiomyocyte-specific deletion of topoisomerase-Ⅱ β prevented Dox-induced cardiotoxicity. Furthermore, the deletion prevented Dox-induced DNA damage and transcriptional changes that are responsible for impaired mitochondrial biogenesis, ROS formation, and apoptosis. Thus, the mechanism of Dox-induced cardiotoxicity may involve molecular targeting of topoisomerase-Ⅱ β as well as the potential contribution of semiquinone redox cycling.

PROTECTIVE STRATEGIES AGAINST DOX-INDUCED CARDIOTOXICITY

Currently, available therapies to effectively prevent cardiotoxicity in patients treated with Dox are limited. Thus, the first line of defense is to limit the cumulative dose of Dox. However, lowering cumulative dose may translate to reduced treatment efficacy[19]. Another strategy to protect against cardiotoxicity has been to alter the mode of delivery of Dox, such as encapsulation in liposomes, which aims to target the delivery to the tumor, thereby, reducing plasma concentrations of Dox. The United States Food and Drug Administration has approved one liposomal doxorubicin, Doxil[19]. Shorter-term clinical trials have shown that liposomal doxorubicin can reduce early cardiotoxicity while having the same antineoplastic efficacy as conventional doxorubicin[19]. Although, liposomal doxorubicin has shown promise in reducing cardiotoxicity, currently, it is still mainstream to use conventional Dox. Utilizing antioxidants or iron chelators to reduce Dox-induced oxidative stress has been another tested strategy, but with limited success[19]. Dexrazoxane, an iron chelating agent, has shown the most promise in reducing oxidative stress and cardiotoxicity, however, with some limitations. Most studies have shown that Dexrazoxane is safe, however, some have shown that dexrazoxane may cause myelosuppression and also increase the risk of second malignancies[20,21]. Furthermore, it has been shown that the efficacy of dexrazoxane may vary between sexes, with less benefit in males[19,22]. Despite extensive investigation and numerous tested strategies to prevent cardiotoxicity, success has been limited. Dox-induced cardiotoxicity still remains a major cause of morbidity and mortality in cancer survivors[19]. Thus, exploration of additional strategies to prevent Dox-induced cardiotoxicity is paramount.

A cardioprotective strategy that warrants further exploration is fasting. Fasting and/or caloric restriction (CR) has been shown to protect the heart from a variety of conditions and insults. For example, intermittent fasting protects the heart from ischemic damage and attenuates post-MI cardiac remodeling[23]. Furthermore, calorie restriction has proven protective against coronary artery disease, the process of aging on the cardiovascular system, as well as drug toxicities, including doxorubicin-induced cardiotoxicity[24-27]. Mitra *et al*[26] demonstrated that 40+ days of a 35% calorie restricted diet lead to 100% protection against Dox-induced cardiotoxicity and death while all of the rodents administered with Dox in the *ad libitum* fed group died. However, long term CR regimens, such as this, are not feasible in cancer patients since they typically suffer from malnutrition and other complications. Therefore, short-term fasting may be an alternative approach. Indeed, Raffaghello *et al*[28] reported that 48-60 h of complete fasting prevented organ toxicity induced by chemotherapy in various species of female mice, however, etoposide rather than Dox was used in the study. Kawaguchi *et al*[29] demonstrated that 48 h of complete fasting prior to Dox administration mitigated the Dox-induced impairment in cardiac function in adult GFP-LC-3 transgenic mice, as determined by left ventricular ejection fraction (LVEF), systolic pressure (LVSP), end diastolic pressure (LVEDP), and +dP/dt. Microscopy revealed attenuation

Table 1 Summary of unpublished data

Dependent variable	Control (mean ± SEM)	Dox (mean ± SEM)	*P* value
Aconitase activity (nmol/min per milligram protein)	14.46 ± 3.68	23.74 ± 3.25	0.08
SOD activity (units/mg protein)	0.026 ± 0.003	0.026 ± 0.002	0.904
SOD1 content	1025 ± 110.2	949 ± 91.6	0.603
SOD2 content	275.6 ± 23.25	288.1 ± 23.71	0.715
Procaspase-12 content	36.90 ± 6.14	24.28 ± 4.19	0.1
Procaspase-9 content	28.59 ± 1.57	25.33 ± 3.61	0.5
Procaspase-8 content	68.10 ± 11.90	82.90 ± 0.93	0.34
Caspase-3 activity (arbitrary OD/mg protein)	0.951 ± 0.676	0.490 ± 0.295	0.524
Caspase-9 activity (arbitrary OD/mg protein)	1.084 ± 0.809	0.462 ± 0.255	0.451
FIS1 content	563.6 ± 76.6	474.3 ± 68.8	0.4
DRP1 content	1294.9 ± 109.8	1187.5 ± 73.5	0.421
MFN1 content	5443.5 ± 786.8	4607.8 ± 627.0	0.417
MFN2 content	2001.5 ± 456.8	2053.6 ± 330.2	0.926
OPA1 content	6019.5 ± 739.3	6143.6 ± 601.0	0.897
PINK1 content	3343.0 ± 206.9	3422.0 ± 263.4	0.824
Parkin content	4192.0 ± 1009.0	4157.0 ± 1629.0	0.986
p62 content	1895.7 ± 272.7	1896.7 ± 252.2	0.998

Protein content determined by Western blot (units are "normalized OD"). SOD: Superoxide dismutase.

of LV dilatation, myocardial atrophy, and fibrosis[29]. *In vitro*, a caloric restriction mimetic, 2-deoxyglucose (2-DG), was shown to exhibit cardioprotective properties against Dox using neonatal rat cardiomyocytes isolated from 0-2 d old Harlan Sprague-Dawley rat neonates[30]. Thus, the literature supports that fasting may be an effective regimen to protect against Dox-induced cardiotoxicity.

Unpublished data from our laboratory (Table 1) may also suggest that short-term fasting may provide cardioprotection against Dox. Six-week old male F344 rats were treated with a single injection of Dox (20 mg/kg) or saline. Tissues were harvested for analysis 24-h post injection with the aim of determining the effects of Dox on the mitochondrial dynamics and mitophagy machinery. In order to remove the external variable of Dox-induced anorexia, we fasted both groups of animals upon treatment. Studies have shown that animals treated with Dox reduce their food and water intake by up to 70% for several days[31]. Using this experimental design, the results were unexpected. Dox did not affect any markers of oxidative stress or apoptosis that were assessed in the heart. Dox did not affect aconitase activity, superoxide dismutase (SOD) activity, nor the protein content of cytosolic SOD1 and mitochondrial SOD2. Expression and activation of caspase-12, caspase-9, and caspase-8 were assessed *via* Western analysis, as well as caspase-3 and -9 enzyme activities, and were not affected by Dox. As previously mentioned, the original aim of the study was to investigate the effects of Dox on the mitochondrial dynamics and mitophagy machinery with the hypothesis that Dox treatment would increase the protein content of FIS1 and DRP1 (fission regulators) and decrease the content of MFN1, MFN2 and OPA (fusion regulators) thus favoring mitochondrial fission, which is most often associated with oxidative stress, mitochondrial dysfunction and apoptosis[32-34]. Under the current fasting conditions, Dox did not affect the content of any of these primary regulators. Regulators of mitophagy were also

assessed. Dox did not affect the content of PINK1, Parkin, or p62 (a marker of mitophagy) under these fasting conditions. We do know that Dox exerted a biological effect in these animals since many of these variables were altered in the liver. Furthermore, the treatment significantly affected the proteome lysine acetylation status in the heart, inducing deacetylation (Figure 1), although the significance of this observation is currently unknown. Because previously published studies have reported that acute Dox treatment does affect many of these variables and processes[8-11,35-37], we believe that complete fasting of the animals in our study may have exerted an unintended cardioprotective effect against the Dox-induced insult. However, further investigation is required to confirm our interpretation of the data. Although this work was done using an acute model of Dox cardiotoxicity, since short-term fasting may be able to protect against the high dose used in the acute model, it is likely that it may also be protective against lower doses used in chronic models of Dox cardiotoxicity which mimics more closely the clinical use of Dox in patients. In summary, short-term fasting may extend similar benefits as longer term CR in regards to cardioprotection against Dox-induced injury.

MECHANISM OF FASTING-INDUCED CARDIOPROTECTION AGAINST DOX TOXICITY

Fasting and caloric deprivation affect a plethora of cellular processes such as mitochondrial dynamics and biogenesis, energy metabolism, oxidative stress, autophagy, and survival signaling pathways, thus making it difficult to discern the mechanism(s) responsible for the cardioprotection[38-42]. Kawaguchi *et al*[29] concluded that the protection against Dox-induced injury extended by 48-h of fasting prior to treatment was due to restoration of autophagy. Autophagy is a conserved process among eukaryotic cells
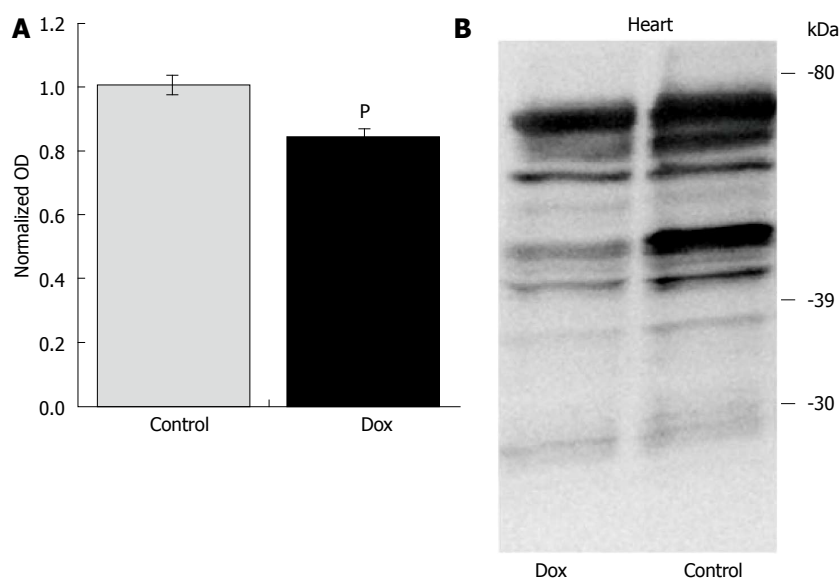
Figure 1 Acute doxorubicin treatment induces proteome lysine deacetylation in the hearts of fasted animals. A: Graphical representation of results; B: Representative Western blot. *P* = 0.0016.

that sequesters cellular material *via* formation of a multi-membrane-bound vacuole, an autophagosome, followed by degradation of the material *via* fusion of the autophagosome with a lysosome[43]. Autophagy can enhance cellular function and survival by degrading damaged or unwanted proteins and organelles such as mitochondria, as well as by modulating apoptosis[44]. Indeed, stimulation of autophagy has been shown to be cardioprotective from a variety of damaging stimuli[44]. Kawaguchi *et al*[29] reported that the inhibition of autophagy by Dox was due to inhibition of AMP-activated protein kinase (AMPK). Prior fasting prevented the Dox-induced inhibition of AMPK. Although fasting was able to reverse the effects of Dox on autophagy, no experimental methods were employed to identify restoration of autophagy as the underlying factor for cardioprotection. Furthermore, no other processes known to be affected by fasting were assessed in the study. Moreover, several studies have shown that stimulation of autophagy contributes to Dox-induced cardiotoxicity and protection is provided *via* inhibition of autophagy[43]. Thus the role of autophagy in Dox-induced cardiotoxicity, whether protective or pathological, is still under question. Therefore, the underlying mechanism(s) of fasting-induced cardioprotection against Dox remains to be determined and is likely due to a combination of mechanisms[30].

EFFECTS OF FASTING ON TUMOR CELL KILLING

It is critical that a potential cardioprotective agent or regimen does not interfere with the goal of cancer treatment. CR has long been shown to have antineoplastic effects. CR can slow the intrinsic rate of aging and prevent the onset of age-related pathologies, including cancer[45,46]. Furthermore, CR mimetics, such as 2-DG, have been shown to inhibit tumor growth[47]. Moreover, 2-DG has been shown to enhance the antitumor efficacy of Dox both *in vitro* and *in vivo*[48,49]. Short-term (48-60 h) fast-

ing was shown to enhance death of cancer cells, prevent organ toxicity, and increase survival in chemotherapy treated mice, however the chemotherapy tested was etoposide, not Dox[28]. Interestingly, Raffaghello *et al*[28] noted that fasting longer than 60 h worsened outcomes. Thus, there may be a window of optimal duration of fasting to maximize beneficial effects. Many of the benefits of fasting and caloric restriction have been shown to be, at least in part, due to decreased circulating levels of insulin and reduced insulin-like growth factor-1 receptor (IGF-1R) signaling[50,51]. Seventy-two hours of fasting reduced circulating IGF-1 by 70% and increased the level of the IFG-1 binding protein (IGFBP) by 11x[52]. Survival time, after Dox treatment, was extended by delaying metastasis of highly aggressive melanoma and prevented Dox-induced toxicity in liver-specific IGF-1-deficient (LID) mice compared to non-LID mice[52]. Ninety days after inoculation with the melanoma cancer cells, all non-LID mice that were treated with Dox had died from either cancer metastases or Dox toxicity. 60% of LID mice treated with Dox were cancer-free with no signs of toxicity[52]. Thus, the evidence supports that fasting may be a safe regimen to use in conjunction with Dox in order to prevent cardiotoxicity.

CONCLUSION

In conclusion, Dox-induced cardiotoxicity remains a significant cause of morbidity and mortality in cancer survivors despite the intensive investigation of potential protective strategies. Studies have shown that short-term fasting induces cardioprotective effects against Dox-induced injury. Importantly, evidence suggests that fasting may enhance the antitumor effects of Dox. It seems that short-term fasting would be a feasible practice that can easily be incorporated into the treatment plans of cancer patients. Thus, short-term fasting is a strategy warranting further exploration. Further studies, both preclinical and clinical, should reveal the optimal regimen of fasting,

confirmation that this regimen does not interfere with the antitumor properties of Dox, as well as the underlying mechanisms exerting the cardioprotective effects.

REFERENCES

1 **Tewey KM**, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; **226**: 466-468 [PMID: 6093249 DOI: 10.1126/science.6093249]

2 **Kalender Y**, Yel M, Kalender S. Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats. The effects of vitamin E and catechin. *Toxicology* 2005; **209**: 39-45 [PMID: 15725512 DOI: 10.1016/j.tox.2004.12.003]

3 **Patel N**, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. *Toxicol Appl Pharmacol* 2010; **245**: 143-152 [PMID: 20144634 DOI: 10.1016/j.taap.2010.02.002]

4 **Takemura G**, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis* 2007; **49**: 330-352 [PMID: 17329180 DOI: 10.1016/j.pcad.2006.10.002]

5 **Lefrak EA**, Pitha J, Rosenheim S, Gottlieb JA. A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* 1973; **32**: 302-314 [PMID: 4353012]

6 **Bugger H**, Guzman C, Zechner C, Palmeri M, Russell KS, Russell RR. Uncoupling protein downregulation in doxorubicin-induced heart failure improves mitochondrial coupling but increases reactive oxygen species generation. *Cancer Chemother Pharmacol* 2011; **67**: 1381-1388 [PMID: 20809120 DOI: 10.1007/s00280-010-1441-7]

7 **Danz ED**, Skramsted J, Henry N, Bennett JA, Keller RS. Resveratrol prevents doxorubicin cardiotoxicity through mitochondrial stabilization and the Sirt1 pathway. *Free Radic Biol Med* 2009; **46**: 1589-1597 [PMID: 19303434 DOI: 10.1016/j.freeradbiomed.2009.03.011]

8 **Pointon AV**, Walker TM, Phillips KM, Luo J, Riley J, Zhang SD, Parry JD, Lyon JJ, Marczylo EL, Gant TW. Doxorubicin in vivo rapidly alters expression and translation of myocardial electron transport chain genes, leads to ATP loss and caspase 3 activation. *PLoS One* 2010; **5**: e12733 [PMID: 20856801 DOI: 10.1371/journal.pone.0012733]

9 **Parra V**, Eisner V, Chiong M, Criollo A, Moraga F, Garcia A, Härtel S, Jaimovich E, Zorzano A, Hidalgo C, Lavandero S. Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis. *Cardiovasc Res* 2008; **77**: 387-397 [PMID: 18006463 DOI: 10.1093/cvr/cvm029]

10 **Marechal X**, Montaigne D, Marciniak C, Marchetti P, Hassoun SM, Beauvillain JC, Lancel S, Neviere R. Doxorubicin-induced cardiac dysfunction is attenuated by ciclosporin treatment in mice through improvements in mitochondrial bioenergetics. *Clin Sci* (Lond) 2011; **121**: 405-413 [PMID: 21605084 DOI: 10.1042/CS20110069]

11 **Ascensão A**, Magalhães J, Soares JM, Ferreira R, Neuparth MJ, Marques F, Oliveira PJ, Duarte JA. Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol* 2005; **289**: H722-H731 [PMID: 15792986 DOI: 10.1152/ajpheart.01249.2004]

12 **Minotti G**, Ronchi R, Salvatorelli E, Menna P, Cairo G. Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. *Cancer Res* 2001; **61**: 8422-8428 [PMID: 11731422]

13 **Childs AC**, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2: Bax ratio. *Cancer Res* 2002; **62**: 4592-4598 [PMID: 12183413]

14 **Ueno M**, Kakinuma Y, Yuhki K, Murakoshi N, Iemitsu M, Miyauchi T, Yamaguchi I. Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. *J Pharmacol Sci* 2006; **101**: 151-158 [PMID: 16766856 DOI: 10.1254/jphs.FP0050980]

15 **Myers C**, Bonow R, Palmeri S, Jenkins J, Corden B, Locker G, Doroshow J, Epstein S. A randomized controlled trial assessing the prevention of doxorubicin cardiomyopathy by N-acetylcysteine. *Semin Oncol* 1983; **10**: 53-55 [PMID: 6340204]

16 **Martin E**, Thougaard AV, Grauslund M, Jensen PB, Bjorkling F, Hasinoff BB, Tjørnelund J, Sehested M, Jensen LH. Evaluation of the topoisomerase II-inactive bisdioxopiperazine ICRF-161 as a protectant against doxorubicin-induced cardiomyopathy. *Toxicology* 2009; **255**: 72-79 [PMID: 19010377 DOI: 10.1016/j.tox.2008.10.011]

17 **Zhang S**, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012; **18**: 1639-1642 [PMID: 23104132 DOI: 10.1038/nm.2919]

18 **Capranico G**, Tinelli S, Austin CA, Fisher ML, Zunino F. Different patterns of gene expression of topoisomerase II isoforms in differentiated tissues during murine development. *Biochim Biophys Acta* 1992; **1132**: 43-48 [PMID: 1380833 DOI: 10.1016/0167-4781(92)90050-A]

19 **Lipshultz SE**, Adams MJ, Colan SD, Constine LS, Herman EH, Hsu DT, Hudson MM, Kremer LC, Landy DC, Miller TL, Oeffinger KC, Rosenthal DN, Sable CA, Sallan SE, Singh GK, Steinberger J, Cochran TR, Wilkinson JD. Long-term cardiovascular toxicity in children, adolescents, and young adults who receive cancer therapy: pathophysiology, course, monitoring, management, prevention, and research directions: a scientific statement from the American Heart Association. *Circulation* 2013; **128**: 1927-1995 [PMID: 24081971 DOI: 10.1161/CIR.0b013e3182a88099]

20 **Tebbi CK**, London WB, Friedman D, Villaluna D, De Alarcon PA, Constine LS, Mendenhall NP, Sposto R, Chauvenet A, Schwartz CL. Dexrazoxane-associated risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies in pediatric Hodgkin's disease. *J Clin Oncol* 2007; **25**: 493-500 [PMID: 17290056 DOI: 10.1200/JCO.2005.02.3879]

21 **Curran CF**, Narang PK, Reynolds RD. Toxicity profile of dexrazoxane (Zinecard, ICRF-187, ADR-529, NSC-169780), a modulator of doxorubicin cardiotoxicity. *Cancer Treat Rev* 1991; **18**: 241-252 [PMID: 1842575 DOI: 10.1016/0305-7372(91)90015-R]

22 **Lipshultz SE**, Scully RE, Lipsitz SR, Sallan SE, Silverman LB, Miller TL, Barry EV, Asselin BL, Athale U, Clavell LA, Larsen E, Moghrabi A, Samson Y, Michon B, Schorin MA, Cohen HJ, Neuberg DS, Orav EJ, Colan SD. Assessment of dexrazoxane as a cardioprotectant in doxorubicin-treated children with high-risk acute lymphoblastic leukaemia: long-term follow-up of a prospective, randomised, multicentre trial. *Lancet Oncol* 2010; **11**: 950-961 [PMID: 20850381 DOI: 10.1016/S1470-2045(10)70204-7]

23 **Ahmet I**, Wan R, Mattson MP, Lakatta EG, Talan M. Cardioprotection by intermittent fasting in rats. *Circulation* 2005; **112**: 3115-3121 [PMID: 16275865 DOI: 10.1161/CIRCULATIONAHA.105.563817]

24 **Varady KA**, Bhutani S, Church EC, Klempel MC. Short-term modified alternate-day fasting: a novel dietary strategy for weight loss and cardioprotection in obese adults. *Am J Clin Nutr* 2009; **90**: 1138-1143 [PMID: 19793855 DOI: 10.3945/ajcn.2009.28380]

25 **Ahmet I**, Tae HJ, de Cabo R, Lakatta EG, Talan MI. Effects of calorie restriction on cardioprotection and cardiovascular

health. *J Mol Cell Cardiol* 2011; **51**: 263-271 [PMID: 21586294 DOI: 10.1016/j.yjmcc.2011.04.015]

26   **Mitra MS**, Donthamsetty S, White B, Latendresse JR, Mehendale HM. Mechanism of protection of moderately diet restricted rats against doxorubicin-induced acute cardiotoxicity. *Toxicol Appl Pharmacol* 2007; **225**: 90-101 [PMID: 17904602 DOI: 10.1016/j.taap.2007.07.018]

27   **Vucevic D**, Mladenovic D, Ninkovic M, Aleksic V, Stankovic MN, Stankovic M, Jorgacevic B, Vukicevic RJ, Radosavljevic T. The effects of caloric restriction against ethanol-induced oxidative and nitrosative cardiotoxicity and plasma lipids in rats. *Exp Biol Med* (Maywood) 2013; **238**: 1396-1405 [PMID: 24157589 DOI: 10.1177/1535370213506806]

28   **Raffaghello L**, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, Longo VD. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci USA* 2008; **105**: 8215-8220 [PMID: 18378900 DOI: 10.1073/pnas.0708100105]

29   **Kawaguchi T**, Takemura G, Kanamori H, Takeyama T, Watanabe T, Morishita K, Ogino A, Tsujimoto A, Goto K, Maruyama R, Kawasaki M, Mikami A, Fujiwara T, Fujiwara H, Minatoguchi S. Prior starvation mitigates acute doxorubicin cardiotoxicity through restoration of autophagy in affected cardiomyocytes. *Cardiovasc Res* 2012; **96**: 456-465 [PMID: 22952253 DOI: 10.1093/cvr/cvs282]

30   **Chen K**, Xu X, Kobayashi S, Timm D, Jepperson T, Liang Q. Caloric restriction mimetic 2-deoxyglucose antagonizes doxorubicin-induced cardiomyocyte death by multiple mechanisms. *J Biol Chem* 2011; **286**: 21993-22006 [PMID: 21521688 DOI: 10.1074/jbc.M111.225805]

31   **Gilliam LA**, Ferreira LF, Bruton JD, Moylan JS, Westerblad H, St Clair DK, Reid MB. Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. *J Appl Physiol* (1985) 2009; **107**: 1935-1942 [PMID: 19779154 DOI: 10.1152/japplphysiol.00776.2009]

32   **Palmer CS**, Osellame LD, Stojanovski D, Ryan MT. The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. *Cell Signal* 2011; **23**: 1534-1545 [PMID: 21683788 DOI: 10.1016/j.cellsig.2011.05.021]

33   **Lee YJ**, Jeong SY, Karbowski M, Smith CL, Youle RJ. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell* 2004; **15**: 5001-5011 [PMID: 15356267 DOI: 10.1091/mbc.E04-04-0294]

34   **Chen H**, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 2005; **280**: 26185-26192 [PMID: 15899901 DOI: 10.1074/jbc.M503062200]

35   **Kavazis AN**, Smuder AJ, Min K, Tümer N, Powers SK. Short-term exercise training protects against doxorubicin-induced cardiac mitochondrial damage independent of HSP72. *Am J Physiol Heart Circ Physiol* 2010; **299**: H1515-H1524 [PMID: 20833957 DOI: 10.1152/ajpheart.00585.2010]

36   **Smuder AJ**, Kavazis AN, Min K, Powers SK. Doxorubicin-induced markers of myocardial autophagic signaling in sedentary and exercise trained animals. *J Appl Physiol* (1985) 2013; **115**: 176-185 [PMID: 23703114 DOI: 10.1152/japplphysiol.00924.2012]

37   **Abd El-Aziz MA**, Othman AI, Amer M, El-Missiry MA. Potential protective role of angiotensin-converting enzyme inhibitors captopril and enalapril against adriamycin-induced acute cardiac and hepatic toxicity in rats. *J Appl Toxicol* 2001;

21: 469-473 [PMID: 11746193 DOI: 10.1002/jat.782]

38   **Sorensen M**, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, Barja G. Effects of fasting on oxidative stress in rat liver mitochondria. *Free Radic Res* 2006; **40**: 339-347 [PMID: 16517498 DOI: 10.1080/10715760500250182]

39   **Rambold AS**, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci USA* 2011; **108**: 10190-10195 [PMID: 21646527 DOI: 10.1073/pnas.1107402108]

40   **Houtkooper RH**, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 2012; **13**: 225-238 [PMID: 22395773 DOI: 10.1038/nrm3293]

41   **Di Simplicio P**, Rossi R, Falcinelli S, Ceserani R, Formento ML. Antioxidant status in various tissues of the mouse after fasting and swimming stress. *Eur J Appl Physiol Occup Physiol* 1997; **76**: 302-307 [PMID: 9349643 DOI: 10.1007/s004210050252]

42   **Hayashida S**, Arimoto A, Kuramoto Y, Kozako T, Honda S, Shimeno H, Soeda S. Fasting promotes the expression of SIRT1, an NAD+ -dependent protein deacetylase, via activation of PPARalpha in mice. *Mol Cell Biochem* 2010; **339**: 285-292 [PMID: 20148352 DOI: 10.1007/s11010-010-0391-z]

43   **Dirks-Naylor AJ**. The role of autophagy in doxorubicin-induced cardiotoxicity. *Life Sci* 2013; **93**: 913-916 [PMID: 24404586 DOI: 10.1016/j.lfs.2013.10.013]

44   **Gottlieb RA**, Finley KD, Mentzer RM. Cardioprotection requires taking out the trash. *Basic Res Cardiol* 2009; **104**: 169-180 [PMID: 19242643 DOI: 10.1007/s00395-009-0011-9]

45   **Dirks AJ**, Leeuwenburgh C. Caloric restriction in humans: potential pitfalls and health concerns. *Mech Ageing Dev* 2006; **127**: 1-7 [PMID: 16226298 DOI: 10.1016/j.mad.2005.09.001]

46   **Longo VD**, Fontana L. Calorie restriction and cancer prevention: metabolic and molecular mechanisms. *Trends Pharmacol Sci* 2010; **31**: 89-98 [PMID: 20097433 DOI: 10.1016/j.tips.2009.11.004]

47   **Zhang XD**, Deslandes E, Villedieu M, Poulain L, Duval M, Gauduchon P, Schwartz L, Icard P. Effect of 2-deoxy-D-glucose on various malignant cell lines in vitro. *Anticancer Res* 2006; **26**: 3561-3566 [PMID: 17094483]

48   **Zhang F**, Aft RL. Chemosensitizing and cytotoxic effects of 2-deoxy-D-glucose on breast cancer cells. *J Cancer Res Ther* 2009; **5** Suppl 1: S41-S43 [PMID: 20009293 DOI: 10.4103/0973-1482.55140]

49   **Maschek G**, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, De Young LR, Lampidis TJ. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res* 2004; **64**: 31-34 [PMID: 14729604 DOI: 10.1158/0008-5472.CAN-03-3294]

50   **Katic M**, Kahn CR. The role of insulin and IGF-1 signaling in longevity. *Cell Mol Life Sci* 2005; **62**: 320-343 [PMID: 15723168 DOI: 10.1007/s00018-004-4297-y]

51   **Sell Ch**. Caloric restriction and insulin-like growth factors in aging and cancer. *Horm Metab Res* 2003; **35**: 705-711 [PMID: 14710349 DOI: 10.1055/s-2004-814156]

52   **Lee C**, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, Hwang D, Cohen P, Bianchi G, Longo VD. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res* 2010; **70**: 1564-1572 [PMID: 20145127 DOI: 10.1158/0008-5472.CAN-09-3228]

EDITORIAL

Life is more than a computer running DNA software

František Baluška, Guenther Witzany

František Baluška, IZMB, University of Bonn, 53113 Bonn, Germany

Guenther Witzany, Telos-Philosophische Praxis, 5111 Buermoos, Austria

Author contributions: Both of the authors contributed to this paper.

Correspondence to: Guenther Witzany, PhD, Telos-Philosophische Praxis, Vogelsangstr. 18c, 5111 Buermoos, Austria. witzany@sbg.at

Telephone: +43-6274-6805 Fax: +43-6274-6805

Abstract

In his recent interview for the Guardian Craig Venter is elaborating about a household appliance for the future, Digital Biological Converter (DBC). Current prototype, which can produce DNA, is a box attached to the computer which receives DNA sequences over the internet to synthesize DNA; later in future also viruses, proteins, and living cells. This would help the household members to produce, *e.g.*, insulin, virus vaccines or phages that fight antibiotic resistant bacteria. In more distant future, Craig Venter's hope is that the DBC will generate living cells *via* so-called "Universal Recipient Cell". This platform will allow digitally transformed genomes, downloaded from the internet, to form new cells fitted for the particular needs such as therapeutics, food, fuel or cleaning water. In contrast to this, the authors propose that DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes. In light of the variety of epigenetic markings, DNA can store a multitude of further meanings hidden under the superficial grammar of nucleic acid sequences.

Core tip: Craig Venter is elaborating a box attached to a computer that receives DNA sequences over the internet to synthesize DNA. As a leading expert in the field of synthetic biology, he is convinced that "life is a DNA software system", and all living things are reducible to DNA sequences. In contrast to this, the authors propose that DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes. In light of the variety of epigenetic markings, DNA can store a multitude of further meanings hidden under the superficial grammar of nucleic acid sequences.

Baluška F, Witzany G. Life is more than a computer running DNA software. *World J Biol Chem* 2014; 5(3): 275-278 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/275.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.275

INTRODUCTION

In his recent interview for the Guardian[1], Craig Venter is elaborating about a household appliance for the future, the Digital Biological Converter (DBC). The current prototype, which can produce DNA, is a box attached to a computer that receives DNA sequences over the internet to synthesize DNA; in future, it will be able to do the same for viruses, proteins and living cells. This would help the household members to produce, for example, insulin, virus vaccines or phages that fight antibiotic-resistant bacteria. Additionally, it could help future Martian colonists, giving them vaccines, antibiotics or personalized drugs that they would need on Mars. If there should be DNA-based life, a digital version could be transmitted to earth without danger of contaminating the home-planet's biosphere[1]. In the more distant future, Craig Venter's hope is that the DBC will generate living cells *via* the so-called "Universal Recipient Cell". This platform will allow digitally transformed genomes, downloaded from

the internet, to form new cells for particular needs (therapeutics, food, fuel or cleaning water)[1]. The final aim is to establish a "Digital Life Sending Unit" allowing biological teleportation. This unit will sample unknown organisms, perhaps on Mars, then analyse their sequences and generate digital DNA files that will be used by the receiving DBC to re-generate these organisms at new places, such as on Earth.

NOTHING ELSE THAN A SOFTWARE SYSTEM?

Some readers might be reminded of Goethe's Dr Faustus' pact with Mephisto and its goal to create a "homunculus" or similar dreams of living robots that would invade space and time, covered by an abundance of science fiction poetries. But Craig Venter is a leading expert in the field of synthetic biology, in which scientists design new biological systems, *i.e.*, synthetic life. He insists that his insertion of a synthetic bacterial (*Mycoplasma genitalium*) genome into a living recipient bacterium (*Mycoplasma capricolum*) represents the "world's first synthetic life" because the synthetic cell replicated itself into a colony of bacteria, containing only the synthetic DNA.

We do not want to enter here the debate of whether his indisputable achievements represent true creation of new life or represent just some kind of a copy-paste approach. We can also be pretty sure that he and his company will achieve several further technological breakthroughs in the near future. However, we would like to make very clear that his conclusions about the nature of Life are not justified. For example, Craig Venter is convinced that it would be possible, in principle, to synthetically create most complex organisms: "I can't explain consciousness yet, but like anything else it will be explainable at the molecular level, the cellular level and therefore the DNA coding level"[1]. In his view, the question of Erwin Schrödinger What is life? has been answered. "Life is a DNA software system", and all living things are reducible to DNA sequences[1]. The DNA-based software creates as well as directs the more visible "hardware" of life, such as proteins and cells[1-3].

This DNA-centric concept looks clear and straightforward. However, it can work only if the theoretical background on which Craig Venter makes his conclusions is correct. In his view[1-3], organisms are mechanistic apparatus-like molecular structures that work as computing machines according the algorithm-based programs encoded in the DNA storage medium. The syntax structure of DNA follows Francis Crick's central dogma of molecular biology "DNA-RNA-anything else". But is this view coherent with recent empirical knowledge? Are cellular organisms only robot-like computing machines that function strictly according to their algorithm-based programming? Or, rather, are they coordinated complex entities that share bio-communication properties that may vary according to different context-specific needs? Is DNA the unequivocal syntax for sequences out of which

one can construct living cells, viruses and phages for a household appliance? Or is the superficial molecular syntax of DNA solely the result of evolution's long inserts and deletions of an abundance of various genetic parasites that shape host genomes? The most crucial questions are: do DNA sequences contain a hidden deep grammar structure that varies according to the meaning and context of environmental insults; do DNA sequences match with high fidelity environmental circumstances that led to epigenetic markings and memory? If yes, this would then mean that the identical DNA sequence may have various-even contradictory-meanings. In fact, this scenario is emerging as true[4-8].

EPIGENETICS: HIDDEN DEEP GRAMMAR

Interestingly, in complex genomes like humans, the coding genes are about 1.5% of the total genome whereas the abundance of non-coding RNAs are about 98.5%. This means Craig Venter's household appliance box could focus only on the 1.5% coding sequences. The DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes, but in light of the variety of epigenetic markings-with its executives RNA editing and alternative splicing-can store a multitude of further meanings[4-8].

This means epigenetic marking saves energy costs like in human language. A limited repertoire of signs, and a limited number of rules to combine these signs correctly, enables signs using agents to generate an unlimited number of sentences with a superficial grammar in the visible text and an abundance of connotations by marking through gestures and other conscious and unconscious bodily expressions such as the movements of three hundred different eye muscles[9].

Are organisms computing machines that fulfil what the DNA program determines? The machine metaphor in molecular biology is an old-fashioned narrative[9] that would like to reduce life to physics and chemistry. Manfred Eigen and Sydney Brenner expanded the concept by adding also information: "Life = physics + chemistry + information"[10,11]. But they defined information according to the mathematical theory of language as used by John von Neumann and Alan Turing in their concept of self-reproducing automata, a chimera that has remained for the last 80 years at a conceptual stage without any functional realization[9,12].

Similar to the algorithm-based computing machines of Turing and von Neumann, Venter's concept of DNA as a software system relies on these computation models. However, these models cannot explain: (1) *de novo* generation of new functional nucleic acid sequences; (2) their context-dependent recombination; and (3) the abundance of mobile regulatory elements being active in all essential processes of life such as replication, transcription, translation, repair and immune defence, all of which are organized by an abundance of small and large RNAs[4-8].

Today, we know that these RNAs predated the emer-

gence of DNA and many of these RNA-world descendants-even RNA viruses-remain as defective parts of genetic parasites in host cellular genomes as exapted and endogenized tools to regulate gene functions[13-17].

RNA-WORLD AGENT ACTIVITIES

Endogenous viruses, transposons, retrotransposons, long terminal repeats, non-long terminal repeats, long interspersed nuclear elements, short interspersed nuclear elements, group Ⅰ introns, group Ⅱ introns, phages and plasmids are currently investigated examples that use genomic DNA as their preferred live habitat. This means that DNA is not solely a genetic storage medium that serves as a read and write medium as an evolutionary protocol, but it is also a (quasi-)species-specific ecological niche[4,17]. A great variety of such mobile genetic elements infect, insert, delete, cut and paste, copy and paste and spread within the genome. They change host genetic identities either by insertion, recombination or the epigenetic regulation of genetic content, and co-evolve with the host and interact in a module-like manner. In this respect, they play vital roles in evolutionary and developmental processes. In contrast to accidental point mutations, integration at various preferred sites is not a randomly occurring process but is coherent with the genetic content of the host; otherwise, important protein-coding regions would be damaged, causing disease or even lethal consequences for the host organism[17].

Therefore, DNA organized in chromatin is far more complex than the human-made "software system", except that we are confusing the algorithm-based simulation of real-life storage with the real life, the computer machines with the living cells and organisms, and the self-reproducing automatons with the real-life organisms that can replicate since the origins of life[5,9,14].

Although various complex attempts to simulate early evolution and emergence of life have been accomplished, no complete living cell with all of its components (cell membranes, organelles, microtubules, chromosomes, *etc*.) has yet been engineered. Although hundreds of announcements have been made within the last 60 years, not one of them has been successfully completed.

BIOCOMMUNICATION AND NATURAL GENOME EDITING

The logical alternatives to the concepts of synthetic biology are not "guilty of a kind of modern day vitalism" as suggested by Craig Venter[1,2]. The alternative is the full range of nucleic acid sequence-based life and the agents that are competent to arrange and rearrange DNA information according to their real-life needs. Communication between cells, tissues, organs and organisms cannot be predicted or simulated by computing machines, because biocommunication does not function mechanistically and is not algorithm dependent[18]. The genome itself, *via* natural genome editing[19], generates large amounts of coher-

ent new sequences and inserts these into DNA genomes without damaging essential protein-coding regions. This is not possible for any human-made software. Therefore, despite the bold visions of Craig Venter, it will not be possible to create digital life in the future. The 20th century DNA-based models and concept cannot integrate current empirical data into a coherent picture of how the real life functions. We need new concepts that will be able to integrate all the currently available empirical data on viruses, mobile genetic elements and the abundance of non-coding RNAs most relevant for genome shaping, regulation and evolution[20-26].

CONCLUSION

Despite the theoretical concepts of Turing and von Neumann, and the abundance of announcements of self-reproducing machines, the vision of digital life files that can be used as modules for generating life units will remain on the theoretical stage. The main reason is that the 20th century DNA-based models cannot integrate current empirical data into a coherent picture of how the real life functions: nucleic acid sequences do not represent unequivocal meanings that can be expressed in protein bodies, but depend on context, *i.e.*, epigenetic markings, RNA-editing and alternative splicing that vary according to environmental circumstances, even though the DNA remains identical.

REFERENCES

1   **Corby Z**. Craig Venter: 'This isn't a fantasy look at the future. We are doing the future'. The Guardian/The Observer 13 October, 2013. Available from: URL: http: //www.theguardian.com/science/2013/oct/13/craig-ventner-mars

2   **Venter C**. Life at the Speed of Light. From the Double Helix to the Dawn of Digital Life. New York: Viking, 2013

3   **Roosth S**. The Godfather, Part II. *Science* 2013; **342**: 312-313

4   **Villarreal LP**. Viruses and the Evolution of Life. Washington: ASM Press, 2005

5   **Baluška F**, Witzany G. At the dawn of a new revolution in life sciences. *World J Biol Chem* 2013; **4**: 13-15 [PMID: 23710294 DOI: 10.4331/wjbc.v4.i2.13]

6   **Mattick JS**, Taft RJ, Faulkner GJ. A global view of genomic information--moving beyond the gene and the master regulator. *Trends Genet* 2010; **26**: 21-28 [PMID: 19944475 DOI: 10.1016/j.tig.2009.11.002.Epub]

7   **Mercer TR**, Mattick JS. Understanding the regulatory and transcriptional complexity of the genome through structure. *Genome Res* 2013; **23**: 1081-1088 [PMID: 23817049 DOI: 10.1101/gr.156612.113]

8   **Qureshi IA**, Mehler MF. Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease. *Nat Rev Neurosci* 2012; **13**: 528-541 [PMID: 22814587 DOI: 10.1038/nrn3234]

9   **Witzany G**, Baluška F. Life's code script does not code itself. The machine metaphor for living organisms is outdated. *EMBO Rep* 2012; **13**: 1054-1056 [PMID: 23146891 DOI: 10.1038/embor.2012.166]

10 **Eigen M**. From Strange Simplicity to Complex Familiarity A Treatise on Matter, Information, Life, and Thought. Oxford: Oxford University Press, 2013

11 **Brenner S**. Turing centenary: Life's code script. *Nature* 2012; **482**: 461 [PMID: 22358811 DOI: 10.1038/482461a]

12   **Witzany G**. Can mathematics explain the evolution of human language? *Commun Integr Biol* 2011; **4**: 516-520 [PMID: 22046452 DOI: 10.4161/cib.4.5.16426.Epub]

13   **Zuckerkandl E**, Cavalli G. Combinatorial epigenetics, "junk DNA", and the evolution of complex organisms. *Gene* 2011; **390**: 232-242 [PMID: 17223284]

14   **Lozada-Chávez I**, Stadler PF, Prohaska SJ. "Hypothesis for the modern RNA world": a pervasive non-coding RNA-based genetic regulation is a prerequisite for the emergence of multicellular complexity. *Orig Life Evol Biosph* 2011; **41**: 587-607 [PMID: 22322874 DOI: 10.1007/s11084-011-9262-1]

15   **Koonin EV**. On the origin of cells and viruses: primordial virus world scenario. *Ann N Y Acad Sci* 2009; **1178**: 47-64 [PMID: 19845627 DOI: 10.1111/j.1749-6632.2009.04992.x]

16   **Baluska F**. Cell-cell channels, viruses, and evolution: via infection, parasitism, and symbiosis toward higher levels of biological complexity. *Ann N Y Acad Sci* 2009; **1178**: 106-119 [PMID: 19845631 DOI: 10.1111/j.1749-6632.2009.04995.x]

17   **Shapiro JA.** Evolution: A view from the 21st century. Washington: FT Press

18   **Witzany G.** Biocommunication and Natural Genome Editing. Dortrecht: Springer, 2010

19   **Witzany G, editor.** Natural Genetic Engineering and Natural Genome Editing. Cambridge: Wiley & Sons, 2010

20   **Brosius J**. The contribution of RNAs and retroposition to evolutionary novelties. *Genetica* 2003; **118**: 99-116 [PMID: 12868601]

21   **Venner S**, Feschotte C, Biémont C. Dynamics of transposable elements: towards a community ecology of the genome. *Trends Genet* 2009; **25**: 317-323 [PMID: 19540613 DOI: 10.1016/j.tig.2009.05.003.Epub]

22   **Witzany G.** From Molecular Entities to Competent Agents: Viral Infection-derived Consortia act as Natural Genetic Engineers. In: Witzany G, editor. Viruses: Essential Agents of Life. Dortrecht: Springer, 2012: 407-419

23   **Slotkin RK**, Martienssen R. Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* 2007; **8**: 272-285 [PMID: 17363976 DOI: 10.1038/nrg2072]

24   **Villarreal LP**. Viral ancestors of antiviral systems. *Viruses* 2011; **3**: 1933-1958 [PMID: 22069523 DOI: 10.3390/v3101933.Epub]

25   **Baluška F.** Evolution in revolution. Paradigm shift in our understanding of life and biological evolution. *Commun Integr Biol* 2011; **4**: 521-523 [DOI: 10.4161/cib.4.5.17702]

26   **Witzany G**. The agents of natural genome editing. *J Mol Cell Biol* 2011; **3**: 181-189 [PMID: 21459884 DOI: 10.1093/jmcb/mjr005.Epub]

FIELD OF VISION

Pragmatic turn in biology: From biological molecules to genetic content operators

Guenther Witzany

Guenther Witzany, Telos-Philosophische Praxis, 5111 Buermoos, Austria

Author contributions: Witzany G solely contributed to this paper.

Correspondence to: Guenther Witzany, PhD, Telos-Philosophische Praxis, Vogelsangstraße 18c, 5111 Buermoos, Austria. witzany@sbg.at

Telephone: +61-43-62746805 Fax: +61-43-62746805

Abstract

Erwin Schrödinger's question "What is life?" received the answer for decades of "physics + chemistry". The concepts of Alain Turing and John von Neumann introduced a third term: "information". This led to the understanding of nucleic acid sequences as a natural code. Manfred Eigen adapted the concept of Hammings "sequence space". Similar to Hilbert space, in which every ontological entity could be defined by an unequivocal point in a mathematical axiomatic system, in the abstract "sequence space" concept each point represents a unique syntactic structure and the value of their separation represents their dissimilarity. In this concept molecular features of the genetic code evolve by means of self-organisation of matter. Biological selection determines the fittest types among varieties of replication errors of quasi-species. The quasi-species concept dominated evolution theory for many decades. In contrast to this, recent empirical data on the evolution of DNA and its forerunners, the RNA-world and viruses indicate cooperative agent-based interactions. Group behaviour of quasi-species consortia constitute de novo and arrange available genetic content for adaptational purposes within real-life contexts that determine epigenetic markings. This review focuses on some fundamental changes in biology, discarding its traditional status as a subdiscipline of physics and chemistry.

Core tip: Meaning in natural languages/codes and communication is context dependent. In contrast, artificial formalizable (algorithm based) languages employ a "universal" syntax in order to determine meaning independent of the contextual circumstances. It is empirically evident that no natural language speaks itself as no natural code codes itself. It always requires living agents that share a competence to generate and interpret these natural codes. Therefore I suppose that changes in the genetic code, which are of evolutionary relevance, are rather the result of fine-tuned processes by a large network of mobile genetic elements, persistent viruses, its defectives and other genetic parasites that alter DNA sequences. In this respect DNA remains as ecosphere habitat for social interacting RNA inhabitants. This represents a pragmatic turn in biology from syntax centered molecular biology to pragmatics centered agents interactions.

INTRODUCTION

Sydney Brenner recently described the radical revolution in life sciences in the early 1950s: the occupation of biology by quantum mechanics examining the fundamental questions of matter and energy followed by the rise of genetics that showed that chromosomes were the carriers of genes. The discovery of the double helix resulted in

the new paradigm that information is physically embodied in DNA sequences of four different types[1]. In contrast to the years before 1953, the question of "information" now became central: the components of DNA are simple chemicals, but the biological complexity that can be generated by the information of different sequences is revolutionary. The fundamental concept that integrated this new biological "information" with matter and energy was enshrined in the universal Turing machine and von Neumann's self-reproducing machines[2-4]. Consequently it follows that biology is physics with computation[5]. This was the core paradigm of molecular biology for almost the next half-century. The crucial step in the serious discussion of "information" as an essential part of definitions of "life" was taken by Manfred Eigen.

MANFRED EIGEN COMBINES PHYSICS, CHEMISTRY, MATHEMATICS AND INFORMATION THEORY

In a series of articles and books Manfred Eigen developed a model of how the essential features of life and its inherent complexity can be explained by physical properties of matter[6,7]. If certain chemical properties exist on a planet and certain physical conditions obtain, life will start by self-reproducing macromolecular cycles which act in a complementary way. On the one hand there are "information"-carrying nucleic acids which build a reproductive cycle. On the other there are functional amino acids which build the protein bodies. Both code-systems together can build a catalytic "hypercycle" which is the basis of the self-reproductivity of life.

Both parts can be reconstructed physically. Nucleic acids (information) and proteins (function) represent a closed system, because there is no function without information, and information gets meaning from function. "Mutations" are replication errors with selective advantage, *i.e.*, instabilities in this system represent irreversible thermodynamic processes. A series of such mutations in nucleic acid sequences leads to quasi-species that are mutant distributions of primitive replicating entities. Such dynamic distributions of genomes that share genetic variation, competition and selection generate the fittest types ("master copies") and therefore avoid "error thresholds", *i.e.*, excessively high mutation rates, in that information cannot further reproduce. The resulting evolution of life is an optimising process in that Darwinian selection evaluates the fittest results of mutations[8,9].

Manfred Eigen adapted the concept of Hammings "sequence space" to explain hypercycle concept by physical properties of matter. Similar to Hilbert space, in which every ontological entity could be defined by an unequivocal point in a mathematical axiomatic system, in the abstract , information space" concept each point represents a unique syntactic structure and the value of their separation represents their dissimilarity. In this concept molecular features of the genetic code evolve by means of self-organisation of

matter. Each point in the sequence space can be occupied by one of four different nucleotides. But each point can also be represented by digital computation (1 and 0)[7].

BIOLINGUISTICS, BIOINFORMATICS, SYSTEMS BIOLOGY AND SYNTHETIC BIOLOGY

A series of varieties of mathematical theories of language emerged such as Biolinguistics, Bioinformatics, and systems biology. They all interpret and investigate genetic structures in the light of linguistic categories as quantifiable sets of signs[10-12] and use statistical methods and algorithms to identify genetic sequence orders.

An emerging hybrid of information-theoretical aspects of nucleic acid language is synthetic biology. Its theoretical assumptions clearly derive from systems biology and information theory and generally from a mathematical theory of language. Proponents of synthetic biology want to deconstruct complex biological systems into its parts and artificially reconstruct and even evolve biological systems[13]. This kind of artificial molecular design could serve as an appropriate tool in genetic engineering for, *e.g.*, new vaccines, immune functions, *etc*. This rather mechanistic concept depends on syntax structure identification that represents meaning/function. The context-dependent epigenetic imprinting which represents a deep grammar hidden in the superficial grammar of nucleic acid sequences is not the focus of synthetic biology approaches. In contrast to predominant genetic engineering synthetic biology tries to construct complex biological systems which are then subject to selection processes. They are expected to be mutation-resistant in a certain sense.

UNEXPECTED EARTHQUAKE IN THE FOUNDATIONS OF MATHEMATICS

The original mainstream assumptions regarding the several mathematical theories of language are still present in concepts, curricula and even the definition of life and animated nature[14,15]. The conviction of an exact science based on exact definitions of scientific sentences in contrast to non-scientific ones is at the basis of scientific communities and their self-understanding[16].

The history of science or even sociology of knowledge evidences the interesting fact that it is still largely ignored that 50 years ago the basis of this world view was shaken to the core. The belief that mathematics was the best tool for depicting the physical reality of matter and natural laws marginalised world views other than mathematical ones[16-19].

In his Unvollständigkeitssatz (incompleteness theorem) Manfred Gödel investigated a formal system converting a meta-theoretical statements into an arithmetical one[20]. He strove to convert the statements formulated in a meta-language into the object language S. This led

Gödel to two prominent and critical conclusions: (1) If system S is consistent, then it will contain at least one formally indeterminable sentence. This means that one sentence is inevitably present that can be neither proved nor disproved within the system; and (2) If system S is consistent, then this consistency of S cannot be proved within S.

The consequence of the incompleteness theorem for the automaton theory of Turing and von Neumann was significant: a machine can principally calculate only those functions for which an algorithm can be provided. Sign-mediated interactions between living organisms in which the meaning of the signs depends on real life circumstances relay on non-formalizable sequence generation, for which no algorithm can be provided. Essential functions of every natural language, such as non-formalizable features are not object of algorithm based calculations. Living organisms are no machines[21].

PRAGMATIC TURN IN BIOLOGY: NATURAL GENETIC CONTENT OPERATORS EDIT GENOMES

Manfred Eigen's concept of natural languages/codes and the current concepts embraced by bioinformatics, biolinguistics, systems biology and synthetic biology are not coherent with current knowledge about key features of natural languages or codes, *i.e.*, the three levels of rules that govern natural code use by competent code-using groups: combinatorial rules (syntax), contextual rules (semantics) and context-dependent rules (pragmatics). In all mathematical theories of language the syntax determines semantics (function), but in natural codes pragmatics (context) determines semantics. Pragmatic rules do not exist in Eigen's concept. Natural code-inherent rules are absent in abiotic matter that is determined strictly by natural laws: no syntax, pragmatic or semantic rules are present if water freezes to ice. Therefore the explanation of the evolution of biological macromolecules in Eigen's concept as well as in other mathematical theories of language cannot explain the evolution of natural codes and its inherent rules[22-25].

RNAS THAT ORGANIZE GENETIC CONTENT COMPOSITION

The change from a read-only-memory genome with copying errors to a read-and-write genome with active change operators is fundamental. In contrast to the decades long assumption that the driving forces of evolution were chance mutation (statistical replication errors) and selection it is now recognised that although mutation is an empirical fact it does not contribute very much to genetic novelty. Key roles now act as non-random genetic change operators in the production of complex evolutionary inventions[26-28].

Now we can investigate several key players that or-

ganise the genetic content compositions of host organisms such as, *e.g.*, endogenous viruses and its defectives, transposons, retrotransposons, LTRs (long terminal repeats), non-LTRs (non-long terminal repeats), LINEs (long interspersed nuclear elements), SINEs (short interspersed nuclear elements), ALUs, group I introns, group II introns, phages, plasmids[29-31]. We now recognize that DNA is not solely a genetic storage medium but is also a kind of ecological habitat. Many of such mobile genetic elements have been found within the last 40 years as inhabitants of all genomes[32-35]. Some cut and paste, others copy and paste and both spread within the genome. They modify host genetic identities through insertion, recombination, or the epigenetic regulation of genetic content. They co-evolve with the host, interact in a modular manner and additionally generate highly adaptive immune systems for host organisms from the simplest prokaryotes (CRISPRs/Cas system) to the most complex eukaryotes (VJD-Systems). Such mobile genetic elements shape both genome architecture and regulation. Therefore they are agents of change not only over evolutionary time but also in real time as domesticated agents[36-38].

FROM MOLECULAR BIOLOGICAL ENTITIES TO SOCIAL GROUPS

The question arises how these RNA populations, it's closely related RNA viruses and their complex interactions can be explained and understood without mathematical theories of language. How should we investigate non-coding RNA interactions, competencies and even their role in epigenetic imprinting without formalisable tools? This world of life processes is dominated by RNA, whereas DNA remains a habitat, an ecosphere of interacting RNAs that behave like inhabitants and as genetic information storage[39-43].

All these these RNAs, share a secondary structure like a hairpin, or a stem-loop. In more complex ligated consortia of such stem loops we can look at tRNAs, or ribosomal subunits, RNA polymerases or a great variety of RNA viruses and its defectives as listed above. The RNA stem loops have two characteristic parts: stems that consist of base-paired nucleic acids and loops, bulges and junctions that consist of unpaired regions limited by stems. Most interesting from an evolutionary perspective are two recently found key features[44-46]: (1) Randomly associated RNAs that have no evolutionary history show the same structure-dependent compositional bias as ribosomal RNAs. This means that the differences do not depend on selection processes but on the overall composition of the RNA consortium; and (2) The singular RNA stem loop behaves like a random assembly of nucleotides without selective forces and underlies physico-chemical laws exclsusively Only if stem loops build groups, they share a culture of interactional patterns and a history of defined timescales, *i.e.*, they underlie biological selective forces.

This looks like the true split of life and non-life processes. To better understand behavioral motifs of RNA

stem-loop swarms and viruses, one should add group membership features that are absent in the inanimate world. The basic tool of such RNAs is their complementary composition of base-pairing stems and not base-pairing loops, the result of an inherent property of RNA chemicals, the foldback of polyRNAs. The variety of regulations on protein coding genes as well as the processing of these regulatory RNAs by phases of splicing and editing RNA transcripts makes its algorithm based predictability nearly impossible because of its complexity[39].

These populations of RNAs share properties with RNA viruses, which have defined capabilities. In contrast to DNA viruses RNA viruses have much smaller genomes on RNA bases without proofreading and repair. In contrast to the previous perspective (mutation, *i.e.*, replication error) the new perspective assembles the property of invention of new sequence contents, *de novo*, that have not existed before and for which no algorithms are available in principle. This is important for variation and innovation, as well as infection, immunity and identity, for both diversified viral and cellular populations[47-50].

RNA STEM LOOP STRUCTURES CONSTITUTE LIFE

This change in perspective from molecules to agent-based behaviour will look at interactions of RNA viruses, DNA viruses, RNA-DNA viruses, viral swarms, and sub-viral groups like any ligated RNA stem loop groups that cooperate and coordinate (regulate) within cellular genomes as replication-relevant co-players[51-53]. Or they interact as suppression-relevant silencers or as infection-derived modular tools of non-coding RNAs that have built consortia of complementary agents that function together such as retrovirus-derived remnants, such as LTRs, non-LTRs, group II introns, rRNAs, tRNAs, spliceosomes, editosomes, and other counterbalancing modules[54-58]. Such populations determine regulations in many ways and may newly adapt different functions. The use of a natural language or code depends on consortia of living agents, because natural languages and codes function according to rules. In contrast to the inevitability of natural laws rule-following is a feature of social interaction and not solely one of physico-chemical necessity[58].

Investigating syntactic sequences without knowing something about the real-life context of code-using agents is senseless because syntactic structures do not represent unequivocally semantic meaning. Quantifiable analyses of signs, words and sequences cannot extract meaning. Only in a restricted (statistical) sense this is possible through sequence comparison.

EVOLUTIONARY GENETIC INVENTION IS NOT THE RESULT OF REPLICATION ERRORS

The virosphere in particular exemplifies how genetic in-

novation derives from novel nucleic acid sequences and their combination[59]. If cells are infected by more than one virus, the genomes of different viruses are copackaged into the viral progeny. During reverse transcription the reverse transcriptase switches between two or more templates, generating a new DNA sequence[60]. Similar sequence generations are known in various co-infection events such as the combination of external RNA viruses and persistent endogenous retrovirus, infectious RNA viruses with former viruses, retaining defective parts which can be combined into new sequence orders of still functioning viruses[52,53]. Interestingly, not only viruses generate de novo, or combine and recombine sequences. With this innovation competence quasispecies-consortia (qs-c) transfer this adaptive principle also to all forms of cellular life. The defective parts of infectious genetic parasites represent an abundance of appropriate tools for cellular needs, documented in the variety of non-coding RNAs which are essential actors in all stages of cellular life such as transcription, translation, repair, recombination and immune functions[60-65].

REMEMBER GÖDEL: NATURAL CODES ARE OPEN "SYSTEMS"

RNA group membership can be described by its various features. But this membership can never be completely specified, since it can always be further parasitised by unknown and even unpredictable parasites. This essential feature renders the ability to specify membership absolutely impossible. Additionally this means absolute immunity in this open "system" is impossible in principle. This "insecurity" provides the inherent capacity for novelty, that is, the precondition for greater complexity. It seems we are here at the core competence of variation the essential feature for biological selection.

How do agents emerge from ribozymes to form identity of replicators and then form groups that learn membership? The emergence of single RNA stem-loops solely depends on physico-chemical properties. As mentioned above, if stem-loop groups build complex consortia biological selection and social interactions emerge that are not present in a purely chemical world[44-46]. This looks coherent with the results of sociology and the evolution of natural languages. Natural languages and codes depend on competent agents that followsemiotic (syntactic, pragmatic, semantic) rules, and rule-following are social interactions. This means one agent alone cannot generate or follow a rule. Evolution of identity implies emergence of self/non-self differentiation competence. This is a crucial step from single RNA stem loops to RNA stem loop groups[28,36,45,46].

RNA GROUP BUILDING: CONTEXT DETERMINES MEANING

If we apply some interactional motifs of RNA agents

to form biotic structures that follow biological selection processes and not mere physico-chemical reaction patterns we must also look at the group-building of RNA stem-loop structures.

As previously mentioned it has been found that single stem loops react in a purely physico-chemical reaction mode without selective forces, regardless of whether they derived randomly or are constructed under in vitro conditions[39,46]. Conversely, if these single RNA stem loops build groups they overrule pure physico-chemical reaction patterns and emerge as biological selection forces: biological identities of self/non-self recognition and preclusion, immune functions, dynamically changing (adapting) membership roles. A single alteration in a base-pairing RNA stem that leads to a new bulge may dynamically alter not only a single stem loop but the whole group identity from which this stem loop containing the newly emerged bulge derives[39,46].

Simple self-ligating RNA stem loops can build much larger groups of RNA stem loops that serve to increase complexity[66]. This may lead to ribozymatic consortia, which later on build success stories, such as the merger of the two subunits of transfer RNAs or RNA-dependent RNA-polymerases for replication of RNA through RNA or the subunits of ribosomal RNAs, all of them being former groups that evolved and functioned for different reasons than those applicable to subsequent conserved modes[67-69].

If RNA fragments self-ligate into self-replicating ribozymes they constitute networks. For example, three-membered networks represent highly cooperative growth behavior. If such networks compete directly with selfish autocatalytic cycles, the former grow faster. This clearly indicates the ability of RNA populations to evolve into higher complexity through cooperation which clearly outruns selfishness[46].

Another intriguing example of the biological (selective) group-building competence of RNA stem loop consortia is the chemical interaction based on the molecular syntax in stem-loop "kissing", in that single-stranded regions of RNA stem loops bind according to Chargaff rules to other single-stranded stem loop structures to unite and build more complex group identities for several functions, such as dimerisation of genomic RNA in viruses, *e.g.*, HIV 1. Such complementary interactions are also important in RNA replication of the hepatitis C virus[70-72].

Complex three-dimensional structures can be built by consortia of single RNA sequence strings. One of the most interesting structures is the pseudoknot composed of two helical segments connected by single-stranded regions or loops. Bases in the single-stranded loop are base-pairings with bases outside the loop. This interaction pattern clearly depends on the rules of molecular syntax but is initiated for adaptational purposes by different ecosphere habitat dynamics. So the results of these interactions may lead to structurally diverse groups with important different biological roles such as the catalytic

core of key players of the present RNA world, *i.e.*, ribozymes, self-splicing introns, telomerase and its context-dependent altering gene expression by inducing ribosomal frameshifting in several viruses[73-75].

Most interestingly, the base-pairing in pseudoknots is strictly context-sensitive and base-pairs overlap with one another in sequence positions. This leads to the limits of algorithm-based prediction models such as dynamic programming or stochastic context-free grammars. This indicates the natural language nature of nucleic acid code3aw which represents the possibility of coherent de novo generation and context-dependent alterations for a diversity of different meanings (functions) for the same syntax structures.

CONCLUSION

How long will biology remain a subdiscipline of physics and chemistry? As I have tried to demonstrate, the investigation methods of natural languages/codes such as the genetic code (in terms of both its superficial syntax and the deep grammar hidden as a result of epigenetic imprintings) in the light of mathematical theories of language and its derivatives such as biolinguistics, bioinformatics, systems biology and synthetic biology can lead to quantifiable, *i.e.*, statistical, results which can be compared, measured and computed. The question remains whether it is sensible, to measure, investigate and compare the wavelength and modulations of phonetic utterances of humans to extract a meaning? Can we extract semantics from investigations of certain features of syntax structure?

In natural languages/codes it is not the structure of syntax that determines the meaning of sequences. In nearly all cases it is the hidden deep grammar which determines meaning for the recipient of the message. The deep grammar depends on how the superficial syntax is marked: in the genetic code by epigenetic imprintings or in sign sequences of utterances by gestures and emphasis. In all cases the hidden deep grammar decides whether a competent recipient can understand the intended meaning of the sender or not.

The real-life world in which natural sign users are included decides the meaning of a natural language or code, not the in vitro experimental set ups, the universal grammar or similar algorithm-based components. In contrast with previous approaches the real action between interactors determines what signs of communication and coordination are used to express what should be transported, what is intended, and what is focused. The real actions are the driving force of content and represent the context which determines the meaning of thoughts and interpretations. Therefore pragmatics is of essential relevance to identify the meaning of natural languages/codes, not syntax or semantics.

This aspect is missing completely in Eigens concept of a sequence space in which each nucleotide sequence occupies a unique position that can be computed by digi-

tal units. Because each nucleotide sequence can have several meanings, depending on the contextual use, sequence space position can not explain the variety of its functions.

This means the mathematical concept of language and its derivatives is based upon a fundamental error. Natural languages/codes are not the core objects of natural sciences because the latter''s tools for appropriate investigations are rather limited and cannot lead to a full explanation or understanding. As a consequence we need a pragmatic turn in biology to liberate this discipline from its role as a subdiscipline of physics and chemistry.

REFERENCES

1   **Brenner S**. History of science. The revolution in the life sciences. *Science* 2012; **338**: 1427-1428 [PMID: 23239722 DOI: 10.1126/science.1232919]
2   **Turing A**. Computing machinery and intelligence. *Mind* 1950; **59**: 433-460
3   **Neumann J**. Theory of Self-Reproducing Automata. Urbana/London: University of Illinois Press, 1966
4   **Shannon CE**, Weaver W. The Mathematical Theory of Communication. Urbana: University of Illinois Press, 1949
5   **Brenner S**. Turing centenary: Life's code script. *Nature* 2012; **482**: 461 [PMID: 22358811 DOI: 10.1038/482461a]
6   **Eigen M**. Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 1971; **58**: 465-523 [PMID: 4942363]
7   **Eigen M**. The origin of genetic information: viruses as models. *Gene* 1993; **135**: 37-47 [PMID: 8276276]
8   **Biebricher CK**, Eigen M. The error threshold. *Virus Res* 2005; **107**: 117-127 [PMID: 15649558]
9   **Eigen M**. From Strange Simplicity to Complex Familiarity A Treatise on Matter, Information, Life, and Thought. Oxford: Oxford University Press, 2013
10 **Dong S**, Searls DB. Gene structure prediction by linguistic methods. *Genomics* 1994; **23**: 540-551 [PMID: 7851881]
11 **Chomsky N**. Current Issues in Linguistic Theory. London: The Hague: Mouton, 1964
12 **Zhang HY**. The evolution of genomes and language. *EMBO Rep* 2006; **7**: 748-749 [PMID: 16880816 DOI: 10.1038/sj.embor.7400756]
13 **Venter C**. Life at the Speed of Light. From the Double Helix to the Dawn of Digital Life. New York: Viking, 2013
14 **Nowak MA**, Krakauer DC. The evolution of language. *Proc Natl Acad Sci USA* 1999; **96**: 8028-8033 [PMID: 10393942]
15 **Nowak MA**, Komarova NL, Niyogi P. Evolution of universal grammar. *Science* 2001; **291**: 114-118 [PMID: 11141560]
16 **Carnap R**. Foundations of Logic and Mathematics. Chicago: University of Chicago Press, 1939
17 **Hilbert D**, Bernays P, Grundlagen der Mathematik. Vol I. Berlin, New York: Springer, 1934
18 **Hilbert D**, Bernays P, Grundlagen der Mathematik. Vol II. Berlin, New York: Springer, 1939
19 **Whitehead AN**, Russell B. Principia Mathematica, 3 vols. Cambridge: Cambridge University Press, 1910, 1912, 1913
20 **Gödel K**. Über formal unentscheidbare Sätze der Principia Mathematica und verwandter Systeme. *Monatshefte für Mathematik und Physik* 1931; **38**: 173-198
21 **Witzany G**. Life: The Communicative Structure. Norderstedt: Libri BoD, 2000
22 **Witzany G**. Biocommunication and Natural Genome Editing. Dordrecht: Springer, 2010
23 **Witzany G**. Can mathematics explain the evolution of human language? *Commun Integr Biol* 2011; **4**: 516-520 [PMID: 22046452 DOI: 10.4161/cib.4.5.16426]
24 **Witzany G**, Baluška F. Turing: A formal clash of codes. *Nature* 2012; **483**: 541 [PMID: 22460888 DOI: 10.1038/483541c]
25 **Witzany G**, Baluška F. Life's code script does not code itself. The machine metaphor for living organisms is outdated. *EMBO Rep* 2012; **13**: 1054-1056 [PMID: 23146891 DOI: 10.1038/embor.2012.166]
26 **Ben-Jacob E**. Bacterial wisdom, Goedel's theorem and creative genomic webs. *Physica A* 1998; **248**: 57-76
27 **Shapiro JA**. How life changes itself: the Read-Write (RW) genome. *Phys Life Rev* 2013; **10**: 287-323 [PMID: 23876611 DOI: 10.1016/j.plrev.2013.07.001]
28 **Villarreal LP**. Origin of Group Identity: Viruses, addiction and cooperation. New York: Springer, 2009
29 **Witzany G**. Natural Genetic Engineering and Natural Genome Editing. New York: Wiley & Blackwell, 2010
30 **Witzany G**. Noncoding RNAs: persistent viral agents as modular tools for cellular needs. *Ann N Y Acad Sci* 2009; **1178**: 244-267 [PMID: 19845641 DOI: 10.1111/j.1749-6632.2009.04989.x]
31 **Weiner AM**. SINEs and LINEs: troublemakers, saboteurs, benefactors, ancestors. In: Gesteland RF, Cech TR, Atkins JF, editors. The RNA World, 3rd ed. New York: Cold Spring Harbor Laboratory Press, 2006: 507-534
32 **Witzany G**. Viruses: Essential Agents of Life. Dortrecht: Springer, 2012
33 **Koonin EV**, Senkevich TG, Dolja VV. The ancient Virus World and evolution of cells. *Biol Direct* 2006; **1**: 29 [PMID: 16984643]
34 **Koonin EV**. On the origin of cells and viruses: primordial virus world scenario. *Ann N Y Acad Sci* 2009; **1178**: 47-64 [PMID: 19845627 DOI: 10.1111/j.1749-6632.2009.04992.x]
35 **Villarreal LP**, Witzany G. The DNA Habitat and its RNA Inhabitants. At the dawn of RNA sociology. *Genom Ins* 2013; **6**: 1-12[DOI: 10.4137/GEI.S11490]
36 **Villarreal L**. Viruses and host evolution: virus-mediated self identity. *Adv Exp Med Biol* 2012; **738**: 185-217 [PMID: 22399381 DOI: 10.1007/978-1-4614-1680-7_12]
37 **Villarreal LP**. The source of self: genetic parasites and the origin of adaptive immunity. *Ann N Y Acad Sci* 2009; **1178**: 194-232 [PMID: 19845639 DOI: 10.1111/j.1749-6632.2009.05020.x]
38 **Villarreal LP**. Viral ancestors of antiviral systems. *Viruses* 2011; **3**: 1933-1958 [PMID: 22069523 DOI: 10.3390/v3101933]
39 **Villarreal LP,** Witzany G. Rethinking quasispecies theory: From fittest type to cooperative consortia. *World J Biol Chem* 2013; **4**: 79-90 [PMID: 24340131 DOI: 10.4331/wjbc.v4.i4.79]
40 **Moelling K**. What contemporary viruses tell us about evolution: a personal view. *Arch Virol* 2013; **158**: 1833-1848 [PMID: 23568292]
41 **Forterre P**, Prangishvili D. The major role of viruses in cellular evolution: facts and hypotheses. *Curr Opin Virol* 2013; **3**: 558-565 [PMID: 23870799 DOI: 10.1016/j.coviro.2013.06.013]
42 **Witzany G**. From Molecular Entities to Competent Agents: Viral Infection-derived Consortia act as Natural Genetic Engineers. In: Witzany G, editor. Viruses: Essential Agents of Life. Dortrecht: Springer. 2012: 407-419
43 **Le Rouzic A**, Capy P. Population genetics models of competition between transposable element subfamilies. *Genetics* 2006; **174**: 785-793 [PMID: 16888345]
44 **Smit S**, Yarus M, Knight R. Natural selection is not required to explain universal compositional patterns in rRNA secondary structure categories. *RNA* 2006; **12**: 1-14 [PMID: 16373489]
45 **Gwiazda S**, Salomon K, Appel B, Müller S. RNA self-ligation: from oligonucleotides to full length ribozymes. *Biochimie* 2012; **94**: 1457-1463 [PMID: 22465106 DOI: 10.1016/j.biochi.2012.03.015]
46 **Vaidya N**, Manapat ML, Chen IA, Xulvi-Brunet R, Hayden EJ, Lehman N. Spontaneous network formation among cooperative RNA replicators. *Nature* 2012; **491**: 72-77 [PMID: 23075853 DOI: 10.1038/nature11549]
47 **Briones C**, Stich M, Manrubia SC. The dawn of the RNA

World: toward functional complexity through ligation of random RNA oligomers. *RNA* 2009; **15**: 743-749 [PMID: 19318464 DOI: 10.1261/rna.1488609]

48 **Villarreal LP**. The Addiction Module as a Social Force. In: Viruses: Essential Agents of Life. In: Witzany G, editor. Dordrecht: Springer, 2012: 107-145

49 **Venner S**, Feschotte C, Biémont C. Dynamics of transposable elements: towards a community ecology of the genome. *Trends Genet* 2009; **25**: 317-323 [PMID: 19540613 DOI: 10.1016/j.tig.2009.05.003]

50 **Stoddard B**, Belfort M. Social networking between mobile introns and their host genes. *Mol Microbiol* 2010; **78**: 1-4 [PMID: 20545861 DOI: 10.1111/j.1365-2958.2010.07217.x]

51 **Dinter-Gottlieb G**. Viroids and virusoids are related to group I introns. *Proc Natl Acad Sci USA* 1986; **83**: 6250-6254 [PMID: 3462692]

52 **Evans LH**, Alamgir AS, Owens N, Weber N, Virtaneva K, Barbian K, Babar A, Malik F, Rosenke K. Mobilization of endogenous retroviruses in mice after infection with an exogenous retrovirus. *J Virol* 2009; **83**: 2429-2435 [PMID: 19116259 DOI: 10.1128/JVI.01926-08]

53 **Diemer GS**, Stedman KM. A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses. *Biol Direct* 2012; **7**: 13 [PMID: 22515485 DOI: 10.1186/1745-6150-7-13]

54 **Witzany G**. The agents of natural genome editing. *J Mol Cell Biol* 2011; **3**: 181-189 [PMID: 21459884 DOI: 10.1093/jmcb/mjr005]

55 **Mruk I**, Kobayashi I. To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems. *Nucleic Acids Res* 2014; **42**: 70-86 [PMID: 23945938 DOI: 10.1093/nar/gkt711]

56 **Kobayashi I**. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res* 2001; **29**: 3742-3756 [PMID: 11557807]

57 **Garrett RA**, Vestergaard G, Shah SA. Archaeal CRISPR-based immune systems: exchangeable functional modules. *Trends Microbiol* 2011; **19**: 549-556 [PMID: 21945420 DOI: 10.1016/j.tim.2011.08.002]

58 **Villarreal LP**, Witzany G. Viruses are essential agents within the roots and stem of the tree of life. *J Theor Biol* 2010; **262**: 698-710 [PMID: 19833132 DOI: 10.1016/j.jtbi.2009.10.014]

59 **Witzany G**. Viruses: Essential Agents of Life. Springer, 2012

60 **Hu WS**, Temin HM. Retroviral recombination and reverse transcription. *Science* 1990; **250**: 1227-1233 [PMID: 1700865]

61 **Sciamanna I**, Vitullo P, Curatolo A, Spadafora C. Retrotransposons, reverse transcriptase and the genesis of new genetic information. *Gene* 2009; **448**: 180-186 [PMID: 19631262 DOI: 10.1016/j.gene.2009.07.011]

62 **Pathak KB**, Nagy PD. Defective Interfering RNAs: Foes of Viruses and Friends of Virologists. *Viruses* 2009; **1**: 895-919 [PMID: 21994575 DOI: 10.3390/v1030895]

63 **Cheng LK**, Unrau PJ. Closing the circle: replicating RNA with RNA. *Cold Spring Harb Perspect Biol* 2010; **2**: a002204 [PMID: 20554706 DOI: 10.1101/cshperspect.a002204]

64 **Li D**, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J. Defective interfering viral particles in acute dengue infections. *PLoS One* 2011; **6**: e19447 [PMID: 21559384 DOI: 10.1371/journal.pone.0019447]

65 **Brosius J**. RNAs from all categories generate retrosequences that may be exapted as novel genes or regulatory elements. *Gene* 1999; **238**: 115-134 [PMID: 10570990]

66 **Müller S**, Appel B, Krellenberg T, Petkovic S. The many faces of the hairpin ribozyme: structural and functional variants of a small catalytic RNA. *IUBMB Life* 2012; **64**: 36-47 [PMID: 22131309 DOI: 10.1002/iub.575]

67 **Maizels N**, Weiner AM. Phylogeny from function: evidence from the molecular fossil record that tRNA originated in replication, not translation. *Proc Natl Acad Sci USA* 1994; **91**: 6729-6734 [PMID: 8041690]

68 **Dick TP**, Schamel WA. Molecular evolution of transfer RNA from two precursor hairpins: implications for the origin of protein synthesis. *J Mol Evol* 1995; **41**: 1-9 [PMID: 7608982]

69 **Sun FJ**, Caetano-Anollés G. Evolutionary patterns in the sequence and structure of transfer RNA: early origins of archaea and viruses. *PLoS Comput Biol* 2008; **4**: e1000018 [PMID: 18369418 DOI: 10.1371/journal.pcbi.1000018]

70 **Paillart JC**, Skripkin E, Ehresmann B, Ehresmann C, Marquet R. A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. *Proc Natl Acad Sci USA* 1996; **93**: 5572-5577 [PMID: 8643617]

71 **Friebe P**, Boudet J, Simorre JP, Bartenschlager R. Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 2005; **79**: 380-392 [PMID: 15596831]

72 **Lodmell JS**, Ehresmann C, Ehresmann B, Marquet R. Convergence of natural and artificial evolution on an RNA loop-loop interaction: the HIV-1 dimerization initiation site. *RNA* 2000; **6**: 1267-1276 [PMID: 10999604]

73 **Rivas E**, Eddy SR. A dynamic programming algorithm for RNA structure prediction including pseudoknots. *J Mol Biol* 1999; **285**: 2053-2068 [PMID: 9925784]

74 **Staple DW**, Butcher SE. Pseudoknots: RNA structures with diverse functions. *PLoS Biol* 2005; **3**: e213 [PMID: 15941360 DOI: 10.1371/journal.pbio.0030213]

75 **Gupta A**, Rahman R, Li K, Gribskov M. Identifying complete RNA structural ensembles including pseudoknots. *RNA Biol* 2012; **9**: 187-199 [PMID: 22418849 DOI: 10.4161/rna.18386]

TOPIC HIGHLIGHT

In 2014, can we do better than CA125 in the early detection of ovarian cancer?

Joshua G Cohen, Matthew White, Ana Cruz, Robin Farias-Eisner

Joshua G Cohen, Matthew White, Ana Cruz, Robin Farias-Eisner, Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of California, Los Angeles Medical Center, Los Angeles, CA 90095, United States
Author contributions: Cohen JG, White M and Cruz A performed research and acquisition of data; Cohen JG, White M, Cruz A and Farias-Eisner R performed synthesis of data, developed tables and figures, and authored manuscript.
Correspondence to: Robin Farias-Eisner MD, PhD, Department of Obstetrics and Gynecology, University of California, Los Angeles Medical Center, Peter Morton Medical Building, 200 Medical Plaza, Suite 220, Los Angeles, CA 90095, United States. rfeisner@mednet.ucla.edu
Telephone: +1-310-2068914 Fax: +1-310-2062057
Received: January 17, 2014   Revised: March 12, 2014
Accepted: May 14, 2014
Published online: August 26, 2014

Abstract

Ovarian cancer is a lethal gynecologic malignancy with greater than 70% of women presenting with advanced stage disease. Despite new treatments, long term outcomes have not significantly changed in the past 30 years with the five-year overall survival remaining between 20% and 40% for stage Ⅲ and Ⅳ disease. In contrast patients with stage Ⅰ disease have a greater than 90% five-year overall survival. Detection of ovarian cancer at an early stage would likely have significant impact on mortality rate. Screening biomarkers discovered at the bench have not translated to success in clinical trials. Existing screening modalities have not demonstrated survival benefit in completed prospective trials. Advances in high throughput screening are making it possible to evaluate the development of ovarian cancer in ways never before imagined. Data in the form of human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. With the correct pooling of resources including prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery, we are poised to make progress in ovarian cancer screening. This review will summarize current biomarkers, imaging, and multimodality screening strategies in the context of emerging technologies.

Core tip: Ovarian cancer is a lethal gynecologic malignancy with five-year survival of only 20% to 40% for advanced stage disease. Detection at an early stage would likely have significant impact on mortality rate. Advances in high throughput screening with the human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. To make progress in screening we need greater emphasis on prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery.

Cohen JG, White M, Cruz A, Farias-Eisner R. In 2014, can we do better than CA125 in the early detection of ovarian cancer? *World J Biol Chem* 2014; 5(3): 286-300 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/286.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.286

INTRODUCTION

Ovarian cancer is a lethal gynecologic malignancy with greater than 70% of women presenting with advanced stage disease[1]. Worldwide it is estimated there are 225500 new cases of ovarian cancer and 140200 deaths every

year including 14030 deaths in the United States alone[2,3]. Primary treatment for advanced stage disease involves both surgery and chemotherapy.

Despite new treatments, long term outcomes have not significantly changed in the past 30 years with the five-year overall survival remaining between 30 and 40%[3]. Greater than 60% of advanced stage patients will develop recurrent disease[4]. Patients with advanced stage disease have a five-year overall survival between 20% and 40%, in stark contrast to the greater than 90% five-year overall survival of patients identified and treated with stage I disease[5-7].

Given the poor prognosis for patients with advanced stage disease, effective screening modalities are needed to identify patients with early stage disease. The majority of women with early stage disease are asymptomatic, and unfortunately when they do present for diagnosis, three quarters are found to have regional or distant metastases[7]. Preliminary evaluations of screening with serum markers, pelvic ultrasounds, and multimodality strategies have demonstrated potential benefit in the earlier identification of ovarian cancer[8,9]. With these encouraging results, prospective screening trials have been undertaken as the scientific community continues to increase the number of potential biomarkers and imaging tests which might assist with identification of early stage ovarian cancer in asymptomatic women.

In the United States a woman's lifetime risk of developing ovarian cancer is 1 in 70 and the prevalence of ovarian cancer in postmenopausal women over the age of 50 is 1 in 2500[10]. To minimize harms while identifying women at risk, a positive predictive value (PPV) of 10% is needed, requiring a sensitivity of greater than 75% and specificity of 99.6% to identify one case of ovarian cancer for every ten operations[10]. It is unlikely that one biomarker test will meet this criteria given the high specificity needed[11].

The ideal biomarker or panel of biomarkers is obtained through noninvasive means such as a bodily fluid: blood, saliva, urine, and cervical mucous are possibilities[12]. Advances in high throughput screening have made it possible to evaluate the human genome with the hope of better understanding genetic and epigenetic changes associated with the development of ovarian cancer. Enormous amounts of data in the form of human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. The Cancer Genome Atlas (TCGA) recently completed a comprehensive genomic and epigenomic evaluation of over three hundred high-grade serous ovarian cancer samples with microarray analyses and massively parallel sequencing coupled with hybrid affinity capture[13].

Ovarian cancer represents a very diverse group of tumors. Scientific endeavors such as TCGA are now making it possible to better delineate characteristics of various subtypes. The continued quest for a strategy that meets the need to identify asymptomatic women in the general population may depend on the ability to parse out the origins of ovarian cancer. The epithelial category, which accounts for 90% of all ovarian cancers, consists of the following subtypes: (1) serous (50%); (2) endometrioid (10%-25%); (3) mucinous (5%-10%); (4) clear cell (4%-5%); (5) undifferentiated carcinomas (5%); and (6) transitional cells (rare)[14]. These ovarian tumors are likely distinct diseases with different cells of origin and driver mutations, united under one term due to their predilection for dissemination to the ovary and related pelvic organs[15]. A pitfall of the past may be failure to develop screening strategies based on differences among these tumors. It is not yet clear if one screening strategy or separate approaches will be needed to identify patients with these tumor types at an early stage of disease. This review will summarize current biomarkers, imaging, and multimodality screening strategies in the context of emerging technologies.

BIOMARKERS AND EXISTING ALGORITHMS

Initially described by Bast *et al*[16] in 1981, cancer antigen 125 (CA125) was recognized by the murine monoclonal antibody OC-125 as an antigenic determinant on a high molecular-weight glycoprotein. It is the most widely studied biomarker in ovarian cancer screening. Measurement of CA125 can be performed with different commercial assays resulting in a certain degree of variation. The majority of assays appear to be both clinically reliable and correlative, nonetheless, new quantitative methods including mass spectrometry are under investigation[17,18]. As part of its development, CA125 underwent molecular cloning and was found to have characteristics of mucin, receiving the name MUC16[19].

In adults, CA125 is expressed in tissues derived from coelomic epithelium (mesothelial cells of the peritoneum, pleura, and pericardium) and Mullerian (tubal, endometrial, and endocervical) epithelia, as well as epithelia of the pancreas, colon, gall bladder, lung, kidney, and stomach[20,21]. CA125 can be elevated in a number of conditions unrelated to ovarian cancer, resulting in decreased specificity and PPV. Diverticulitis, endometriosis, liver cirrhosis, uterine fibroids, menstruation, pregnancy, benign ovarian neoplasms, and other malignancies (pancreatic, bladder, breast, liver, lung) can all result in an elevated CA125[11].

When values below 35 U/L are designated as normal, CA125 is elevated in 80% of epithelial ovarian cancers[22]. CA125 is elevated in approximately 50%-60% of stage I epithelial ovarian cancers and 75%-90% of patients with advanced stage disease[21,23]. The sensitivity of CA125 to identify early stage disease is limited as a screening tool. With evaluation of 22000 volunteers and over 50000 serum CA125 samples with a median follow up of 8.6 years, Jacobs *et al*[24] demonstrated CA125 levels in women without ovarian cancer remained static or decreased over time while levels associated with malignancy tended to increase[8,24]. Based on these findings, the Risk

of Ovarian Cancer Algorithm (ROCA) was developed incorporating an individual's age specific incidence of ovarian cancer and CA125 profile to triage women into various risk categories[8]. ROCA increased the sensitivity of CA125 from 62% to 86% for detection of preclinical ovarian cancer while maintaining a specificity of 98%[25]. A randomized control trial to evaluate ROCA consisted of 13582 postmenopausal women over the age of 50 and demonstrated a specificity of 99.8% (CI: 99.7% to 99.9%) and positive predictive value of 19% (CI: 4.1% to 45.6%)[26]. This model has been incorporated into various multimodality screening strategies in an attempt to optimize sensitivity, specificity, and positive predictive value.

The quest for other biomarker candidates has continued because a single CA125 value at a given time point will not reach a specificity of 99.6%, and approximately 20% of ovarian cancers may not express this antigen. Human epididymis protein (HE4), found primarily in the epithelia of normal genital tissues and made up of two whey acidic protein (WAP) domains and a four disulfide core, is elevated in epithelial ovarian cancer[27,28]. HE4 is overexpressed in 50% of clear cell, 93% of serous, and 100% of endometrioid cancers but is not overexpressed in mucinous tumors[28]. Identified initially as an mRNA transcript specific to the distal epididymal tissue, genomic advances with microarray gene expression profiling demonstrated HE4 is highly-expressed in ovarian cancer[29,30]. HE4 has greater specificity in the premenopausal age group than CA125 given it does not appear to be expressed at high levels in the setting of benign conditions such as endometriomas[31-33]. HE4 represents a victory for genomic strategies in the search for potentially effective biomarkers with microarray gene expression[34].

In a systemic review of women with suspected gynecologic disease HE4 demonstrated a higher specificity (93% *vs* 78%) and similar sensitivity (79%) to CA125 when distinguishing benign disease from ovarian cancer[35]. Studies have demonstrated a potential benefit in combining HE4 and CA125 when quantifying risk potential malignancy in the evaluation of a pelvic mass[36,37]. Even with new technology, it is unlikely that an individual biomarker will reach a specificity of 99.6%, positive predictive value of 10%, and sensitivity greater than 75% when screening an asymptomatic general population.

In efforts to further triage women in the detection of ovarian cancer, progress has been made in the development of algorithms to delineate malignancy in the setting of an adnexal mass. Woman appropriately referred to a gynecologic oncologist have better outcomes including survival, demonstrating the potential importance of these triage tests[38,39]. The Risk of Malignancy Index (RMI), developed by Jacobs *et al*[40] in 1990, is a formula which incorporates a woman's CA125 level, ultrasound score, and menopausal status to determine her likelihood of malignancy in the setting of an adnexal mass. Since that time two other algorithms have been developed for assessment of malignancy risk in women with adnexal masses: the Risk of Malignancy Algorithm (ROMA) and

the OVA1 test[41,42]. The ROMA algorithm is based on serum levels of HE4 and CA125 with menopausal status[41]. OVA1, with the exception of CA125, is made up of biomarkers discovered through mass spectrometry: β-2 microglobulin, transferrin, transthyretin, and apolipoprotein[42,43]. Various studies have been published evaluating the effectiveness of RMI, ROMA, and OVA1, as well as other strategies to help delineate the likelihood of malignancy in the setting of a pelvic mass. Table 1 provides a summary of various algorithms and assays used to predict likelihood of malignancy.

OVA1 and ROMA each have benefits and disadvantages. Prospective multi-institutional trials and cost-benefit analysis are needed before definitive conclusions can be drawn regarding these tests[34]. Table 2 lists sensitivities and specificities for various modalities in the setting of a pelvic mass. Based on available data, OVA1 and ROMA likely have similar sensitivities, but ROMA appears to have greater specificity (75% *vs* 43%) which may impact cost-effectiveness and referral patterns from general gynecologists reticent to lose patients with benign masses to gynecologic oncologists[53]. OVA1, based largely on mass spectrometry with proteomics, and ROMA, made possible by the incorporation of a microarray gene-expression based discovery in HE4, represent hopeful advancements in the ability to identify women with malignancy earlier than had been in the past. These are not screening tests for the general population, but represent potential tools to further triage of women to the appropriate providers once the decision for surgical intervention has been made.

Various other biomarkers and biomarker panels are currently under development for both the prediction of malignancy in the setting of a pelvic mass and in asymptomatic women. Table 3 lists various single biomarker and multi-biomarker panels with sensitivities and specificities for ovarian cancer detection. An important consideration with all of these tests is the ultimate need to demonstrate benefit for patients through reduction in morbidity and mortality while minimizing harm. The advancements of technology combined with our exponentially growing knowledge of the human "-omes" have outpaced our ability to reliably test these discoveries through clinical settings in a timely fashion.

AREAS OF GROWTH IN BIOMARKER DISCOVERY

High throughput technology in conjunction with TCGA has now made it possible to combine multiplex assays with data from the proteome, genome, metabolome, and transcriptome. Within proteomics, biomarker panels have been developed in an attempt to increase sensitivity for ovarian cancer detection due to the heterogeneous make up of subtypes (Table 3). Biomarker discovery in proteomics is usually based on two-dimensional gel electrophoresis, mass spectrometry (MS), and/or protein microarrays in combination with bioinformatics analysis[65].

Table 1 Screening algorithms and commercially available assays

Algorithm or assay *(Screening population)*	How it works
ROCA *(asymptomatic general population)*	1 Compares a woman's longitudinal CA-125 pattern to the change-point CA-125 profile seen in women with ovarian cancer and the flat CA-125 profiles seen in women without ovarian cancer[1] 2 Based on the ROCA result, women get triaged into one of three groups[1]: (1) Low Risk: continue annual CA-125 testing (2) Intermediate Risk: repeat CA-125 test 3 mo later (3) High Risk: receive TVS and referral to a gynecologic oncologist 3 After each additional CA-125 value, ROCA is recalculated and a new recommendation is made[1]
ROMA *(known pelvic mass)*	1 Uses both HE-4 and CA-125 test levels to evaluate patients as low or high risk for ovarian cancer[8] 2 A predictive index (PI) is calculated using different equations for pre-menopausal and post-menopausal women[8] 3 The PI is then inserted into the ROMA algorithm to predict the probability of ovarian cancer[8]
RMI *(known pelvic mass)*	Uses menopausal status, ultrasound findings, and serum CA-125 levels to determine malignancy risk[40]
OVA1 *(known pelvic mass)*	1 A multivariate index assay that incorporates CA-125, transferrin, transthyretin (prealbumin), apolipoprotein A1, and beta-2-microglobulin[41] 2 An algorithm is used to generate an ovarian malignancy risk score between 0 and 10[41] 3 OVA1 scores greater than or equal to 5.0 (premenopausal) or 4.4 (postmenopausal) result in high risk stratification and referral to a gynecologic oncologist[41]
LR-1 *(known pelvic mass)*	1 An ultrasound-based prediction model 2 Twelve variables are used to calculate a probability of malignancy[88]: (1) personal history of ovarian cancer (2) current hormonal therapy (3) age of the patient (4) maximum diameter of the lesion (5) pain during examination (6) ascites (7) blood flow within a solid papillary projection (8) a purely solid tumor (9) maximum diameter of the solid component (10) irregular internal cyst walls (11) acoustic shadows (12) color score
LR-2 *(known pelvic mass)*	1 An ultrasound-based prediction model 2 Uses six variables to calculate a probability of malignancy[90]: (1) patient's age (2) presence of ascites (3) presence of blood flow within a papillary projection (4) maximal diameter of solid components (5) irregular internal cyst walls (6) presence of acoustic shadows

ROCA: Risk of ovarian cancer algorithm; ROMA: Risk of ovarian malignancy algorithm; RMI: Risk of malignancy index; OVA1: Vermillion Inc. OVA1 ® blood test; LR-1: International ovarian tumor analysis logistic regression model 1; LR-2: International ovarian tumor analysis logistic regression model 2; ROCA: Risk of ovarian cancer algorithm; ROMA: Risk of Malignancy Algorithm.

MS with matrix assisted laser desorption and ionization time of flight (MALDI-TOF) and surface-enhanced laser desorption and ionization time of flight (SELDI-TOF) allow for the entire protein complement of a patient sample to be evaluated in rapid high throughput fashion[12,64]. Protein microarrays can be used to profile the proteome of cell populations using antigen-antibody interactions[66]. Protein microarrays are made up of two major classes: (1) forward-phase arrays (FPA) with antibodies arrayed and probed with cell lysates; and (2) reverse-phase arrays (RPA) with cell lysates arrayed and probed with antibodies[65].

Unfortunately, proteomics has not resulted in the major breakthroughs previously anticipated. An important consideration here is the biological samples used when identifying potential biomarkers. Various studies have demonstrated protein biomarkers perform very differently in the detection of ovarian cancer when analyzed in prospectively collected samples from asymptomatic patients[67,68]. Future proteomic discovery may best focus on samples from patients prospectively followed until diagnosis in larger population based trials. Incorporation of methods aimed at depletion of abundant serum proteins such as acute phase reactants, and the use of multiplex bead-based immunossays may allow for identification of low abundance or low concentration proteins not previously identified[12].

MS continues to serve as an important tool to explore the thousands of proteins relevant to ovarian cancer and has now been extended to use in glycomics, metabolomics, MALDI-MS imaging, and autoantibody signatures for biomarker discovery[69]. Glycosylation or the addition of carbohydrates to nascent proteins is a common post-translational modification that is potentially altered in a

Table 2 Specificity and sensitivity results of various screening strategies in the setting of a pelvic mass

Algorithm or assay	Ref.	Sensitivity (%)	Specificity (%)
ROMA	Karlsen *et al*[44]	94.4	76.5
	Moore *et al*[45]	94.3	75
	Sandri *et al*[46]	91.2	75
		89.3	81.7
	Van Gorp *et al*[89]	84.7	76.8
	Sandri *et al*[46]	84.4	90
	Chan *et al*[47]	89.2	87.3
	Kaijser *et al*[90]	84	80
RMI	Karlsen *et al*[44]	94.4	81.5
	Håkansson *et al*[48]	92	82
	Moore *et al*[45]	84.6	75
	Van den Akker[49]	81	85
	Van Gorp *et al*[89]	76	92.4
OVA1	Bristow *et al*[50]	92.4	53.5
	Longoria *et al*[52]	92.2	49.4
OVA1 +	Bristow *et al*[50]	95.7	50.7
Clinical assessment	Longoria *et al*[52]	95.3	44.2
LR-1	Kaijser *et al*[88]	93	77
LR-2	Nunes *et al*[51]	97	69
	Kaijser *et al*[88]	92	75
	Kaijser *et al*[90]	93.8	81.9
TVS	van Nagell *et al*[8]	86.4	98.8

ROMA: Risk of ovarian malignancy algorithm; RMI: Risk of malignancy index; OVA1: Vermillion Inc. OVA1® blood test; LR-1: International ovarian tumor analysis logistic regression model 1; LR-2: International ovarian tumor analysis logistic regression model 2; TVS: Transvaginal ultrasonography.

malignant state[69-71]. There is evidence to indicate various histologic subtypes of ovarian cancer exhibit different glycoproteins[72]. This is encouraging given the significant heterogeneity of ovarian cancer. The differences seen in glycomics may assist in screening algorithms which can be developed with this heterogeneity in mind.

Evaluation of the metabolome through MS has demonstrated differences in metabolites in patients with and without epithelial ovarian cancer[73,74]. Existing concerns with the study of metabolites include the significant variation in metabolic response and extensive biotransformation from the site of malignancy to fluids such as serum or plasma[75]. Study of the peptidome within the low-molecular weight proteome in ovarian cancer has been limited by the potential loss of peptides bound to carrier proteins during sample processing, although attempts have been made to mitigate this with isolation and enrichment of carrier proteins prior to MS evaluation[69,76]. Ovarian cancer diagnoses may also be aided with the uses of anti-tumor autoantibody signatures and MALDI-MS imaging; however, these areas of research are preliminary with MS[77,78].

Separate from the use of MS, there is a growing role for microRNAs in the development of ovarian cancer biomarkers[79]. MicroRNAs are a class of small noncoding RNAs which impact gene expression by targeting multiple messenger RNAs and triggering translation repression and/or RNA degradation[80]. Aberrant expression of microRNAs in ovarian cancer indicate they may act as a novel class of oncogenes or tumor-suppressor genes[79]. Five microRNAs (miR-200a, miR-100, miR-141, miR-200b, and miR-200c) have been found to be consistently differentially regulated in epithelial ovarian cancer and may assist in the development of biomarkers[81]. The future is promising with these techniques; however, validation strategies and appropriate patient samples are vital to improving success in clinical testing. No individual biomarker or biomarker panel has been developed which meets the sensitivity, specificity, and PPV criteria desired for screening in a general population.

IMAGING

There has been an immense effort placed in the evaluation of screening with radiologic technology. A systematic approach to the diagnosis of ovarian tumors with imaging is necessary given the majority of women have benign lesions, and unnecessary interventions should be avoided without placing patients at risks for advanced stage disease[82]. Available imaging modalities include ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET). Pelvic ultrasound has been the most studied imaging modality in ovarian cancer screening. Of 48053 postmenopausal women in the ultrasound group of the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), 4367 asymptomatic women (9.1%CI: 8.8%-9.3%) had abnormal adnexal morphology with an overall absolute risk of epithelial ovarian cancer of 1.08% (95%CI: 0.79%-1.43%) and a 1 in 22 risk of epithelial ovarian cancer if the abnormal findings included solid elements[83].

In a single arm prospective screening cohort, the University of Kentucky Ovarian Cancer Screening Trial,

Table 3 Results of serum marker panels for the detection of ovarian cancer

Serum marker(s)	Ref.	Sensitivity (%)	Specificity (%)
CA-125	[1]Karlsen et al[44]	91.7	75
	[1]Chan et al[47]	90.8	67.2
	[1]Leung et al[123]	89	90
	[1]Sandri et al[46]	84.4	80
	[1]Montagnana et al[54]	83	100
	[1]Sandri et al[46]	73.1	90
	Yang et al[55]	62.5	80
	Havrilesky et al[56]	45.9-58.5	98.2
	[1]Moore et al[37]	43.3	95
	Jacob et al[57]	12.5	90.1-93.9
HE-4	[1]Montagnana et al[54]	98	100
	Yang et al[55]	96.2	83.8
	[1]Karlsen et al[44]	91.3	75
	[1]Sandri et al[46]	83.1	90
	Havrilesky et al[56]	82.7-92.5	86.3
	[1]Moore et al[37]	72.9	95
	Jacob et al[57]	62.5	81.8-85.9
	[1]Chan et al[47]	56.9	96.9
CA-125, HE-4	[1]Moore et al[37]	76.4	95
	[1]Moore et al[41]	88.7	74.7
CA 125, leptin, PRL, OPN, IGFII, MIF	Visintin et al[58]	95.3	99.4
CA 125, CRP, SAA, IL-6, IL-8	Edgell et al[59]	94.1	91.3
CA-125, apoA-I, TTR, TF	Su et al[60]	89-97	91-99
CA 125, HE4, CEA, VCAM-1	Yurkovetsky et al[61]	86–93	98
CA 125, ApoA1, TTR	Kim et al[62]	93.9	95
	Zhang et al[42]	74	97
CA 125, CA 19-9, EGFR, CRP, myoglobin, ApoA1, ApoCIII, MIP-1a, IL-6, IL-18, tenascin C	[1]Amonkar et al[63]	91.3	88.5
CA-125, OVX1[r], LASA,CA15-3, CA72-4	Nossov et al[11]	90.6	93.2
CA 125, CA 72-4, CA 15-3, M-CSF	[1]Skates et al[64]	70	98
LPA	Nossov et al[11]	90-100	90
FOLR1	[1]Leung et al[123]	62	90
M-CSF	Nossov et al[11]	61-68	93
SMRP	[1]Moore et al[37]	53.7	95

[1]Study involved patients presenting with a pelvic mass; CA: Cancer antigen; HE-4: Human epididymis protein 4; PRL: Prolactin; OPN: Osteopontin; IGFII: Insulin-like growth factor II; MIF: Macrophage inhibitory factor; CRP: C-reactive protein; SAA: Serum amyloid A; IL: Interleukin; apoA-I: Apolipoprotein A-I; TTR: Transthyretin; TF: Transferrin; CEA: Carcinoembryonic antigen; VCAM-1: Vascular cell adhesion protein 1; EGFR: Epidermal growth factor receptor; ApoCIII: Apolipoprotein CIII; MIP-1a: Macrophage inflammatory protein-1alph; OVX1: Mouse antibody generated by immunizing mice with antigenic preparations from multiple OC cell lines[21]; LASA: Lipid-associated sialic acid; M-CSF: Macrophage colony-stimulating factor; LPA: Lipoprotein A; FOLR1: Folate receptor 1; SMRP: Soluble mesothelin-related peptide.

asymptomatic women 25 years or older with a documented family history of ovarian cancer and asymptomatic women 50 years or older were screened with annual transvaginal ultrasound[84]. Serial ultrasonography in this trial demonstrated many ovarian abnormalities resolve in follow up: 63.2% of women with an initially abnormal ultrasound were found to have resolution on subsequent imaging[85]. Observation with serial imaging may help improve positive predictive value and decrease false positive results in screening trials[85]. Of 37293 women who underwent annual screening, the five-year disease-free survival rate for women with ovarian cancer in the screening group, including those who developed ovarian cancer within one year of a normal ultrasound (false negative), was 74.8% ± 6.6%. In contrast, a group of unscreened women with ovarian cancer treated at the same institution with the same surgical and chemotherapeutic protocols had a five-year disease free survival of 53.7% ± 2.3%, P-value < 0.01[86]. Ultrasound screening does not impact disease-free survival by itself. Ultimately, the goal of ultrasound screening is to identify patients with early

stage disease who can be treated before the malignancy becomes advanced. While the results from this study are encouraging, the mortality benefit may have been impacted by a healthy volunteer effect and lead time detection rather than impact on the natural history of ovarian cancer[87].

Although screening in an asymptomatic population ultimately provides the best opportunity to improve survival in women with ovarian cancer, there has been progress made in the development of imaging algorithms designed for those women with a known adnexal mass. The International Ovarian Tumor Analysis (IOTA) group has developed various approaches to characterize adnexal masses as malignant or benign with ultrasound guidelines. These approaches can be divided into two strategies: the first consisting of risk prediction with two logistic regression models (LR1 and LR2) based on demographic and ultrasound variables (Table 1), and the second based on simple ultrasound features that are descriptors of benign or malignant masses[88]. In women with a pelvic mass the sensitivity and specificity of ROMA and the RMI were

compared to subjective assessment by skilled ultrasonographers in a prospective cohort study of women[89]. The sensitivity of ROMA, RMI, and expert ultrasonographers were 84.7% (77.9% to 90.0%), 76.0% (68.4% to 82.6%), and 96.7% (92.4% to 98.9%) respectively, and the specificity was 76.8% (70.7% to 82.2%), 92.4% (88.1% to 95.5%), and 90.2% (85.5% to 93.7%) respectively[89]. Generalizability of these results may not be possible based on its location, the cohort, and the ultrasonographers used. The study took place at one single tertiary care center in Europe with experienced ultrasonographers and a high prevalence of malignant disease in the cohort[89].

In a different study, a cross-sectional cohort of 360 patients with adnexal masses undergoing surgery was retrospectively evaluated with ROMA and LR2[90]. This study demonstrated decreased sensitivity and specificity for ROMA *vs* LR2 in both premenopausal and postmenopausal patients, with overall sensitivity 84.0% *vs* 93.8%, and specificity 80% *vs* 81.9%, respectively[90]. While this result indicates LR2 may be a more effective screening test in the setting of adnexal mass, prospective randomized control trials are needed before conclusions can be made regarding the use of algorithms which include biomarkers such as HE4 and CA125 (ROMA) *vs* ultrasound-based prediction models such as LR2. Table 2 lists sensitivities and specificities for various modalities in the setting of a pelvic mass.

Currently no prospective randomized studies support the use of imaging as a single strategy in screening for ovarian cancer. At this time, given ultrasound is relatively inexpensive, available widely, and can provide tissue specific information with a presumptively risk-free technology; it is the method of choice for initial evaluation of an adnexal mass and estimating risk of malignancy[82]. In asymptomatic postmenopausal women, the ultrasound screening arm results of the UKCTOCS expected in 2015 will help elucidate the role of ultrasound in population-based screening strategies. At this time is it unlikely ultrasound will significantly reduce mortality in primary screening, but it may be extremely important in reducing false positive rates in multimodality screening[91]. Existing ultrasound-based strategies evaluating the likelihood of malignancy in the setting of a known adnexal mass are based on those who have already been scheduled for surgery. Comparative prospective studies are needed to determine efficacy and effect on survival in women who have surgery based on prediction models using proposed ultrasound-based strategies with and without biomarkers such as CA125 and HE4.

MULTIMODALITY SCREENING

The promising results of imaging in population-based screening for ovarian cancer have led to large scale multimodality strategies. Prior prospective studies demonstrating CA125 and ultrasound were feasible screening modalities have given way to prospective randomized multimodality screening trials involving ultrasound, serum biomarkers, and risk calculations using patient demographics[92,93]. The Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) is a multicenter randomized control trial of 78216 asymptomatic women aged 55 to 74 years who underwent multimodality screening or usual care between November 1993 and July 2001 with management of positive screens left to the discretion of the patient's physician[94]. Multimodality screening consisted of annual testing for three years with transvaginal ultrasound and serum CA125 with a cutoff of 35 U/mL followed by CA125 alone for an additional two years[94]. After four rounds of screening, the PPV and cancer yield per 10000 women screened in the multimodality screening arm remained similar across screening rounds at 1.0% to 1.3% and 4.7 to 6.2 cancers respectively with the overall ratio of surgeries to screen-detected cancers 19.5 to 1[95]. After a median follow up of 12.4 years (25th% to 75th%, 10.9 to 13.0), no mortality benefit was found with combination transvaginal ultrasound and CA125 using an absolute cutoff: 118 deaths due to ovarian cancer (3.1 per 10000 person-years) in the intervention group and 100 deaths (2.6 per 10000 person-years) in the usual care group (mortality rate ratio, 1.18; 95%CI: 0.82-1.71)[96].

The Japanese Shizuoka Cohort Study of Ovarian Cancer Screening is a randomized control trial of 82487 low risk postmenopausal women between 1985 and 1999 with the intervention arm consisting of annual ultrasound and CA125 with a cutoff value[97]. The strategy achieved a sensitivity of 77.1% and specificity of 99.9% with a nonsignificant difference in the proportion of stage I ovarian cancers identified, 63% in the screened group *vs* 38% in the control group, *P*-value = 0.2285[97]. Mortality results from this trial have not yet been published and, as such, conclusions cannot be drawn from this trial regarding the benefit of screening in an asymptomatic population.

The UKCTOCS is a randomized prospective multiarm ovarian cancer screening study in the United Kingdom. This trial, made up of 202638 post-menopausal women aged 50 to 74, randomizes women in a 2:1:1 format to three arms: (1) control; (2) annual screening with ultrasound; and (3) a multimodality strategy that takes advantage of ROCA to triage women to various substrategies[98]. These substrategies include transvaginal ultrasound and/or repeat CA125 at defined time points[98]. In the prevalence screen of the UKCTOCS, ultrasonography alone was compared to multimodality screening (ROCA as a primary test followed by transvaginal ultrasound as a secondary test or repeat CA125 if indicated). With regard to primary invasive epithelial and tubal cancers, the multimodality screening arm demonstrated a higher specificity compared to the ultrasonography arm (99.8% *vs* 98.2%), *P*-value < 0.001, while the difference in sensitivity was not statistically significant (89.4% *vs* 84.9%), *P*-value = 0.564[98].

A single-arm prospective cohort study of 4051 average-risk postmenopausal women in the United States was performed over 11 years using a two-stage ovarian cancer

screening strategy (CA125 interpreted through ROCA with subsequent repeat CA125 or transvaginal ultrasound as indicated) with a PPV of 40% for invasive ovarian cancer and specificity of 99.9% (95%CI: 99.7%-100%)[1]. The results from both the UKCTOCS and two-stage strategy in the United States indicate the use of ROCA to interpret CA125 may be effective in triaging women to subsequent follow-up categories that impact both screening outcomes.

When comparing the UKCTOCS prevalence screen results to the PLCO trial results, both the UKCTOCS multimodality arm (89.4% *vs* 51.7%) and the ultrasound arm (75.0% *vs* 67.4%) had higher sensitivities[99]. When CA125 values were retrospectively evaluated with ROCA within the PLCO data set no mortality benefit was seen; best-case and stage-shift scenarios resulted in 25 and 19 deaths prevented with ROCA for relative risks of 0.90 (95%CI: 0.69-1.17) and 0.95 (95%CI: 0.74-1.23), respectively[100]. In addition to the use of absolute cutoff value for CA125, other concerns have been raised regarding the PCLO trial design including leaving management of positive screens to the discretion of the treating physician and 40.6% of ovarian cancer diagnosis took place after the screening ended[101]. Use of an individualized algorithm that tracks a patient over time will likely provide the best combination of sensitivity, specificity, and PPV. For this reason, the UKCTOCS and its incorporation of ROCA in the multimodality screening arm, represents the best opportunity yet to identify a potential screening strategy. The results from the final mortality analysis in the UKCTOCS will be reported in 2015 and provide significant insight into whether population-based screening in asymptomatic women is possible with currently available imaging and biomarkers.

SYMPTOM-BASED SCREENING

Screening efforts in ovarian cancer have largely focused on asymptomatic women in the general population or women with known adnexal masses requiring further dichotomization for treatment purposes. Women with ovarian cancer do have physical symptoms such as abdominal pain, bloating, and bowel irregularity that may serve as a potential trigger for diagnosis. In a case-control study comparing woman with ovarian cancer to age and race matched controls, more than 90% of cases reported at least one symptom and symptoms were cited as the most common reason for the doctor visit leading to diagnosis (74%)[102]. Two feasibility studies have been performed demonstrating symptom-based screening in women is possible[103,104]. A symptom index was created with a sensitivity of 56.7% for early stage disease and 79.5% for advanced stage disease, and a specificity of 90% for women greater than 50 years of age and 86.7% for women less than 50 years of age[105]. Based on patient interviews performed with 812 women with ovarian cancer and 1313 population-based controls, the symptom index and symptoms established in consensus recommendations had a

PPV of 0.6%-1.1% overall and less than 0.5% for early-stage disease[106]. The identification of specific symptoms associated with ovarian cancer has value, but recognition of symptoms alone will not significantly improve overall survival from ovarian cancer[107]. A cross sectional study of 160 women evaluated with use of this symptom index found that the addition of CA125, HE4, or the ROMA to a positive symptom index increased PPV when determining malignancy *vs* benign process in patients with a known adnexal mass[108]. At this time, given no effective screening tool has been proven in a prospective model, physicians should continue to discuss potential symptoms with their patients in an effort to increase self-awareness regarding warning signs for ovarian cancer.

SCREENING IN HIGH RISK PATIENTS

Familial genetic predisposition makes up approximately 10% of ovarian cancers with germline mutations in *BRCA1*/*BRCA2* and mismatch repair (*MMR*) genes in Lynch syndrome being the most common[109]. Women with *BRCA1* and *BRCA2* mutations have a cumulative lifetime risk of ovarian cancer of 40%-50% and 20%-30% respectively, while the DNA MMR genes, including those that predispose to Lynch syndrome, result in a cumulative lifetime risk of ovarian cancer ranging from 6.7% to 12%[110]. As seen with improved survival in BRCA-associated ovarian cancers, inherited ovarian cancers may have biological differences which allow treatment at time of screen detection to have significant benefit[111].

Currently, no prospective studies exist which demonstrate a mortality benefit by screening high risk asymptomatic patients. The United Kingdom Familial Ovarian Cancer Screening Study (FOCSS) has recently completed a phase 1 trial in which 3563 women at greater than a 10% risk of ovarian or fallopian tube cancer were screened with annual transvaginal ultrasound and CA125 for a mean of 3.2 years[109]. Sensitivity for detection of incident ovarian and fallopian tube cancers at one year after last annual screen was 81.3% (95%CI: 54.3%-96.0%) if occult cancers were classified as false negatives, and the PPV was 25.5% (95%CI: 14.3%-40.0%) with only four women undergoing surgery for each case of detected cancer[109]. As part of phase Ⅱ of the FOCSS, screening frequency will increase to every four months, ROCA will be incorporated into the decision tree, and the threshold and work-up for repeat tests will be per protocol. The Gynecologic Oncology Group (GOG) and Cancer Genetics Network have recently completed GOG 199, a prospective study screening women at high risk of ovarian cancer with the use of ROCA and transvaginal ultrasound[112].

For women with Hereditary Breast/Ovarian Cancer Syndrome who have not undergone risk reducing bilateral salpingo-oophorectomy, the National Comprehensive Cancer Network recommends screening with transvaginal ultrasound and CA125 every 6 mo starting at age 30 or 5 to 10 years prior to the earliest age at diagnosis of ovarian can-
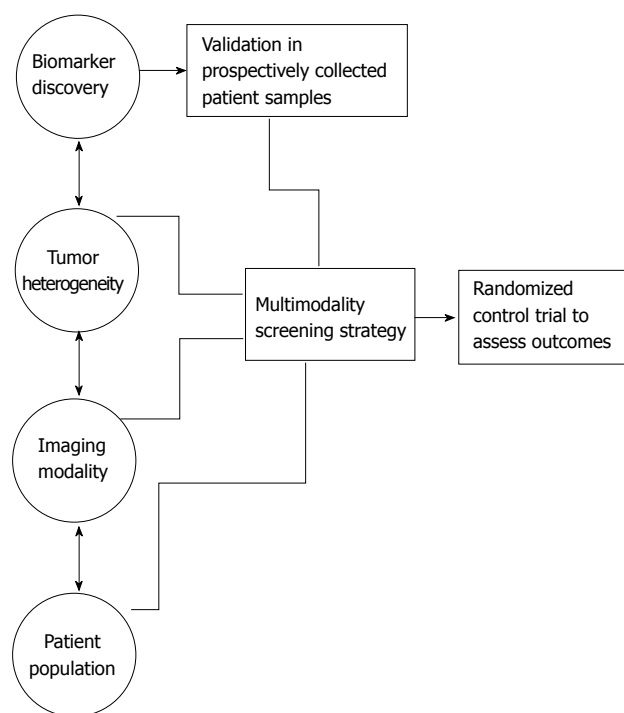
Figure 1 Biomarker candidates must be tested in patient samples collected prior to the onset of physical symptoms in ovarian cancer screening strategies.

cer in relatives[113]. Given the potential biologic differences associated with high risk patients, screening asymptomatic women within this population may have greater benefit than in the general population. The FOCSS phase II results and GOG 199 will provide evidence regarding potential screening benefits and assist with strategy optimization.

FUTURE CONSIDERATIONS

Despite the technological advances which have been made, our current approach to screening strategies in ovarian cancer has inherent difficulties which need to be overcome. Directly impacting our ability to screen asymptomatic women for ovarian cancer is the evolving reclassification of this heterogeneous group of tumors. Results from the recently completed study of serous ovarian cancer through TCGA demonstrate significant genomic heterogeneity even within one subtype of epithelial ovarian cancer, high grade serous carcinoma[13].

As in colorectal cancer and cervical cancer, identification of a precursor lesion or lesions will improve our ability to screen for the disease. These precursor lesions are likely varied based on the subtype of ovarian cancer. With high grade serous carcinoma, a precursor lesion may develop in the fimbria of the fallopian tube (serous tubal intraepithelial carcinoma also known as a STIC) or in an ovarian cortical inclusion cyst during implantation of fimbrial epithelium on the denuded ovarian surface with ovulation[114]. Genetic evaluation links both clear cell and endometrioid carcinomas to precursor lesions within endometriosis[115]. A new model that considers both mor-

phologic and molecular characteristics separates epithelial ovarian tumors into two categories: type I tumors are low-grade serous, low-grade endometrioid, clear cell, and mucinous tumors which usually present as large cystic masses within one ovary, while type II tumors are composed of high-grade serous, high-grade endometrioid, malignant mixed mesodermal (carcinosarcoma), and undifferentiated carcinomas which commonly present as advanced stage disease[116].

A focus on identification of the origins of these groups of tumors will lead to more effective screening strategies in an asymptomatic population. For example, evaluating blood samples of patients found to have STICs at the time of prophylactic bilateral salpingo-oophorectomy may prove useful in identifying biomarkers for preclinical serous carcinoma[12]. Type I tumors tend to be genetically stable with mutations in various genes including *PTEN*, *BRAF*, β-*catenin* and *KRAS*, while type II tumors have a high level of genetic instability and commonly have a *TP53* mutation[117]. Biomarker panels and multimodality screening may achieve better sensitivity and specificity with screening strategies based on differences in both cell origin and genetics among these varied tumors.

In addition to the varied origin and molecular heterogeneity, the time course of ovarian cancer development still eludes understanding. The time required for development of invasive disease or progression from stage I to stage III remains unknown[118]. This information is likely specific to the various ovarian tumors, and improved categorization through molecular advances will better elucidate the time course of disease. For example, type I tumors appear to follow a developed path of transformation with stepwise progression from a benign lesion to a malignant tumor[119]. It has been proposed that ovarian cancer screening strategies should focus on type II tumors with the goal to identify low volume disease rather than early stage, as high grade serous carcinomas represent 75% of all ovarian cancers and result in the majority of deaths[113,120]. Low volume advanced stage disease may be more easily resectable at the time of tumor debulking, but advanced stage patients still have a worse prognosis than those patients who are treated with early stage disease. Identification of early stage disease will have the greatest benefit on mortality, and will require a shift from current approaches to incorporate advances made in the understanding of tumor heterogeneity in this malignancy.

Five phases of biomarker development have been previously proposed: (1) the preclinical exploratory phase; (2) the clinical assay and validation stage; (3) the retrospective longitudinal study; (4) prospective screening evaluation; and (5) randomized control trials[121]. The preclinical exploratory phase must take advantage of developments in high-throughput screening technologies to more effectively identify potential biomarkers among the thousands of candidate molecules. For example, a biomarker discovery platform which incorporates proteome and transcriptome comparisons of serum, tissue, ascites,

cancer cell lines, and animal models through mass spectrometry and microarray technology makes it possible to take advantage of these immense data sets[122]. Folate receptor 1 protein, developed through use of proteomics, transcriptomics, and bioinformatics, demonstrates the incorporation of various technologic platforms that make it possible to identify new biomarkers[123].

Further efforts must be devoted to the collection of appropriate patient specimens in prospective trials. Within ovarian cancer, the majority of biomarkers are evaluated with patient samples taken at the time of diagnosis, usually advanced stage disease. It is not surprising that biomarkers discovered in an advanced disease setting do not perform with the same sensitivity or specificity in a prospective trial in which the goal is diagnosis of early stage disease. Prospectively collected samples in asymptomatic women provide a better understanding of the ability of candidate biomarkers to detect cancer prior to physical symptoms[124]. The prospective specimen collection retrospective blinded evaluation (PRoBE) study design mandates samples are collected prospectively, stored in a similar fashion, and once outcome status is defined, used to validate biomarkers in a blinded fashion with randomly selected cases and controls[125]. Given the low prevalence of ovarian cancer in the general population, pooling of resources is necessary to make advances in biomarker discovery. The National Cancer Institute's Early Detection Research Network assists with development of prospective patient samples under the PRoBE study design[126]. PLCO samples have been used in this fashion to test potential biomarkers[67,68]. Development of a large scale collection of samples prospectively in asymptomatic women on a national or international level would provide the ability to validate biomarkers and predict lead time in the discovery of ovarian cancer prior to physical symptoms (Figure 1).

The final phase of biomarker design is a randomized control trial, with the goal of ovarian cancer screening to demonstrate a mortality benefit in the studied population. This mortality benefit must be considered in the context of the number needed to treat to reach such a benefit. A systematic review and meta-analysis of available screening trials involving asymptomatic women found no reduction in ovarian cancer-specific or all-cause mortality [relative risk (RR), 1.08; 95%CI: 0.84-1.38; and 1.0; 95%CI: 0.84-1.38 respectively][127]. While this analysis does not include results from the UKCTOCS which will not be available until 2015, it does demonstrate that prospective trials within current paradigms have failed to meet major goals.

In the PLCO trial 1080 women underwent surgery in the setting of false positive results and 163 (15%) experienced a complication[96]. Based on review of available clinical trials, 6% of women with false positive screening results experienced a severe complication while undergoing surgery[127]. These patients underwent potential harm without benefit. A mortality benefit is necessary to justify the potential harm associated with false positives. If the UKCTOCS and/or the Japanese cohort fail to show a benefit in mortality, this may be explained by lead time bias in which slow growing tumors are detected more commonly by screening than fast growing lethal serous epithelial ovarian cancers[91]. Type I tumors, which tend to be slow growing and more indolent than type II tumors, were detected twice as often as type II tumors in the ultrasound arm of the UKCTOCS (32 borderline or type I tumors *vs* 15 type II tumors) despite a higher prevalence of type II tumors in epithelial ovarian cancer[83]. If this same pattern is seen through the UKCTOCS in 2015, it is unlikely there will be a mortality benefit given the better prognosis associated with the majority of borderline and type I tumors compared to type II tumors.

While further prospective screening trials will take place, ovarian cancer screening in the asymptomatic general population results in potential harms without proven benefit at this time. Guidelines from the American College of Obstetrics and Gynecology, the Society of Gynecologic Oncologists, the United States Preventive Services Task Force, and the American Cancer Society do not recommend screening for ovarian cancer in asymptomatic low-risk women in the general population[128-130].

CONCLUSION

Ovarian cancer is deadly at advanced stage, and as the quest for an optimal screening strategy continues, it is apparent there are risks associated with false positives and invasive tests. When surveyed, 80% of women without risk factors or symptoms for ovarian cancer in the University of Kentucky cohort felt that they would definitely want to participate in ovarian cancer screening starting at age 50[131]. Various avenues continue to be investigated in ovarian cancer screening including imaging, protein profiles, specific symptoms, and combinations of these, as well as other modalities. An expanded and shared biobank of patient specimens collected before development of symptoms and advanced disease is needed. It is with these precious samples that high throughput technology and human "-omes" will have the most positive impact on identification of screening modalities. Emerging technology will allow science to evaluate biological data in ways never imagined. With the correct pooling of resources, including prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery, we are poised to make progress in ovarian cancer screening. If we are prudent in trial design and altruistic in the sharing of resources such as biological samples, identification of an effective screening modality for ovarian cancer is within our capabilities.

REFERENCES

1   **Lu KH**, Skates S, Hernandez MA, Bedi D, Bevers T, Leeds L, Moore R, Granai C, Harris S, Newland W, Adeyinka O, Gef-

fen J, Deavers MT, Sun CC, Horick N, Fritsche H, Bast RC. A 2-stage ovarian cancer screening strategy using the Risk of Ovarian Cancer Algorithm (ROCA) identifies early-stage incident cancers and demonstrates high positive predictive value. *Cancer* 2013; **119**: 3454-3461 [PMID: 23983047 DOI: 10.1002/cncr.28183]

2   **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

3   **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]

4   **Salani R**, Santillan A, Zahurak ML, Giuntoli RL, Gardner GJ, Armstrong DK, Bristow RE. Secondary cytoreductive surgery for localized, recurrent epithelial ovarian cancer: analysis of prognostic factors and survival outcome. *Cancer* 2007; **109**: 685-691 [PMID: 17219441 DOI: 10.1002/cncr.22447]

5   **Cannistra SA**. Cancer of the ovary. *N Engl J Med* 2004; **351**: 2519-2529 [PMID: 15590954 DOI: 10.1056/NEJMra041842]

6   **Howlader N**, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. SEER Cancer Statistics Review, 1975-2010. SEER online, cited 2013-04. Available from: URL: http: //seer.cancer. gov/csr/1975_2010/

7   **Holschneider CH**, Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin Surg Oncol* 2000; **19**: 3-10 [PMID: 10883018 DOI: 10.1002/1098-2388(200007/08)]

8   **Jacobs IJ**, Skates SJ, MacDonald N, Menon U, Rosenthal AN, Davies AP, Woolas R, Jeyarajah AR, Sibley K, Lowe DG, Oram DH. Screening for ovarian cancer: a pilot randomised controlled trial. *Lancet* 1999; **353**: 1207-1210 [PMID: 10217079 DOI: 10.1016/S0140-6736(98)10261-1]

9   **van Nagell JR**, DePriest PD, Reedy MB, Gallion HH, Ueland FR, Pavlik EJ, Kryscio RJ. The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer. *Gynecol Oncol* 2000; **77**: 350-356 [PMID: 10831341 DOI: 10.1006/gyno.2000.5816]

10   **Bast RC**, Brewer M, Zou C, Hernandez MA, Daley M, Ozols R, Lu K, Lu Z, Badgwell D, Mills GB, Skates S, Zhang Z, Chan D, Lokshin A, Yu Y. Prevention and early detection of ovarian cancer: mission impossible? *Recent Results Cancer Res* 2007; **174**: 91-100 [PMID: 17302189 DOI: 10.1007/978-3-540-37696-5_9]

11   **Nossov V**, Amneus M, Su F, Lang J, Janco JM, Reddy ST, Farias-Eisner R. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? *Am J Obstet Gynecol* 2008; **199**: 215-223 [PMID: 18468571 DOI: 10.1016/j.ajog.2008.04.009]

12   **Nolen BM**, Lokshin AE. Protein biomarkers of ovarian cancer: the forest and the trees. *Future Oncol* 2012; **8**: 55-71 [PMID: 22149035 DOI: 10.2217/fon.11.135]

13   **Cancer Genome Atlas Research Network.** Integrated genomic analyses of ovarian carcinoma. *Nature* 2011; **474**: 609-615 [PMID: 21720365]

14   **Vaughan S**, Coward JI, Bast RC, Berchuck A, Berek JS, Brenton JD, Coukos G, Crum CC, Drapkin R, Etemadmoghadam D, Friedlander M, Gabra H, Kaye SB, Lord CJ, Lengyel E, Levine DA, McNeish IA, Menon U, Mills GB, Nephew KP, Oza AM, Sood AK, Stronach EA, Walczak H, Bowtell DD, Balkwill FR. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 2011; **11**: 719-725 [PMID: 21941283 DOI: 10.1038/nrc3144]

15   **Chen VW**, Ruiz B, Killeen JL, Coté TR, Wu XC, Correa CN. Pathology and classification of ovarian tumors. *Cancer* 2003; **97**: 2631-2642 [PMID: 12733128 DOI: 10.1002/cncr.11345]

16   **Bast RC**, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981; **68**: 1331-1337 [PMID: 7028788 DOI: 10.1172/JCI110380]

17   **Weiland F**, Martin K, Oehler MK, Hoffmann P. Deciphering the Molecular Nature of Ovarian Cancer Biomarker CA125. *Int J Mol Sci* 2012; **13**: 10568-10582 [PMID: 22949880 DOI: 10.3390/ijms130810568]

18   **Davelaar EM**, van Kamp GJ, Verstraeten RA, Kenemans P. Comparison of seven immunoassays for the quantification of CA 125 antigen in serum. *Clin Chem* 1998; **44**: 1417-1422 [PMID: 9665418]

19   **Yin BW**, Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. *J Biol Chem* 2001; **276**: 27371-27375 [PMID: 11369781 DOI: 10.1074/jbc.M103554200]

20   **Kabawat SE**, Bast RC, Bhan AK, Welch WR, Knapp RC, Colvin RB. Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125. *Int J Gynecol Pathol* 1983; **2**: 275-285 [PMID: 6196309 DOI: 10.1097/00004347-198303000-00005]

21   **Jacobs I**, Bast RC. The CA 125 tumour-associated antigen: a review of the literature. *Hum Reprod* 1989; **4**: 1-12 [PMID: 2651469]

22   **Cramer DW**, O'Rourke DJ, Vitonis AF, Matulonis UA, Dijohnson DA, Sluss PM, Crum CP, Liu BC. CA125 immune complexes in ovarian cancer patients with low CA125 concentrations. *Clin Chem* 2010; **56**: 1889-1892 [PMID: 20943848 DOI: 10.1373/clinchem.2010.153122]

23   **Woolas RP**, Xu FJ, Jacobs IJ, Yu YJ, Daly L, Berchuck A, Soper JT, Clarke-Pearson DL, Oram DH, Bast RC Jr. Elevation of multiple serum markers in patients with stage I ovarian cancer. *J Natl Cancer Inst* 1993; **85**: 1748-1751 [PMID: 8411259 DOI: 10.1093/jnci/85.21.1748]

24   **Jacobs IJ**, Skates S, Davies AP, Woolas RP, Jeyerajah A, Weidemann P, Sibley K, Oram DH. Risk of diagnosis of ovarian cancer after raised serum CA 125 concentration: a prospective cohort study. *BMJ* 1996; **313**: 1355-1358 [PMID: 8956699 DOI: 10.1136/bmj.313.7069.1355]

25   **Gentry-Maharaj A**, Menon U. Screening for ovarian cancer in the general population. *Best Pract Res Clin Obstet Gynaecol* 2012; **26**: 243-256 [PMID: 22182415 DOI: 10.1016/j.bpobgyn.2011.11.006]

26   **Skates SJ**, Menon U, MacDonald N, Rosenthal AN, Oram DH, Knapp RC, Jacobs IJ. Calculation of the risk of ovarian cancer from serial CA-125 values for preclinical detection in postmenopausal women. *J Clin Oncol* 2003; **21**: 206s-210s [PMID: 12743136 DOI: 10.1200/JCO.2003.02.955]

27   **Menon U**, Skates SJ, Lewis S, Rosenthal AN, Rufford B, Sibley K, Macdonald N, Dawnay A, Jeyarajah A, Bast RC, Oram D, Jacobs IJ. Prospective study using the risk of ovarian cancer algorithm to screen for ovarian cancer. *J Clin Oncol* 2005; **23**: 7919-7926 [PMID: 16258091 DOI: 10.1200/JCO.2005.01.6642]

28   **Hellström I**, Raycraft J, Hayden-Ledbetter M, Ledbetter JA, Schummer M, McIntosh M, Drescher C, Urban N, Hellström KE. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. *Cancer Res* 2003; **63**: 3695-3700 [PMID: 12839961]

29   **Drapkin R**, von Horsten HH, Lin Y, Mok SC, Crum CP, Welch WR, Hecht JL. Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. *Cancer Res* 2005; **65**: 2162-2169 [PMID: 15781627 DOI: 10.1158/0008-5472.CAN-04-3924]

30   **Kirchhoff C**, Habben I, Ivell R, Krull N. A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors. *Biol Reprod* 1991; **45**: 350-357 [PMID: 1686187 DOI: 10.1095/biolreprod45.2.350]

31   **Moore RG**, MacLaughlan S, Bast RC. Current state of biomarker development for clinical application in epithelial ovarian cancer. *Gynecol Oncol* 2010; **116**: 240-245 [PMID:

19879639 DOI: 10.1016/j.ygyno.2009.09.041]

32   **Huhtinen K**, Suvitie P, Hiissa J, Junnila J, Huvila J, Kujari H, Setälä M, Härkki P, Jalkanen J, Fraser J, Mäkinen J, Auranen A, Poutanen M, Perheentupa A. Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts. *Br J Cancer* 2009; **100**: 1315-1319 [PMID: 19337252 DOI: 10.1038/sj.bjc.6605011]

33   **Moore RG**, Miller MC, Steinhoff MM, Skates SJ, Lu KH, Lambert-Messerlian G, Bast RC. Serum HE4 levels are less frequently elevated than CA125 in women with benign gynecologic disorders. *Am J Obstet Gynecol* 2012; **206**: 351. e1-351.e8 [PMID: 22284961 DOI: 10.1016/j.ajog.2011.12.029]

34   **Leung F**, Diamandis EP, Kulasingam V. From bench to bedside: discovery of ovarian cancer biomarkers using high-throughput technologies in the past decade. *Biomark Med* 2012; **6**: 613-625 [PMID: 23075239 DOI: 10.2217/bmm.12.70]

35   **Ferraro S**, Braga F, Lanzoni M, Boracchi P, Biganzoli EM, Panteghini M. Serum human epididymis protein 4 vs carbohydrate antigen 125 for ovarian cancer diagnosis: a systematic review. *J Clin Pathol* 2013; **66**: 273-281 [PMID: 23426716 DOI: 10.1136/jclinpath-2012-201031]

36   **Nolen B**, Velikokhatnaya L, Marrangoni A, De Geest K, Lomakin A, Bast RC, Lokshin A. Serum biomarker panels for the discrimination of benign from malignant cases in patients with an adnexal mass. *Gynecol Oncol* 2010; **117**: 440-445 [PMID: 20334903 DOI: 10.1016/j.ygyno.2010.02.005]

37   **Moore RG**, Brown AK, Miller MC, Skates S, Allard WJ, Verch T, Steinhoff M, Messerlian G, DiSilvestro P, Granai CO, Bast RC. The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass. *Gynecol Oncol* 2008; **108**: 402-408 [PMID: 18061248 DOI: 10.1016/j.ygyno.2007.10.017]

38   **Engelen MJ**, Kos HE, Willemse PH, Aalders JG, de Vries EG, Schaapveld M, Otter R, van der Zee AG. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. *Cancer* 2006; **106**: 589-598 [PMID: 16369985 DOI: 10.1002/cncr.21616]

39   **Vernooij F**, Heintz P, Witteveen E, van der Graaf Y. The outcomes of ovarian cancer treatment are better when provided by gynecologic oncologists and in specialized hospitals: a systematic review. *Gynecol Oncol* 2007; **105**: 801-812 [PMID: 17433422 DOI: 10.1016/j.ygyno.2007.02.030]

40   **Jacobs I**, Oram D, Fairbanks J, Turner J, Frost C, Grudzinskas JG. A risk of malignancy index incorporating CA 125, ultrasound and menopausal status for the accurate preoperative diagnosis of ovarian cancer. *Br J Obstet Gynaecol* 1990; **97**: 922-929 [PMID: 2223684 DOI: 10.1111/j.1471-0528.1990.tb02448.x]

41   **Moore RG**, McMeekin DS, Brown AK, DiSilvestro P, Miller MC, Allard WJ, Gajewski W, Kurman R, Bast RC, Skates SJ. A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. *Gynecol Oncol* 2009; **112**: 40-46 [PMID: 18851871 DOI: 10.1016/j.ygyno.2008.08.031]

42   **Zhang Z**, Bast RC, Yu Y, Li J, Sokoll LJ, Rai AJ, Rosenzweig JM, Cameron B, Wang YY, Meng XY, Berchuck A, Van Haaften-Day C, Hacker NF, de Bruijn HW, van der Zee AG, Jacobs IJ, Fung ET, Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004; **64**: 5882-5890 [PMID: 15313933 DOI: 10.1158/0008-5472.CAN-04-0746]

43   **Ueland FR**, Desimone CP, Seamon LG, Miller RA, Goodrich S, Podzielinski I, Sokoll L, Smith A, van Nagell JR, Zhang Z. Effectiveness of a multivariate index assay in the preoperative assessment of ovarian tumors. *Obstet Gynecol* 2011; **117**: 1289-1297 [PMID: 21606739 DOI: 10.1097/AOG.0b013e31821b5118]

44   **Karlsen MA**, Sandhu N, Høgdall C, Christensen IJ, Nedergaard L, Lundvall L, Engelholm SA, Pedersen AT, Hartwell D, Lydolph M, Laursen IA, Høgdall EV. Evaluation of HE4,

CA125, risk of ovarian malignancy algorithm (ROMA) and risk of malignancy index (RMI) as diagnostic tools of epithelial ovarian cancer in patients with a pelvic mass. *Gynecol Oncol* 2012; **127**: 379-383 [PMID: 22835718 DOI: 10.1016/j.ygyno.2012.07.106]

45   **Moore RG**, Jabre-Raughley M, Brown AK, Robison KM, Miller MC, Allard WJ, Kurman RJ, Bast RC, Skates SJ. Comparison of a novel multiple marker assay *vs* the Risk of Malignancy Index for the prediction of epithelial ovarian cancer in patients with a pelvic mass. *Am J Obstet Gynecol* 2010; **203**: 228.e1-228.e6 [PMID: 20471625 DOI: 10.1016/j.ajog.2010.03.043]

46   **Sandri MT**, Bottari F, Franchi D, Boveri S, Candiani M, Ronzoni S, Peiretti M, Radice D, Passerini R, Sideri M. Comparison of HE4, CA125 and ROMA algorithm in women with a pelvic mass: correlation with pathological outcome. *Gynecol Oncol* 2013; **128**: 233-238 [PMID: 23200911 DOI: 10.1016/j.ygyno.2012.11.026]

47   **Chan KK**, Chen CA, Nam JH, Ochiai K, Wilailak S, Choon AT, Sabaratnam S, Hebbar S, Sickan J, Schodin BA, Sumpaico WW. The use of HE4 in the prediction of ovarian cancer in Asian women with a pelvic mass. *Gynecol Oncol* 2013; **128**: 239-244 [PMID: 23063998 DOI: 10.1016/j.ygyno.2012.09.034]

48   **Håkansson F**, Høgdall EV, Nedergaard L, Lundvall L, Engelholm SA, Pedersen AT, Hartwell D, Høgdall C. Risk of malignancy index used as a diagnostic tool in a tertiary centre for patients with a pelvic mass. *Acta Obstet Gynecol Scand* 2012; **91**: 496-502 [PMID: 22229703 DOI: 10.1111/j.1600-0412.2012.01359.x]

49   **van den Akker PA**, Aalders AL, Snijders MP, Kluivers KB, Samlal RA, Vollebergh JH, Massuger LF. Evaluation of the Risk of Malignancy Index in daily clinical management of adnexal masses. *Gynecol Oncol* 2010; **116**: 384-388 [PMID: 19959215 DOI: 10.1016/j.ygyno.2009.11.014]

50   **Bristow RE**, Smith A, Zhang Z, Chan DW, Crutcher G, Fung ET, Munroe DG. Ovarian malignancy risk stratification of the adnexal mass using a multivariate index assay. *Gynecol Oncol* 2013; **128**: 252-259 [PMID: 23178277 DOI: 10.1016/j.ygyno.2012.11.022]

51   **Nunes N**, Yazbek J, Ambler G, Hoo W, Naftalin J, Jurkovic D. Prospective evaluation of the IOTA logistic regression model LR2 for the diagnosis of ovarian cancer. *Ultrasound Obstet Gynecol* 2012; **40**: 355-359 [PMID: 22223587 DOI: 10.1002/uog.11088]

52   **Longoria TC**, Ueland FR, Zhang Z, Chan DW, Smith A, Fung ET, Munroe DG, Bristow RE. Clinical performance of a multivariate index assay for detecting early-stage ovarian cancer. *Am J Obstet Gynecol* 2014; **210**: 78.e1-78.e9 [PMID: 24055582 DOI: 10.1016/j.ajog.2013.09.017]

53   **Nolen BM**, Lokshin AE. Biomarker testing for ovarian cancer: clinical utility of multiplex assays. *Mol Diagn Ther* 2013; **17**: 139-146 [PMID: 23552992 DOI: 10.1007/s40291-013-0027-6]

54   **Montagnana M**, Lippi G, Ruzzenente O, Bresciani V, Danese E, Scevarolli S, Salvagno GL, Giudici S, Franchi M, Guidi GC. The utility of serum human epididymis protein 4 (HE4) in patients with a pelvic mass. *J Clin Lab Anal* 2009; **23**: 331-335 [PMID: 19774626 DOI: 10.1002/jcla.20340]

55   **Yang Z**, Luo Z, Zhao B, Zhang W, Zhang J, Li Z, Li L. Diagnosis and preoperative predictive value of serum HE4 concentrations for optimal debulking in epithelial ovarian cancer. *Oncol Lett* 2013; **6**: 28-34 [PMID: 23946773]

56   **Havrilesky LJ**, Whitehead CM, Rubatt JM, Cheek RL, Groelke J, He Q, Malinowski DP, Fischer TJ, Berchuck A. Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. *Gynecol Oncol* 2008; **110**: 374-382 [PMID: 18584856 DOI: 10.1016/j.ygyno.2008.04.041]

57   **Jacob F**, Meier M, Caduff R, Goldstein D, Pochechueva T, Hacker N, Fink D, Heinzelmann-Schwarz V. No benefit

from combining HE4 and CA125 as ovarian tumor markers in a clinical setting. *Gynecol Oncol* 2011; **121**: 487-491 [PMID: 21420727 DOI: 10.1016/j.ygyno.2011.02.022]

58   **Visintin I**, Feng Z, Longton G, Ward DC, Alvero AB, Lai Y, Tenthorey J, Leiser A, Flores-Saaib R, Yu H, Azori M, Rutherford T, Schwartz PE, Mor G. Diagnostic markers for early detection of ovarian cancer. *Clin Cancer Res* 2008; **14**: 1065-1072 [PMID: 18258665 DOI: 10.1158/1078-0432. CCR-07-1569]

59   **Edgell T**, Martin-Roussety G, Barker G, Autelitano DJ, Allen D, Grant P, Rice GE. Phase II biomarker trial of a multimarker diagnostic for ovarian cancer. *J Cancer Res Clin Oncol* 2010; **136**: 1079-1088 [PMID: 20082099 DOI: 10.1007/s00432-009-0755-5]

60   **Su F**, Lang J, Kumar A, Ng C, Hsieh B, Suchard MA, Reddy ST, Farias-Eisner R. Validation of candidate serum ovarian cancer biomarkers for early detection. *Biomark Insights* 2007; **2**: 369-375 [PMID: 19662218]

61   **Yurkovetsky Z**, Skates S, Lomakin A, Nolen B, Pulsipher T, Modugno F, Marks J, Godwin A, Gorelik E, Jacobs I, Menon U, Lu K, Badgwell D, Bast RC, Lokshin AE. Development of a multimarker assay for early detection of ovarian cancer. *J Clin Oncol* 2010; **28**: 2159-2166 [PMID: 20368574 DOI: 10.1200/JCO.2008.19.2484]

62   **Kim YW**, Bae SM, Lim H, Kim YJ, Ahn WS. Development of multiplexed bead-based immunoassays for the detection of early stage ovarian cancer using a combination of serum biomarkers. *PLoS One* 2012; **7**: e44960 [PMID: 22970327 DOI: 10.1371/journal.pone.0044960]

63   **Amonkar SD**, Bertenshaw GP, Chen TH, Bergstrom KJ, Zhao J, Seshaiah P, Yip P, Mansfield BC. Development and preliminary evaluation of a multivariate index assay for ovarian cancer. *PLoS One* 2009; **4**: e4599 [PMID: 19240799 DOI: 10.1371/journal.pone.0004599]

64   **Skates SJ**, Horick N, Yu Y, Xu FJ, Berchuck A, Havrilesky LJ, de Bruijn HW, van der Zee AG, Woolas RP, Jacobs IJ, Zhang Z, Bast RC. Preoperative sensitivity and specificity for early-stage ovarian cancer when combining cancer antigen CA-125II, CA 15-3, CA 72-4, and macrophage colony-stimulating factor using mixtures of multivariate normal distributions. *J Clin Oncol* 2004; **22**: 4059-4066 [PMID: 15381683 DOI: 10.1200/JCO.2004.03.091]

65   **Zhang B**, Barekati Z, Kohler C, Radpour R, Asadollahi R, Holzgreve W, Zhong XY. Proteomics and biomarkers for ovarian cancer diagnosis. *Ann Clin Lab Sci* 2010; **40**: 218-225 [PMID: 20689132 DOI: 10.1007/s12010-012-9829-y]

66   **Moshkovskii SA**, Serebryakova MV, Kuteykin-Teplyakov KB, Tikhonova OV, Goufman EI, Zgoda VG, Taranets IN, Makarov OV, Archakov AI. Ovarian cancer marker of 11.7 kDa detected by proteomics is a serum amyloid A1. *Proteomics* 2005; **5**: 3790-3797 [PMID: 16121334 DOI: 10.1002/pmic.200401205]

67   **Zhu CS**, Pinsky PF, Cramer DW, Ransohoff DF, Hartge P, Pfeiffer RM, Urban N, Mor G, Bast RC, Moore LE, Lokshin AE, McIntosh MW, Skates SJ, Vitonis A, Zhang Z, Ward DC, Symanowski JT, Lomakin A, Fung ET, Sluss PM, Scholler N, Lu KH, Marrangoni AM, Patriotis C, Srivastava S, Buys SS, Berg CD. A framework for evaluating biomarkers for early detection: validation of biomarker panels for ovarian cancer. *Cancer Prev Res* (Phila) 2011; **4**: 375-383 [PMID: 21372037 DOI: 10.1158/1940-6207.CAPR-10-0193]

68   **Cramer DW**, Bast RC, Berg CD, Diamandis EP, Godwin AK, Hartge P, Lokshin AE, Lu KH, McIntosh MW, Mor G, Patriotis C, Pinsky PF, Thornquist MD, Scholler N, Skates SJ, Sluss PM, Srivastava S, Ward DC, Zhang Z, Zhu CS, Urban N. Ovarian cancer biomarker performance in prostate, lung, colorectal, and ovarian cancer screening trial specimens. *Cancer Prev Res* (Phila) 2011; **4**: 365-374 [PMID: 21372036 DOI: 10.1158/1940-6207.CAPR-10-0195]

69   **Leung F**, Musrap N, Diamandis E, Kulasingam V. Advances in mass spectrometry-based technologies to direct personalized medicine in ovarian cancer. *Translational Proteomics* 2013; **1**: 74-86 [DOI: 10.1016/j.trprot.2013.08.001]

70   **Adamczyk B**, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochim Biophys Acta* 2012; **1820**: 1347-1353 [PMID: 22178561 DOI: 10.1016/j.bbagen.2011.12.001]

71   **Tian Y**, Yao Z, Roden RB, Zhang H. Identification of glycoproteins associated with different histological subtypes of ovarian tumors using quantitative glycoproteomics. *Proteomics* 2011; **11**: 4677-4687 [PMID: 22113853 DOI: 10.1002/pmic.201000811]

72   **Hua S**, Williams CC, Dimapasoc LM, Ro GS, Ozcan S, Miyamoto S, Lebrilla CB, An HJ, Leiserowitz GS. Isomer-specific chromatographic profiling yields highly sensitive and specific potential N-glycan biomarkers for epithelial ovarian cancer. *J Chromatogr A* 2013; **1279**: 58-67 [PMID: 23380366 DOI: 10.1016/j.chroma.2012.12.079]

73   **Zhang T**, Wu X, Ke C, Yin M, Li Z, Fan L, Zhang W, Zhang H, Zhao F, Zhou X, Lou G, Li K. Identification of potential biomarkers for ovarian cancer by urinary metabolomic profiling. *J Proteome Res* 2013; **12**: 505-512 [PMID: 23163809 DOI: 10.1021/pr3009572]

74   **Fan L**, Zhang W, Yin M, Zhang T, Wu X, Zhang H, Sun M, Li Z, Hou Y, Zhou X, Lou G, Li K. Identification of metabolic biomarkers to diagnose epithelial ovarian cancer using a UPLC/QTOF/MS platform. *Acta Oncol* 2012; **51**: 473-479 [PMID: 22283470 DOI: 10.3109/0284186X.2011.648338]

75   **Schmidt CW**. Metabolomics: what's happening downstream of DNA. *Environ Health Perspect* 2004; **112**: A410-A415 [PMID: 15159214 DOI: 10.1289/ehp.112-a410]

76   **Lowenthal MS**, Mehta AI, Frogale K, Bandle RW, Araujo RP, Hood BL, Veenstra TD, Conrads TP, Goldsmith P, Fishman D, Petricoin EF, Liotta LA. Analysis of albumin-associated peptides and proteins from ovarian cancer patients. *Clin Chem* 2005; **51**: 1933-1945 [PMID: 16099937 DOI: 10.1373/clinchem.2005.052944]

77   **Philip R**, Murthy S, Krakover J, Sinnathamby G, Zerfass J, Keller L, Philip M. Shared immunoproteome for ovarian cancer diagnostics and immunotherapy: potential theranostic approach to cancer. *J Proteome Res* 2007; **6**: 2509-2517 [PMID: 17547437 DOI: 10.1021/pr0606777]

78   **El Ayed M**, Bonnel D, Longuespée R, Castelier C, Franck J, Vergara D, Desmons A, Tasiemski A, Kenani A, Vinatier D, Day R, Fournier I, Salzet M. MALDI imaging mass spectrometry in ovarian cancer for tracking, identifying, and validating biomarkers. *Med Sci Monit* 2010; **16**: BR233-BR245 [PMID: 20671603]

79   **Zhang B**, Cai FF, Zhong XY. An overview of biomarkers for the ovarian cancer diagnosis. *Eur J Obstet Gynecol Reprod Biol* 2011; **158**: 119-123 [PMID: 21632171 DOI: 10.1016/j.ejogrb.2011.04.023]

80   **Iorio MV**, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007; **67**: 8699-8707 [PMID: 17875710 DOI: 10.1158/0008-5472.CAN-07-1936]

81   **Chen Y**, Zhang L, Hao Q. Candidate microRNA biomarkers in human epithelial ovarian cancer: systematic review profiling studies and experimental validation. *Cancer Cell Int* 2013; **13**: 86 [PMID: 23978303 DOI: 10.1186/1475-2867-13-86]

82   **Manegold-Brauer G**, Bellin AK, Tercanli S, Lapaire O, Heinzelmann-Schwarz V. The special role of ultrasound for screening, staging and surveillance of malignant ovarian tumors: distinction from other methods of diagnostic imaging. *Arch Gynecol Obstet* 2014; **289**: 491-498 [PMID: 24253338 DOI: 10.1007/s00404-013-3081-8]

83   **Sharma A**, Apostolidou S, Burnell M, Campbell S, Habib M, Gentry-Maharaj A, Amso N, Seif MW, Fletcher G, Singh N, Benjamin E, Brunell C, Turner G, Rangar R, Godfrey K, Oram D, Herod J, Williamson K, Jenkins H, Mould T,

Woolas R, Murdoch J, Dobbs S, Leeson S, Cruickshank D, Fourkala EO, Ryan A, Parmar M, Jacobs I, Menon U. Risk of epithelial ovarian cancer in asymptomatic women with ultrasound-detected ovarian masses: a prospective cohort study within the UK collaborative trial of ovarian cancer screening (UKCTOCS).*Ultrasound Obstet Gynecol* 2012; **40**: 338-344 [PMID: 22911637 DOI: 10.1002/uog.12270]

84 **van Nagell JR**, DePriest PD, Ueland FR, DeSimone CP, Cooper AL, McDonald JM, Pavlik EJ, Kryscio RJ. Ovarian cancer screening with annual transvaginal sonography: findings of 25,000 women screened. *Cancer* 2007; **109**: 1887-1896 [PMID: 17373668 DOI: 10.1002/cncr.22594]

85 **Pavlik EJ**, Ueland FR, Miller RW, Ubellacker JM, DeSimone CP, Elder J, Hoff J, Baldwin L, Kryscio RJ, van Nagell JR. Frequency and disposition of ovarian abnormalities followed with serial transvaginal ultrasonography. *Obstet Gynecol* 2013; **122**: 210-217 [PMID: 23969786 DOI: 10.1097/AOG.0b013e318298def5]

86 **van Nagell JR**, Miller RW, DeSimone CP, Ueland FR, Podzielinski I, Goodrich ST, Elder JW, Huang B, Kryscio RJ, Pavlik EJ. Long-term survival of women with epithelial ovarian cancer detected by ultrasonographic screening. *Obstet Gynecol* 2011; **118**: 1212-1221 [PMID: 22105249 DOI: 10.1097/AOG.0b013e318238d030]

87 **Jacobs I**, Menon U. Can ovarian cancer screening save lives? The question remains unanswered. *Obstet Gynecol* 2011; **118**: 1209-1211 [PMID: 22105248 DOI: 10.1097/AOG.0b013e31823b49b3]

88 **Kaijser J**, Bourne T, Valentin L, Sayasneh A, Van Holsbeke C, Vergote I, Testa AC, Franchi D, Van Calster B, Timmerman D. Improving strategies for diagnosing ovarian cancer: a summary of the International Ovarian Tumor Analysis (IOTA) studies. *Ultrasound Obstet Gynecol* 2013; **41**: 9-20 [PMID: 23065859 DOI: 10.1002/uog.12323]

89 **Van Gorp T**, Veldman J, Van Calster B, Cadron I, Leunen K, Amant F, Timmerman D, Vergote I. Subjective assessment by ultrasound is superior to the risk of malignancy index (RMI) or the risk of ovarian malignancy algorithm (ROMA) in discriminating benign from malignant adnexal masses. *Eur J Cancer* 2012; **48**: 1649-1656 [PMID: 22226481 DOI: 10.1016/j.ejca.2011.12.003]

90 **Kaijser J**, Van Gorp T, Van Hoorde K, Van Holsbeke C, Sayasneh A, Vergote I, Bourne T, Timmerman D, Van Calster B. A comparison between an ultrasound based prediction model (LR2) and the risk of ovarian malignancy algorithm (ROMA) to assess the risk of malignancy in women with an adnexal mass. *Gynecol Oncol* 2013; **129**: 377-383 [PMID: 23360924 DOI: 10.1016/j.ygyno.2013.01.018]

91 **Campbell S**. Ovarian cancer: role of ultrasound in preoperative diagnosis and population screening. *Ultrasound Obstet Gynecol* 2012; **40**: 245-254 [PMID: 22911634 DOI: 10.1002/uog.12281]

92 **Einhorn N**, Sjövall K, Knapp RC, Hall P, Scully RE, Bast RC, Zurawski VR. Prospective evaluation of serum CA 125 levels for early detection of ovarian cancer. *Obstet Gynecol* 1992; **80**: 14-18 [PMID: 1603484 DOI: 10.1016/0378-5122(93)90150-G]

93 **Campbell S**, Bhan V, Royston P, Whitehead MI, Collins WP. Transabdominal ultrasound screening for early ovarian cancer. *BMJ* 1989; **299**: 1363-1367 [PMID: 2513964 DOI: 10.1136/bmj.299.6712.1363]

94 **Buys SS**, Partridge E, Greene MH, Prorok PC, Reding D, Riley TL, Hartge P, Fagerstrom RM, Ragard LR, Chia D, Izmirlian G, Fouad M, Johnson CC, Gohagan JK. Ovarian cancer screening in the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial: findings from the initial screen of a randomized trial. *Am J Obstet Gynecol* 2005; **193**: 1630-1639 [PMID: 16260202 DOI: 10.1016/j.ajog.2005.05.005]

95 **Partridge E**, Kreimer AR, Greenlee RT, Williams C, Xu JL, Church TR, Kessel B, Johnson CC, Weissfeld JL, Isaacs C,

Andriole GL, Ogden S, Ragard LR, Buys SS. Results from four rounds of ovarian cancer screening in a randomized trial. *Obstet Gynecol* 2009; **113**: 775-782 [PMID: 19305319 DOI: 10.1097/AOG.0b013e31819cda77]

96 **Buys SS**, Partridge E, Black A, Johnson CC, Lamerato L, Isaacs C, Reding DJ, Greenlee RT, Yokochi LA, Kessel B, Crawford ED, Church TR, Andriole GL, Weissfeld JL, Fouad MN, Chia D, O'Brien B, Ragard LR, Clapp JD, Rathmell JM, Riley TL, Hartge P, Pinsky PF, Zhu CS, Izmirlian G, Kramer BS, Miller AB, Xu JL, Prorok PC, Gohagan JK, Berg CD. Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial. *JAMA* 2011; **305**: 2295-2303 [PMID: 21642681 DOI: 10.1001/jama.2011.766]

97 **Klompmaker J**, Jansen HW, Veth RP, de Groot JH, Nijenhuis AJ, Pennings AJ. Porous polymer implant for repair of meniscal lesions: a preliminary study in dogs. *Biomaterials* 1991; **12**: 810-816 [PMID: 1764550 DOI: 10.1111/j.1525-1438.2007.01035.x]

98 **Menon U**, Gentry-Maharaj A, Hallett R, Ryan A, Burnell M, Sharma A, Lewis S, Davies S, Philpott S, Lopes A, Godfrey K, Oram D, Herod J, Williamson K, Seif MW, Scott I, Mould T, Woolas R, Murdoch J, Dobbs S, Amso NN, Leeson S, Cruickshank D, McGuire A, Campbell S, Fallowfield L, Singh N, Dawnay A, Skates SJ, Parmar M, Jacobs I. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). *Lancet Oncol* 2009; **10**: 327-340 [PMID: 19282241 DOI: 10.1016/S1470-2045(09)70026-9]

99 **Menon U**, Kalsi J, Jacobs I. The UKCTOCS experience--reasons for hope? *Int J Gynecol Cancer* 2012; **22** Suppl 1: S18-S20 [PMID: 22543913 DOI: 10.1097/IGC.0b013e318251cb47]

100 **Pinsky PF**, Zhu C, Skates SJ, Black A, Partridge E, Buys SS, Berg CD. Potential effect of the risk of ovarian cancer algorithm (ROCA) on the mortality outcome of the Prostate, Lung, Colorectal and Ovarian (PLCO) trial. *Int J Cancer* 2013; **132**: 2127-2133 [PMID: 23065684 DOI: 10.1002/ijc.27909]

101 **Menon U**, Gentry-Maharaj A, Jacobs I. Ovarian cancer screening and mortality. *JAMA* 2011; **306**: 1544; author reply 1544-1545 [PMID: 21990294 DOI: 10.1001/jama.2011.1461]

102 **Vine MF**, Calingaert B, Berchuck A, Schildkraut JM. Characterization of prediagnostic symptoms among primary epithelial ovarian cancer cases and controls. *Gynecol Oncol* 2003; **90**: 75-82 [PMID: 12821345 DOI: 10.1016/S0090-8258(03)00175-6]

103 **Goff BA**, Lowe KA, Kane JC, Robertson MD, Gaul MA, Andersen MR. Symptom triggered screening for ovarian cancer: a pilot study of feasibility and acceptability. *Gynecol Oncol* 2012; **124**: 230-235 [PMID: 22063459 DOI: 10.1016/j.ygyno.2011.10.032]

104 **Rufford BD**, Jacobs IJ, Menon U. Feasibility of screening for ovarian cancer using symptoms as selection criteria. *BJOG* 2007; **114**: 59-64 [PMID: 17233861 DOI: 10.1111/j.1471-0528.2006.01153.x]

105 **Goff BA**, Mandel LS, Drescher CW, Urban N, Gough S, Schurman KM, Patras J, Mahony BS, Andersen MR. Development of an ovarian cancer symptom index: possibilities for earlier detection. *Cancer* 2007; **109**: 221-227 [PMID: 17154394 DOI: 10.1002/cncr.22371]

106 **Rossing MA**, Wicklund KG, Cushing-Haugen KL, Weiss NS. Predictive value of symptoms for early detection of ovarian cancer. *J Natl Cancer Inst* 2010; **102**: 222-229 [PMID: 20110551 DOI: 10.1093/jnci/djp500]

107 **Cass I**, Karlan BY. Ovarian cancer symptoms speak out--but what are they really saying? *J Natl Cancer Inst* 2010; **102**: 211-212 [PMID: 20110550 DOI: 10.1093/jnci/djp525]

108 **Pitta Dda R**, Sarian LO, Barreta A, Campos EA, Andrade LL, Fachini AM, Campbell LM, Derchain S. Symptoms,

CA125 and HE4 for the preoperative prediction of ovarian malignancy in Brazilian women with ovarian masses. *BMC Cancer* 2013; **13**: 423 [PMID: 24044637 DOI: 10.1186/1471-2407-13-423]

109 **Rosenthal AN**, Fraser L, Manchanda R, Badman P, Philpott S, Mozersky J, Hadwin R, Cafferty FH, Benjamin E, Singh N, Evans DG, Eccles DM, Skates SJ, Mackay J, Menon U, Jacobs IJ. Results of annual screening in phase I of the United Kingdom familial ovarian cancer screening study highlight the need for strict adherence to screening schedule. *J Clin Oncol* 2013; **31**: 49-57 [PMID: 23213100 DOI: 10.1200/JCO.2011.39.7638]

110 **Bolton KL**, Ganda C, Berchuck A, Pharoah PD, Gayther SA. Role of common genetic variants in ovarian cancer susceptibility and outcome: progress to date from the Ovarian Cancer Association Consortium (OCAC). *J Intern Med* 2012; **271**: 366-378 [PMID: 22443200 DOI: 10.1111/j.1365-2796.2011.02509.x]

111 **Long KC**, Kauff ND. Screening for familial ovarian cancer: a ray of hope and a light to steer by. *J Clin Oncol* 2013; **31**: 8-10 [PMID: 23213103 DOI: 10.1200/JCO.2012.45.4678]

112 **Greene MH**, Piedmonte M, Alberts D, Gail M, Hensley M, Miner Z, Mai PL, Loud J, Rodriguez G, Basil J, Boggess J, Schwartz PE, Kelley JL, Wakeley KE, Minasian L, Skates S. A prospective study of risk-reducing salpingo-oophorectomy and longitudinal CA-125 screening among women at increased genetic risk of ovarian cancer: design and baseline characteristics: a Gynecologic Oncology Group study. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 594-604 [PMID: 18349277 DOI: 10.1158/1055-9965.EPI-07-2703]

113 **National Comprehensive Cancer Network.** NCCN Clinical Practice Guidelines in Oncology-Genetic/Familial High-Risk Assessment: Breast and Ovarian Cancer. Version 1, 2014

114 **Nik NN**, Vang R, Shih IeM, Kurman RJ. Origin and pathogenesis of pelvic (ovarian, tubal, and primary peritoneal) serous carcinoma. *Annu Rev Pathol* 2014; **9**: 27-45 [PMID: 23937438 DOI: 10.1146/annurev-pathol-020712-163949]

115 **Wiegand KC**, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih IeM, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* 2010; **363**: 1532-1543 [PMID: 20942669 DOI: 10.1056/NEJMoa1008433]

116 **Kurman RJ**, Shih IeM. Molecular pathogenesis and extra-ovarian origin of epithelial ovarian cancer--shifting the paradigm. *Hum Pathol* 2011; **42**: 918-931 [PMID: 21683865 DOI: 10.1016/j.humpath.2011.03.003]

117 **Kurman RJ**, Shih IeM. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008; **27**: 151-160 [PMID: 18317228 DOI: 10.1097/PGP.0b013e318161e4f5]

118 **Rauh-Hain JA**, Krivak TC, Del Carmen MG, Olawaiye AB. Ovarian cancer screening and early detection in the general population. *Rev Obstet Gynecol* 2011; **4**: 15-21 [PMID: 21629494]

119 **Lim D**, Oliva E. Precursors and pathogenesis of ovarian carcinoma. *Pathology* 2013; **45**: 229-242 [PMID: 23478230 DOI: 10.1097/PAT.0b013e32835f2264]

120 **Kurman RJ**, Visvanathan K, Roden R, Wu TC, Shih IeM. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. *Am J Obstet Gynecol* 2008; **198**: 351-356 [PMID: 18395030 DOI: 10.1016/j.ajog.2008.01.005]

121 **Pepe MS**, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001; **93**: 1054-1061 [PMID: 11459866 DOI: 10.1093/jnci/93.14.1054]

122 **Kulasingam V**, Pavlou MP, Diamandis EP. Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer. *Nat Rev Cancer* 2010; **10**: 371-378 [PMID: 20383179 DOI: 10.1038/nrc2831]

123 **Leung F**, Dimitromanolakis A, Kobayashi H, Diamandis EP, Kulasingam V. Folate-receptor 1 (FOLR1) protein is elevated in the serum of ovarian cancer patients. *Clin Biochem* 2013; **46**: 1462-1468 [PMID: 23528302 DOI: 10.1016/j.clinbiochem.2013.03.010]

124 **Anderson GL**, McIntosh M, Wu L, Barnett M, Goodman G, Thorpe JD, Bergan L, Thornquist MD, Scholler N, Kim N, O'Briant K, Drescher C, Urban N. Assessing lead time of selected ovarian cancer biomarkers: a nested case-control study. *J Natl Cancer Inst* 2010; **102**: 26-38 [PMID: 20042715 DOI: 10.1093/jnci/djp438]

125 **Pepe MS**, Feng Z. Improving biomarker identification with better designs and reporting. *Clin Chem* 2011; **57**: 1093-1095 [PMID: 21666069 DOI: 10.1373/clinchem.2011.164657]

126 **Feng Z**, Kagan J, Pepe M, Thornquist M, Ann Rinaudo J, Dahlgren J, Krueger K, Zheng Y, Patriotis C, Huang Y, Sorbara L, Thompson I, Srivastava S. The Early Detection Research Network's Specimen reference sets: paving the way for rapid evaluation of potential biomarkers. *Clin Chem* 2013; **59**: 68-74 [PMID: 23193062 DOI: 10.1373/clinchem.2012.185140]

127 **Reade CJ**, Riva JJ, Busse JW, Goldsmith CH, Elit L. Risks and benefits of screening asymptomatic women for ovarian cancer: a systematic review and meta-analysis. *Gynecol Oncol* 2013; **130**: 674-681 [PMID: 23822892 DOI: 10.1016/j.ygyno.2013.06.029]

128 American College of Obstetrics and Gynecologists Committee on Gynecologic Practice. Committee Opinion No. 477: the role of the obstetrician-gynecologist in the early detection of epithelial ovarian cancer. *Obstet Gynecol* 2011; **117**: 742-746 [PMID: 21343791 DOI: 10.1097/AOG.0b013e31821477db]

129 **Moyer VA**. Screening for ovarian cancer: U.S. Preventive Services Task Force reaffirmation recommendation statement. *Ann Intern Med* 2012; **157**: 900-904 [PMID: 22964825 DOI: 10.7326/0003-4819-157-11-201212040-00539]

130 **Smith RA**, Brooks D, Cokkinides V, Saslow D, Brawley OW. Cancer screening in the United States, 2013: a review of current American Cancer Society guidelines, current issues in cancer screening, and new guidance on cervical cancer screening and lung cancer screening. *CA Cancer J Clin* 2013; **63**: 88-105 [PMID: 23378235 DOI: 10.3322/caac.21174]

131 **Pavlik EJ**, van Nagell JR. Ovarian cancer screening--what women want. *Int J Gynecol Cancer* 2012; **22** Suppl 1: S21-S23 [PMID: 22543915 DOI: 10.1097/IGC.0b013e318251cbc2]

TOPIC HIGHLIGHT

Lei Zheng, MD, PhD, Assistant Professor, *Series Editor*

MicroRNA regulation network in colorectal cancer metastasis

Jiao-Jiao Zhou, Shu Zheng, Li-Feng Sun, Lei Zheng

Jiao-Jiao Zhou, Shu Zheng, Li-Feng Sun, Department of Surgical Oncology, the Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310009, Zhejiang Province, China

Jiao-Jiao Zhou, Shu Zheng, Li-Feng Sun, The Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Hangzhou 310009, Zhejiang Province, China

Lei Zheng, Departments of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, United States

Lei Zheng, Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21287, United States

Lei Zheng, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Bunting-Blaustein Building Room 488, Baltimore, MD 21287, United States

Author contributions: All authors contributed to this work.

Supported by NIH K23 CA148964, to Zheng L; Lefkofsky Family Foundation, to Zheng L; the NCI SPORE in Gastrointestinal Cancers P50 CA062924, to Zheng L; the Zhang Family Gift Fund, to Zheng L

Correspondence to: Lei Zheng, MD, PhD, Assistant Professor, Department of Oncology, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB1 Room 488, Baltimore, MD 21287, United States. lzheng6@jhmi.edu

Telephone: +1-410-5026241   Fax: +1-410-4168216

Received: November 23, 2013   Revised: March 19, 2014

Accepted: May 31, 2014

Published online: August 26, 2014

Abstract

Colorectal cancer is the third most common cancer worldwide. Metastasis is a major cause of colorectal cancer-related death. Mechanisms of metastasis remain largely obscure. MicroRNA is one of the most important epigenetic regulators by targeting mRNAs post-transcriptionally. Accumulated evidence has supported its significant role in the metastasis of colorectal cancer, including epithelial-mesenchymal transition and angiogenesis. Dissecting microRNAome potentially identifies specific microRNAs as biomarkers of colorectal cancer metastasis. Better understanding of the complex network of microRNAs in colorectal cancer metastasis provide new insights in the biological process of metastasis and in the potential targets for colorectal cancer therapies and for diagnosis of recurrent and metastatic colorectal cancer.

Core tip: MicroRNA is one of the most important epigenetic regulators by targeting mRNAs post-transcriptionally. This article has reviewed the new evidence that has supported the significant role of microRNAs in the metastasis of colorectal cancer. Better understanding of the complex network of microRNAs in colorectal cancer metastasis provide new insights in the biological process of metastasis and in the potential targets for colorectal cancer therapies and for diagnosis of recurrent and metastatic colorectal cancer.

INTRODUCTION

Colorectal cancer is the third most common cancer in both males and females worldwide. The major cause of death from colorectal cancer is the development of metastatic diseases. For those patients who have metastatic diseases at the time of diagnosis, five-year survival rate is only 12%[1,2]. However, the molecular mechanism of metastasis still remains obscure. Multiple cellular character-

istics of neoplastic cells including epithelial-mesenchymal transition (EMT), invasion and migration, and changes in the tumor microenvironment such as angiogenesis are believed to be important for the metastatic process. Genetic abnormalities accounting for metastasis have not been identified while accumulated evidence suggests that microRNAs may play a crucial role in the epigenetic regulation of the multi-step process of metastasis.

MicroRNAs are a family of small non-coding 18-22nt RNAs, which function in the post-transcriptional regulation of gene expression, by targeting mRNA for cleavage or translational repression[3]. In the nucleus, the DNA coding sequences of miRNA are firstly transcribed into pri-miRNA by RNA polymerase Ⅱ. After transcription, the enzyme named Drosha cuts pri-miRNA into pre-microRNA, which is then transported into cytoplasm and further cleaved into mature microRNA by Dicer. Only one strand of the mature microRNA will be incorporated into RNA-induced silencing complex (RISC), where microRNA play its silencing functions by interacting its target mRNA[4].

Accumulated evidence has suggested that microRNAs directly participate in the tumorigenesis process of colorectal cancer, especially through the posttranscriptional regulation. Some microRNAs play roles in colorectal tumorigenesis by regulating the functional pathways of tumor suppressor genes. For example, microRNAs including miR-135a/b and miR-122a were able to inactivate the adenomatous polyposis coli (APC) tumor suppressor gene and the APC-mediated pathways, providing an alternative, epigenetic mechanism for the inactivation of this tumor suppressor gene. In the APC-mutated mouse model, microRNAs including miR-31, miR-137 and miR-215 were found to be differentially regulated in association with colorectal adenoma formation, suggesting that these microRNAs may be regulated by the APC pathway and are involved in the early stage of colorectal cancer development[5-7]. Similarly, microRNAs including miR-34, miR-145 and miR-107 are regulated by p53 to mediate the function of p53 in cell survival, proliferation and angiogenesis, respectively[8,9]. Therefore, it has been well established that microRNA is involved in the initial stage of colorectal tumorigenesis through the APC pathway and in the advanced stage of colorectal tumorigenesis through the p53 pathway. More recently, a large body of evidence has also supported the role of microRNAs in colorectal cancer metastasis. Here we will review the function of microRNAs in the regulation of EMT and angiogenesis-two biological processes that are important for colorectal cancer metastasis.

DISSECT MICRORNAOME IN COLORECTAL CANCER AND METASTASIS

To discover aberrantly expressed microRNAs in colorectal cancer, microarray analysis was commonly used for dissecting microRNAome[10]. A microRNA microarray analysis in a discovery cohort of 84 colorectal cancer pa-

tients comparing microRNA expression profiles between colorectal tumors and paired non-tumorous tissues identified 37 microRNAs differentially expressed in colorectal cancer. Five microRNAs (miR-20a, miR-21, miR-106a, miR-181b, and miR-203) that were most differentially expressed between tumors and non-tumorous tissues and whose expression levels were also associated with patient survivals were selected for further validation. In the validation cohort, it was found that miR-21 was preferentially expressed in colorectal tumors at more advanced TNM stages. In both discovery and validation cohorts, higher miR-21 expression level was significantly associated with poorer survival[11]. Similarly, in another study, 49 microRNAs were identified to be significantly differentially expressed comparing between rectal cancer and adjacent non-tumorous mucosa. Among them, miR-135b was shown to be significantly correlated to disease-free and cancer-specific survival in the validation cohort[12].

Although these microRNAome studies included patients across different stages, there is still a lack of identification of microRNAs associated specifically with metastatic colorectal cancer. Shen *et al*[13] compared the miRNA expression profiles between colorectal cancers with liver metastasis and those without metastasis and found 28 differentially expressed microRNAs. Among them, four microRNAs (miR-150*, miR-125b-2*, miR-1179 and miR-139-3p) are up-regulated in colorectal cancer with metastasis. Although this study provided microRNAome data for colorectal cancer with metastasis, the sample size was small, including only 3 paired samples; and the clinical survival data was not available.

While the microRNAome specific for colorectal cancer metastasis is still highly desired, the research on microRNAome continues to strive ahead with microarray analysis on both tumoral and stromal tissues of colorectal cancer and with microarray analysis on colorectal tumors at different locations, of different subtypes, and with different mismatch repair status. Single nucleotide polymorphism (SNP) variations in microRNA coding sequences and 3'-UTR have been studied[14-18]. These new researches have shed lights on the future research direction of mciroRNA in metastatic colorectal cancer.

MICRORNAS IN PATHWAYS OF COLORECTAL CANCER METASTASIS

Despite genetic mutation is still considered as one of the key characteristics in the primary occurrence of colorectal cancer, the metastasis of colorectal cancer appears to be closely associated with epigenetic regulation, such as DNA promoter methylation, histone modification and microRNAs[19,20]. microRNAs have been demonstrated to be involved in two major pathways of colorectal cancer metastasis, EMT and angiogenesis.

MicroRNAs in epithelial-mesenchymal transition
EMT is the biologic process that the polarized epithelial cells transit to a mesenchymal cell phenotype. During
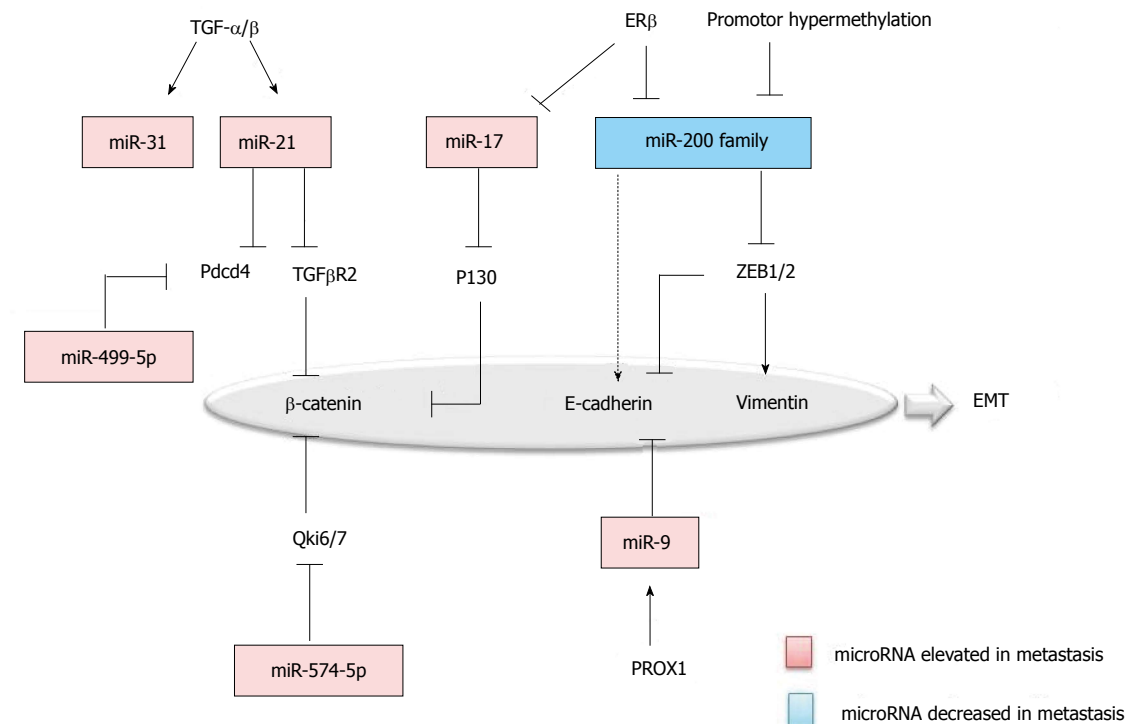
Figure 1 MicroRNAs regulation in epithelial–mesenchymal transition of colorectal cancer metastasis. EMT: Epithelial–mesenchymal transition; PROX1: Prospero Homeobox 1.

the course of metastasis, neoplastic cells undergo EMT, suggesting a mechanistic role of EMT in metastasis. In EMT, proteins such as Vimentin, β-Catenin, TCF8-ZEB1, E-Cadherin, Snail and Slug, are known to be specifically up or down-regulated and have been established as the markers of EMT[21]. Similarly, microRNAs associated with colorectal cancer, were also found to be specifically regulated in EMT (Figure 1).

First, TGF-β/Wnt signaling pathway is one of the prominent pathways in EMT. miR-21 and miR-31 were uncovered as the downstream effectors of TGF-β in colon carcinoma cells[22]. miR-21 is significantly elevated in the colorectal cancer with metastasis and negatively regulates the tumor suppressor programmed cell death 4 (Pdcd4) and TGF-β receptor 2, accompanied by the decrease of the downstreaming β-catenin[23,24]. Second, β-catenin was inversely correlated with Qki6/7 and P130, which are down-regulated by miR-574-5p and miR-17, respectively in colorectal carcinoma cells[25,26]. Third, the miR-200 Family was recognized as a master regulator of the epithelial phenotype, which is decreased in colorectal cancer with metastasis. The miR-200 family repressed the EMT by targeting the ZEB1/2, which down-regulate E-cadherin and up-regulate Vimentin. Furthermore, in the upstream of the miR-200 family, promoter methylation and ERβ can be the cause of decrease in colorectal metastasis[25,27,28]. Fourth, miR-9 has been suggested to be another regulator for E-cadherin. Prospero Homeobox 1 (PROX1) was shown to promote EMT by inhibit-

ing E-cadherin *via* miR-9 in colon carcinoma cells[29,30]. Last, but not the least, microRNAs like miR-499-5p and miR-212 also function in regulating EMT, by targeting Pdcd4 and manganese superoxide dismutase (MnSOD)[31,32].

The mesenchymal to epithelial transition (MET) is a reverse biological process of EMT and has recently been suggested to be important for the metastatic cancer cells, by regaining epithelial properties, to establish their colonization in distant organs. MiR-147 was recently found to be able to induce MET in colon cancer cell lines by targeting the TGF-β signaling pathway[33]. Further exploring the roles of microRNAs in MET may provide a deep insight into how the MET is executed in the cancer metastasis.

Thus, many characteristic markers of EMT such as Vimentin, β-Catenin, TCF8-ZEB1, E-Cadherin, Snail and Slug, are targeted by microRNAs. Moreover, many microRNAs were found to have their targets in different types of cancer. For one example, miR-200, by targeting ZEB-1/2, is a tumor suppressor in both breast cancer and colorectal cancer. For another example, ectopic expression of miR-17 plays a role in regulating cancer cell invasion and migration of other malignancy types including colorectal cancer, breast cancer, head and neck cancer by targeting the TGF-β signaling pathway[34]. Therefore, understanding the roles of microRNAs in colorectal cancer metastasis may identify potential therapeutic targets for the treatment of many different types of malignancies.
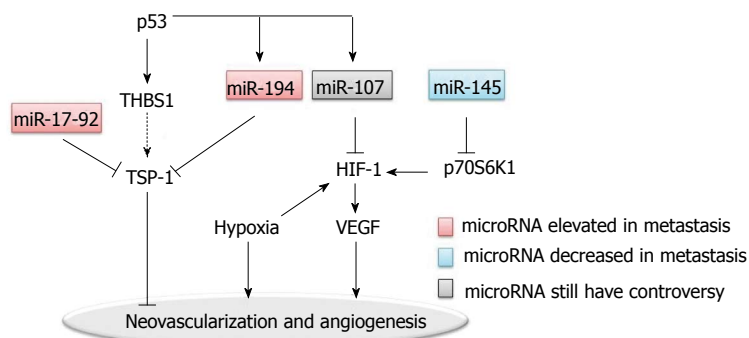
Figure 2 MicroRNAs regulation in the angiogenesis. HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factors; THBS1: Thrombospondin; TSP-1: Thrombospondin-1.

MicroRNAs in angiogenesis

Angiogenesis is considered to be essential for the establishment of blood supply for metastatic lesions in the distant organs. Many pro-angiogenic and anti-angiogenic factors are involved (Figure 2). Tumor angiogenesis is associated with intravasation and extravasation of metastatic cancer cells, suggesting that many crucial molecules in angiogenesis and their corresponding receptors participate in the metastatic process[35]. One of the best studied molecular mechanisms involves the vascular endothelial growth factors (VEGF) and the VEGF receptors. In addition, intratumoral blood vessel is the main route of chemotherapy or targeted drug delivery; but the effectiveness of drug delivery is unsatisfactory due to the disorganized neovascularization in tumors. Clinically, VEGF targeted therapy is used for treating metastatic colorectal cancer, supporting the role of angiogenesis in colorectal cancer metastasis[36,37].

Hypoxia is one of the dominant characteristics of tumor microenvironment; and hypoxia inducible factor (HIF)-1 is one of the key regulators in hypoxia induced angiogenesis[38]. Hypoxic microenvironment drives tumor cells to undergo an angiogenic switch which leads to the production of pro-angiogenesis proteins such as VEGF. The VEGF pathway-mediated neovascularization is also driven partly by additional hypoxia responsive signals including basic FGF (bFGF) and placental growth factor (PlGF).

Epigenetic regulation *via* microRNAs may be one of the major regulatory mechanisms for the disorganization of neovascularization in tumor[39]. MiR-107 was shown to function as a suppresser of HIF-1 and VEGF expression[40]. In addition to miR-107, miR-145 was found to be a regulator of HIF-1 in colon cancer, by targeting p70S6K1 post-transcriptionally[42].

Another well characterized regulator for tumor angiogenesis is thrombospondin-1 (TSP-1), which belongs to the thrombospondins family[43]. TSP-1 acts as a barrier to neovascularization in tumors. MiR-17-92 and miR-194 were both found to repress TSP-1, thereby promoting the angiogenesis in colon cancers[40,44]. Interestingly, MiR-17-92 is a polycistronic microRNA cluster. The precursor transcript derived from this cluster gene produces six mature microRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1[45]. Overexpression of the miR-17-92 cluster was observed in multiple tumor types including colon cancer. As a polycistronic cluster, it can coordinate multiple functions in tumorigenesis including increasing angiogenesis, promoting proliferation and inhibiting differentiation[45,46]. The miR-17-92 microRNA cluster is known to be regulated by the Myc oncogene. The Myc-activated miR-17-92 can stimulate tumor angiogenesis by attenuating the TGF-β signaling pathway, which provides an alternative target for miR-17-92 in addition to TSP-1[47].

Both miR-17-92 and miR-194 are upregulated by the p53 tumor suppressor. miR-194 also negatively regulates the thrombospondin (THBS1) mRNA. On another hand, it is known that p53 induces the transcription of THBS1, which would have further induced the expression of TSP-1, since enhanced expression of THBS1 leads to the induction of TSP-1. Interestingly, p53 does not induce the expression of TSP-1. Thus, it is possible that TSP-1 is suppressed by miR-194 and miR-17-92, which both are regulated by p53[40]. Such a complex regulatory network, by providing multiple feedback mechanisms, allows a more precise regulation in angiogenesis.

MICRORNAS AS POTENTIAL DIAGNOSTIC MARKERS FOR METASTASIS IN COLORECTAL CANCER

MicroRNA is studied as a potential diagnostic marker, considering its commonly conserved existence and remarkable stability[48]. Although plasma CEA has been used as a diagnostic marker for colorectal cancer for decades, one study had shown that only 59% of 417 monitored patients with recurrence had a preceding elevation of CEA concentration[49]. Several recent studies thus have been conducted on the identification of plasma circulating microRNAs, attempting to develop more sensitive and specific detection methods[50-53]. Especially, methods for detecting the circulating microRNAs are attracting because of their noninvasive nature. Recently, a panel of 8 plasma microRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652) was found to be able to distinguish colonic polyps from healthy controls. In addition, this study pointed out that a panel of 3 plasma miRNAs (miR-431, miR-15b, and miR-139-3p) distinguished Stage IV CRC from controls[54].

However, there is still a lack of circulating microR-

Table 1 Summary of microRNAs involved in the tumorigenesis and metastasis of colorectal cancer

	microRNA	Description	Ref.
microRNAs differentially expressed in colorectal cancers	miR-21	Associated to more advanced TNM stages and poorer survival	[11]
	miR-135b	Associated to disease-free and cancer-specific survival	[12]
	miR-150*, miR-125b-2*, miR-1179 and miR-139-3p	Up-regulated in colorectal cancer with metastasis.	[13]
microRNAs as diagnostic markers	Serum miR-29a	Up-regulated in patients with metastasis	[55]
	Circulating miR-221	Associated to poor survival	[53]
	Plasma miR-31	Up-regulated in stage Ⅲ and Ⅳ	[51]
microRNAs involved in epithelial-mesenchymal transition	miR-21	Pdcd4,TGFβ receptor 2	[23,24]
	miR-574-5p,miR-17	Qki6/7,P130	[25,26]
	miR-200	ZEB1/2	[27,28]
	miR-9	E-cadherin	[29,30]
	miR-499-5p	Pdcd4	[31]
	miR-212	MnSOD	[32]
microRNAs involved in Angiogenesis	miR-17-92	TSP-1	[44]
	miR-194	TSP-1,THBS1	[40]
	miR-107	HIF-1,VEGF,DAPK,KLF4	[41]
	miR-145	p70S6K1	[42]

MnSOD: Manganese superoxide dismutase; TSP-1: Thrombospondin-1; THBS1: Thrombospondin; HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factors.

NAs as an ideal biomarker for recurrence and metastasis. Plasma miR-92 and miR-29 were significantly elevated in colorectal cancer patients, but demonstrated no significant difference between different stages of colorectal cancer[50,52]. Nevertheless, in a more recent study by Wang *et al*[55] serum level of the miR-29a was found to be significantly higher in colorectal cancer patients with metastasis comparing to those without metastasis, with a sensitivity of 75% and a specificity of 75%. Similarly, circulating miR-221 was demonstrated to be a significant prognostic factor associated with poor overall survival. Whether it can be used for early detection of recurrence is not studied[53]. Another two more recent studies found several promising microRNAs for the detection of recurrence. Kanaan *et al*[51] demonstrated that plasma miR-31, miR-135b, miR-1 and miR-133a have a 100% sensitivity and a 80% specificity in detecting colorectal cancer, and also found that miR-31 was significantly more upregulated in stage Ⅲ and Ⅳ than stage Ⅰ and Ⅱ. Hofsli *et al*[56] reported that in their "training" study conducted with with serum samples from 30 patients with stage Ⅳ colon cancer and from 10 healthy controls, 375 miRNAs were found to be more abundant in the sera from colon cancer patients than those from healthy controls, including miR-103, miR-107, and miR-221. These miRNAs were also found in sera from patients with stage Ⅰ-Ⅱ colon cancer; however, their roles in stage Ⅳ colon cancer remain interesting to be explored.

CONCLUSION

microRNAs play a substantial role in the epigenetic regulation of colorectal cancer metastasis. Differential expression of microRNAs is reported in the metastasis of colorectal cancer comparing to non-metastatic colorectal cancer (Table 1). More importantly, these microRNAs appear to form a network to coordinate the regulation of the metastatic process. Studies investigating the association of microRNAs with the metastasis process are highly desired. Plasma microRNA remains to be identified as a noninvasive biomarker for early diagnosis of colorectal cancer metastasis. Enlightened by the function of microRNAs in the EMT process and angiogenesis, mechanisms of colorectal cancer metastasis may be revealed by dissecting the regulatory network of microRNAs.

REFERENCES

1   **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]
2   **American Cancer Society**. Cancer Facts & Figures 2013. Atlanta: American Cancer Society, 2013
3   **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297 [PMID: 14744438]
4   **Lee YS**, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol* 2009; **4**: 199-227 [PMID: 18817506 DOI: 10.1146/annurev. pathol.4.110807.092222]
5   **Nagel R**, le Sage C, Diosdado B, van der Waal M, Oude Vrielink JA, Bolijn A, Meijer GA, Agami R. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. *Cancer Res* 2008; **68**: 5795-5802 [PMID: 18632633 DOI: 10.1158/0008-5472.CAN-08-0951]
6   **Necela BM**, Carr JM, Asmann YW, Thompson EA. Differential expression of microRNAs in tumors from chronically inflamed or genetic (APC(Min/+)) models of colon cancer. *PLoS One* 2011; **6**: e18501 [PMID: 21532750 DOI: 10.1371/ journal.pone.0018501]
7   **Wang X**, Lam EK, Zhang J, Jin H, Sung JJ. MicroRNA-122a functions as a novel tumor suppressor downstream of adenomatous polyposis coli in gastrointestinal cancers. *Biochem Biophys Res Commun* 2009; **387**: 376-380 [PMID: 19607815 DOI: 10.1016/j.bbrc.2009.07.034]
8   **Feng Z**, Zhang C, Wu R, Hu W. Tumor suppressor p53 meets microRNAs. *J Mol Cell Biol* 2011; **3**: 44-50 [PMID: 21278451 DOI: 10.1093/jmcb/mjq040]
9   **He L**, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA, Hannon GJ. A microRNA component of the p53 tumour suppressor network. *Nature* 2007; **447**: 1130-1134 [PMID: 17554337 DOI: 10.1038/na-

ture05939]

10   **Cummins JM**, He Y, Leary RJ, Pagliarini R, Diaz LA, Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B, Velculescu VE. The colorectal microRNAome. *Proc Natl Acad Sci USA* 2006; **103**: 3687-3692 [PMID: 16505370 DOI: 10.1073/pnas.0511155103]

11   **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436 [PMID: 18230780 DOI: 10.1001/jama.299.4.425]

12   **Gaedcke J**, Grade M, Camps J, Søkilde R, Kaczkowski B, Schetter AJ, Difilippantonio MJ, Harris CC, Ghadimi BM, Møller S, Beissbarth T, Ried T, Litman T. The rectal cancer microRNAome--microRNA expression in rectal cancer and matched normal mucosa. *Clin Cancer Res* 2012; **18**: 4919-4930 [PMID: 22850566 DOI: 10.1158/1078-0432.CCR-12-0016]

13   **Lin M**, Chen W, Huang J, Gao H, Ye Y, Song Z, Shen X. MicroRNA expression profiles in human colorectal cancers with liver metastases. *Oncol Rep* 2011; **25**: 739-747 [PMID: 21174058 DOI: 10.3892/or.2010.1112]

14   **Balaguer F**, Moreira L, Lozano JJ, Link A, Ramirez G, Shen Y, Cuatrecasas M, Arnold M, Meltzer SJ, Syngal S, Stoffel E, Jover R, Llor X, Castells A, Boland CR, Gironella M, Goel A. Colorectal cancers with microsatellite instability display unique miRNA profiles. *Clin Cancer Res* 2011; **17**: 6239-6249 [PMID: 21844009 DOI: 10.1158/1078-0432.CCR-11-1424]

15   **Landi D**, Gemignani F, Landi S. Role of variations within microRNA-binding sites in cancer. *Mutagenesis* 2012; **27**: 205-210 [PMID: 22294768 DOI: 10.1093/mutage/ger055]

16   **Nishida N**, Nagahara M, Sato T, Mimori K, Sudo T, Tanaka F, Shibata K, Ishii H, Sugihara K, Doki Y, Mori M. Microarray analysis of colorectal cancer stromal tissue reveals upregulation of two oncogenic miRNA clusters. *Clin Cancer Res* 2012; **18**: 3054-3070 [PMID: 22452939 DOI: 10.1158/1078-0432.CCR-11-1078]

17   **Sarver AL**, French AJ, Borralho PM, Thayanithy V, Oberg AL, Silverstein KA, Morlan BW, Riska SM, Boardman LA, Cunningham JM, Subramanian S, Wang L, Smyrk TC, Rodrigues CM, Thibodeau SN, Steer CJ. Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC Cancer* 2009; **9**: 401 [PMID: 19922656 DOI: 10.1186/1471-2407-9-401]

18   **Slattery ML**, Wolff E, Hoffman MD, Pellatt DF, Milash B, Wolff RK. MicroRNAs and colon and rectal cancer: differential expression by tumor location and subtype. *Genes Chromosomes Cancer* 2011; **50**: 196-206 [PMID: 21213373 DOI: 10.1002/gcc.20844]

19   **Kerr D**. Clinical development of gene therapy for colorectal cancer. *Nat Rev Cancer* 2003; **3**: 615-622 [PMID: 12894249 DOI: 10.1038/nrc1147]

20   **Goel A**, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology* 2012; **143**: 1442-1460.e1 [PMID: 23000599 DOI: 10.1053/j.gastro.2012.09.032]

21   **Kalluri R**, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**: 1420-1428 [PMID: 19487818 DOI: 10.1172/JCI39104]

22   **Cottonham CL**, Kaneko S, Xu L. miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. *J Biol Chem* 2010; **285**: 35293-35302 [PMID: 20826792 DOI: 10.1074/jbc.M110.160069]

23   **Asangani IA**, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) posttranscriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2008; **27**: 2128-2136 [PMID: 17968323 DOI: 10.1038/sj.onc.1210856]

24   **Yu Y**, Kanwar SS, Patel BB, Oh PS, Nautiyal J, Sarkar FH, Majumdar AP. MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFβR2) in colon cancer cells. *Carcinogenesis* 2012; **33**: 68-76 [PMID: 22072622 DOI: 10.1093/carcin/bgr246]

25   **Edvardsson K**, Nguyen-Vu T, Kalasekar SM, Pontén F, Gustafsson JÅ, Williams C. Estrogen receptor β expression induces changes in the microRNA pool in human colon cancer cells. *Carcinogenesis* 2013; **34**: 1431-1441 [PMID: 23436804 DOI: 10.1093/carcin/bgt067]

26   **Ji S**, Ye G, Zhang J, Wang L, Wang T, Wang Z, Zhang T, Wang G, Guo Z, Luo Y, Cai J, Yang JY. miR-574-5p negatively regulates Qki6/7 to impact β-catenin/Wnt signalling and the development of colorectal cancer. *Gut* 2013; **62**: 716-726 [PMID: 22490519 DOI: 10.1136/gutjnl-2011-301083]

27   **Chen ML**, Liang LS, Wang XK. miR-200c inhibits invasion and migration in human colon cancer cells SW480/620 by targeting ZEB1. *Clin Exp Metastasis* 2012; **29**: 457-469 [PMID: 22407310 DOI: 10.1007/s10585-012-9463-7]

28   **Davalos V**, Moutinho C, Villanueva A, Boque R, Silva P, Carneiro F, Esteller M. Dynamic epigenetic regulation of the microRNA-200 family mediates epithelial and mesenchymal transitions in human tumorigenesis. *Oncogene* 2012; **31**: 2062-2074 [PMID: 21874049 DOI: 10.1038/onc.2011.383]

29   **Lu MH**, Huang CC, Pan MR, Chen HH, Hung WC. Prospero homeobox 1 promotes epithelial-mesenchymal transition in colon cancer cells by inhibiting E-cadherin via miR-9. *Clin Cancer Res* 2012; **18**: 6416-6425 [PMID: 23045246 DOI: 10.1158/1078-0432.CCR-12-0832]

30   **Zhu L**, Chen H, Zhou D, Li D, Bai R, Zheng S, Ge W. MicroRNA-9 up-regulation is involved in colorectal cancer metastasis via promoting cell motility. *Med Oncol* 2012; **29**: 1037-1043 [PMID: 21562850 DOI: 10.1007/s12032-011-9975-z]

31   **Liu X**, Zhang Z, Sun L, Chai N, Tang S, Jin J, Hu H, Nie Y, Wang X, Wu K, Jin H, Fan D. MicroRNA-499-5p promotes cellular invasion and tumor metastasis in colorectal cancer by targeting FOXO4 and PDCD4. *Carcinogenesis* 2011; **32**: 1798-1805 [PMID: 21934092 DOI: 10.1093/carcin/bgr213]

32   **Meng X**, Wu J, Pan C, Wang H, Ying X, Zhou Y, Yu H, Zuo Y, Pan Z, Liu RY, Huang W. Genetic and epigenetic downregulation of microRNA-212 promotes colorectal tumor metastasis via dysregulation of MnSOD. *Gastroenterology* 2013; **145**: 426-436.e1-6 [PMID: 23583431 DOI: 10.1053/j.gastro.2013.04.004]

33   **Lee CG**, McCarthy S, Gruidl M, Timme C, J. YT. MicroRNA-147 Induces a Mesenchymal-To-Epithelial Transition (MET) and Reverses EGFR Inhibitor Resistance. *PloS One* 2014; **9**: e84597 [DOI: 10.1371/journal.pone.0084597.g001]

34   **Mogilyansky E**, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 2013; **20**: 1603-1614 [PMID: 24212931 DOI: 10.1038/cdd.2013.125]

35   **Weis S**, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* 2004; **167**: 223-229 [PMID: 15504909 DOI: 10.1083/jcb.200408130]

36   **Rmali KA**, Puntis MC, Jiang WG. Tumour-associated angiogenesis in human colorectal cancer. *Colorectal Dis* 2007; **9**: 3-14 [PMID: 17181841 DOI: 10.1111/j.1463-1318.2006.01089.x]

37   **Sun W**. Angiogenesis in metastatic colorectal cancer and the benefits of targeted therapy. *J Hematol Oncol* 2012; **5**: 63 [PMID: 23057939 DOI: 10.1186/1756-8722-5-63]

38   **Liao D**, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 2007; **26**: 281-290 [PMID: 17603752 DOI: 10.1007/s10555-007-9066-y]

39   **Heusschen R**, van Gink M, Griffioen AW, Thijssen VL. MicroRNAs in the tumor endothelium: novel controls on the angioregulatory switchboard. *Biochim Biophys Acta* 2010; **1805**: 87-96 [PMID: 19782719 DOI: 10.1016/j.bbcan.2009.09.005]

40 **Sundaram P**, Hultine S, Smith LM, Dews M, Fox JL, Biyashev D, Schelter JM, Huang Q, Cleary MA, Volpert OV, Thomas-Tikhonenko A. p53-responsive miR-194 inhibits thrombospondin-1 and promotes angiogenesis in colon cancers. *Cancer Res* 2011; **71**: 7490-7501 [PMID: 22028325 DOI: 10.1158/0008-5472.CAN-11-1124]

41 **Chen HY**, Lin YM, Chung HC, Lang YD, Lin CJ, Huang J, Wang WC, Lin FM, Chen Z, Huang HD, Shyy JY, Liang JT, Chen RH. miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. *Cancer Res* 2012; **72**: 3631-3641 [PMID: 22593189 DOI: 10.1158/0008-5472.CAN-12-0667]

42 **Xu Q**, Liu LZ, Qian X, Chen Q, Jiang Y, Li D, Lai L, Jiang BH. MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis. *Nucleic Acids Res* 2012; **40**: 761-774 [PMID: 21917858 DOI: 10.1093/nar/gkr730]

43 **Lawler J**. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med* 2002; **6**: 1-12 [PMID: 12003665]

44 **Dews M**, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, Furth EE, Lee WM, Enders GH, Mendell JT, Thomas-Tikhonenko A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet* 2006; **38**: 1060-1065 [PMID: 16878133 DOI: 10.1038/ng1855]

45 **Olive V**, Jiang I, He L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. *Int J Biochem Cell Biol* 2010; **42**: 1348-1354 [PMID: 20227518 DOI: 10.1016/j.biocel.2010.03.004]

46 **Volinia S**, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006; **103**: 2257-2261 [PMID: 16461460 DOI: 10.1073/pnas.0510565103]

47 **Dews M**, Fox JL, Hultine S, Sundaram P, Wang W, Liu YY, Furth E, Enders GH, El-Deiry W, Schelter JM, Cleary MA, Thomas-Tikhonenko A. The myc-miR-17~92 axis blunts TGF{beta} signaling and production of multiple TGF{beta}-dependent antiangiogenic factors. *Cancer Res* 2010; **70**: 8233-8246 [PMID: 20940405 DOI: 10.1158/0008-5472.CAN-10-2412]

48 **Ichikawa D**, Komatsu S, Konishi H, Otsuji E. Circulating microRNA in digestive tract cancers. *Gastroenterology* 2012; **142**: 1074-1078.e1 [PMID: 22433392 DOI: 10.1053/j.gastro.2012.03.008]

49 **Moertel CG**, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Tangen C. An evaluation of the carcinoembryonic antigen (CEA) test for monitoring patients with resected colon cancer. *JAMA* 1993; **270**: 943-947 [PMID: 8141873 DOI: 10.1001/jama.1993.03510080047030]

50 **Huang Z**, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer* 2010; **127**: 118-126 [PMID: 19876917 DOI: 10.1002/ijc.25007]

51 **Kanaan Z**, Rai SN, Eichenberger MR, Roberts H, Keskey B, Pan J, Galandiuk S. Plasma miR-21: a potential diagnostic marker of colorectal cancer. *Ann Surg* 2012; **256**: 544-551 [PMID: 22868372 DOI: 10.1097/SLA.0b013e318265bd6f]

52 **Ng EK**, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 2009; **58**: 1375-1381 [PMID: 19201770 DOI: 10.1136/gut.2008.167817]

53 **Pu XX**, Huang GL, Guo HQ, Guo CC, Li H, Ye S, Ling S, Jiang L, Tian Y, Lin TY. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. *J Gastroenterol Hepatol* 2010; **25**: 1674-1680 [PMID: 20880178 DOI: 10.1111/j.1440-1746.2010.06417.x]

54 **Kanaan Z**, Roberts H, Eichenberger MR, Billeter A, Ocheretner G, Pan J, Rai SN, Jorden J, Williford A, Galandiuk S. A plasma microRNA panel for detection of colorectal adenomas: a step toward more precise screening for colorectal cancer. *Ann Surg* 2013; **258**: 400-408 [PMID: 24022433 DOI: 10.1097/SLA.0b013e3182a15bcc]

55 **Wang LG**, Gu J. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. *Cancer Epidemiol* 2012; **36**: e61-e67 [PMID: 22018950 DOI: 10.1016/j.canep.2011.05.002]

56 **Hofsli E**, Sjursen W, Prestvik WS, Johansen J, Rye M, Tranø G, Wasmuth HH, Hatlevoll I, Thommesen L. Identification of serum microRNA profiles in colon cancer. *Br J Cancer* 2013; **108**: 1712-1719 [PMID: 23558896 DOI: 10.1038/bjc.2013.121]

REVIEW

KAPtain in charge of multiple missions: Emerging roles of KAP1

Chun-Ting Cheng, Ching-Ying Kuo, David K Ann

Chun-Ting Cheng, Ching-Ying Kuo, David K Ann, Department of Molecular Pharmacology, Beckman Research Institute, City of Hope, Duarte, CA 91010-3000, United States
Chun-Ting Cheng, David K Ann, Irell and Manella Graduate School of Biological Sciences, Beckman Research Institute, City of Hope, Duarte, CA 91010-3000, United States
Author contributions: Cheng CT, Kuo CY and Ann DK contributed solely to this paper.
Correspondence to: David K Ann, PhD, Department of Molecular Pharmacology, Beckman Research Institute, City of Hope, Rm4115, 1500 E Duarte Rd, Duarte, CA 91010-3000, United States. dann@coh.org
Telephone: +1-626-3598111   Fax: +1-626-4717204
Received: November 28, 2014 Revised: March 21, 2014
Accepted: June 20, 2014
Published online: August 26, 2014

Abstract

KAP1/TRIM28/TIF1β was identified nearly twenty years ago as a universal transcriptional co-repressor because it interacts with a large KRAB-containing zinc finger protein (KRAB-ZFP) transcription factor family. Many studies demonstrate that KAP1 affects gene expression by regulating the transcription of KRAB-ZFP-specific loci, trans-repressing as a transcriptional co-repressor or epigenetically modulating chromatin structure. Emerging evidence suggests that KAP1 also functions independent of gene regulation by serving as a SUMO/ubiquitin E3 ligase or signaling scaffold protein to mediate signal transduction. KAP1 is subjected to multiple post-translational modifications (PTMs), including serine/tyrosine phosphorylation, SUMOylation, and acetylation, which coordinately regulate KAP1 function and its protein abundance. KAP1 is involved in multiple aspects of cellular activities, including DNA damage response, virus replication, cytokine production and stem cell pluripotency. Moreover, knockout of KAP1 results in embryonic lethality, indicating that KAP1 is crucial for embryonic development and possibly impacts a wide-range of (patho)physiological manifestations. Indeed, studies from conditional knockout mouse models reveal that KAP1-deficiency significantly impairs vital physiological processes, such as immune maturation, stress vulnerability, hepatic metabolism, gamete development and erythropoiesis. In this review, we summarize and evaluate current literatures involving the biochemical and physiological functions of KAP1. In addition, increasing studies on the clinical relevance of KAP1 in cancer will also be discussed.

Core tip: This review article primarily summarizes the current findings of KAP1/TRIM28/TIF1β, with focuses on its biochemical and physiological functions. Both the canonical transcriptional co-repressor function and the transcriptional-independent roles of KAP1 are discussed in detail. We highlight the post-translational modifications and the compartmentalized localization of KAP1 and suggest that the function of KAP1 could be spatial and temporal regulated in multiple physiological circumstances. Finally, we summarize the clinical relevance of KAP1 in cancer and discuss the possibility to translate the mechanistic studies of KAP1 to human pathophysiology in the future.

Cheng CT, Kuo CY, Ann DK. KAPtain in charge of multiple missions: Emerging roles of KAP1. *World J Biol Chem* 2014; 5(3): 308-320 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/308.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.308

INTRODUCTION

KRAB domain-associated protein 1 (KAP1), also known as

tripartite motif-containing 28 (TRIM28) or transcriptional intermediary factor 1 beta (TIF1β) was identified as an interacting protein for Krüppel-associated box zinc finger proteins (KRAB-ZFPs) in 1996[1-4]. Since then, KAP1 has been reported in regulating multiple aspects of physiology, for examples, cell differentiation, DNA damage response (DDR), virus replication, immune response and tumorigenesis (Figure 1). As KAP1 binds to the conserved KRAB repression domain, which is present in many transcription factors, KAP1 is considered as a critical transcriptional co-repressor[2-4]. For instance, many proteins involved in chromatin remodeling or histone modification, such as heterochromatin-associated protein 1 (HP1), nuclear co-repressor (N-CoR), histone deacetylase (HDAC), chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase (CHD3/NuRD), histone methyltransferases (HMTs), have been identified in KAP1-containing complexes[5-9] (Table 1). Consequently, KAP1 epigenetically regulates gene expression through multiple transcriptional co-repressor complexes. In addition to regulating KRAB-ZFPs, the activity of transcription factors lacking KRAB domain, such as c-Myc and E2F1, can also be regulated by KAP1[10-14]. KAP1 is subjected to multiple post-translational modifications (PTMs), including phosphorylation and SUMOylation (Figure 2). We and others have demonstrated that these PTMs coordinately regulate the gene repressive function of KAP1[15-18].

Deletion of *Kap1* in mouse embryo leads to embryonic lethality[19], suggesting that KAP1 is critical during embryonic development and should be involved in a wide-range of biological/physiological processes. Although most of the KAP1 studies have been focusing on its transcriptional functions, emerging evidence suggests that KAP1 also exerts transcription-independent function. Given that KAP1 functions as a scaffold protein to constitute KAP1-containing complexes that regulate chromatin structure, it also plays an important role in maintaining genome stability by facilitating DNA repair in response to DNA damage through chromatin remodeling[20-22]. Moreover, the RING and the plant homeodomain (PHD) domains of KAP1 possess intrinsic enzymatic activity to potentially catalyze SUMOylation and ubiquitylation[15,23-25]. Interestingly, KAP1 resides in distinct cellular compartments, including the pericentric and centromeric heterochromatin, euchromatin and cytoplasm[6,25,26], implicating its essential functions for different cellular activities. Taken together, the diverse function and regulated subcellular localization suggest that KAP1 impacts multiple aspects of biological processes and it warrants more rigorous investigations of this important protein in the future. In this review, we aim to discuss both the transcriptional and non-transcriptional functions of KAP1. The PTMs of KAP1 involved in these processes will be highlighted. Lastly, the clinical relevance of KAP1 in cancer will also be elaborated.

PROTEIN STRUCTURE AND PTMS OF KAP1

Given that the overall structure of KAP1 has been exten-
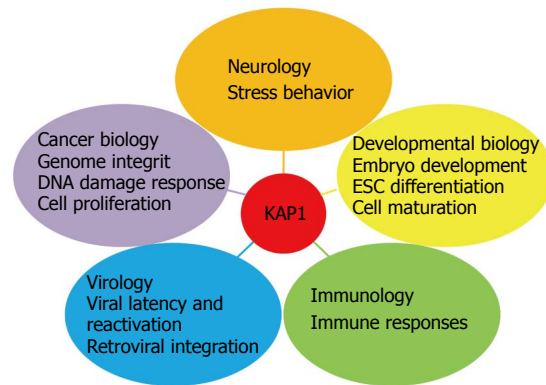
Figure 1 KRAB domain-associated protein 1 is involved in multiple aspects of cellular physiology. Neurology: *kap1* knockout in mouse forebrain induces higher level of anxiety-like behavior. Developmental Biology: Several conditional *kap1* deletions impair normal cell development including embryonic stem cell (ESC) differentiation, spermatogenesis, erythropoiesis, and the development of T-cell and B-cell. Immunology: KAP1 is involved in immune responses by regulating T/B cell activity and immune tolerance. Virology: KAP1 is critical to suppress retroviral activation and prevent HIV integration. Cancer Biology: KAP1 is positively or negatively correlated with prognosis in different cancer types. The roles of KAP1 in maintaining genome stability, mediating DNA damage response and affecting cell proliferation *in vitro* imply its potential roles during tumorigenesis.

sively reviewed previously[27] (and references therein) we will mainly focus on the PTMs of KAP1 and how these PTMs crosstalk to each other and affect the interaction between KAP1 and its partners. KAP1 is a member of TIF1 family, which includes four proteins, TIF1α, TIF1β, TIF1γ and TIF1δ. As the other members in the TIF1 family, KAP1 has an N-terminal tripartite motif (TRIM), RBCC domain, which is composed by a RING finger, 2 B-box zinc fingers and a coiled-coil region. In addition, KAP1 also shares a central TIF1 signature sequence (TSS), an HP1 binding domain (HP1BD), a C-terminal combination of PHD and bromodomain with the other TIF1 members. Different from the other TIF1 proteins, KAP1 does not have a nuclear receptor (NR) box[27] (Figure 2).

The RBCC domain of KAP1 interacts with various KRAB-ZFPs and is considered as an important region for the KAP1 recruitment to KRAB-ZFP binding sites across the genome[28,29]. It has been demonstrated that RBCC domain forms a homotrimer with a single KRAB domain[30]. Interestingly, a recent study suggests that KAP1 can still bind to promoter regions without RBCC domain, suggesting additional mechanisms that might contribute to the KAP1 recruitment to transcription factors on the promoter regions[31]. The TSS domain is adjacent to the RBCC domain, and is required for the transcriptional repressive activity of TIF1γ[32]. However, the function of TSS in KAP1 has yet to been defined.

The hydrophobic PxVxL pentapeptide is located at the central region of KAP1, namely HP1BD. HP1BD interacts with the chromoshadow domain of HP1 proteins and this KAP1-HP1 interaction is critical for the KAP1-mediated gene silencing[5,6,33,34]. It is believed that KAP1-HP1 complex plays a critical role in heterochromatin maintenance and gene silencing. In fact, HP1BD
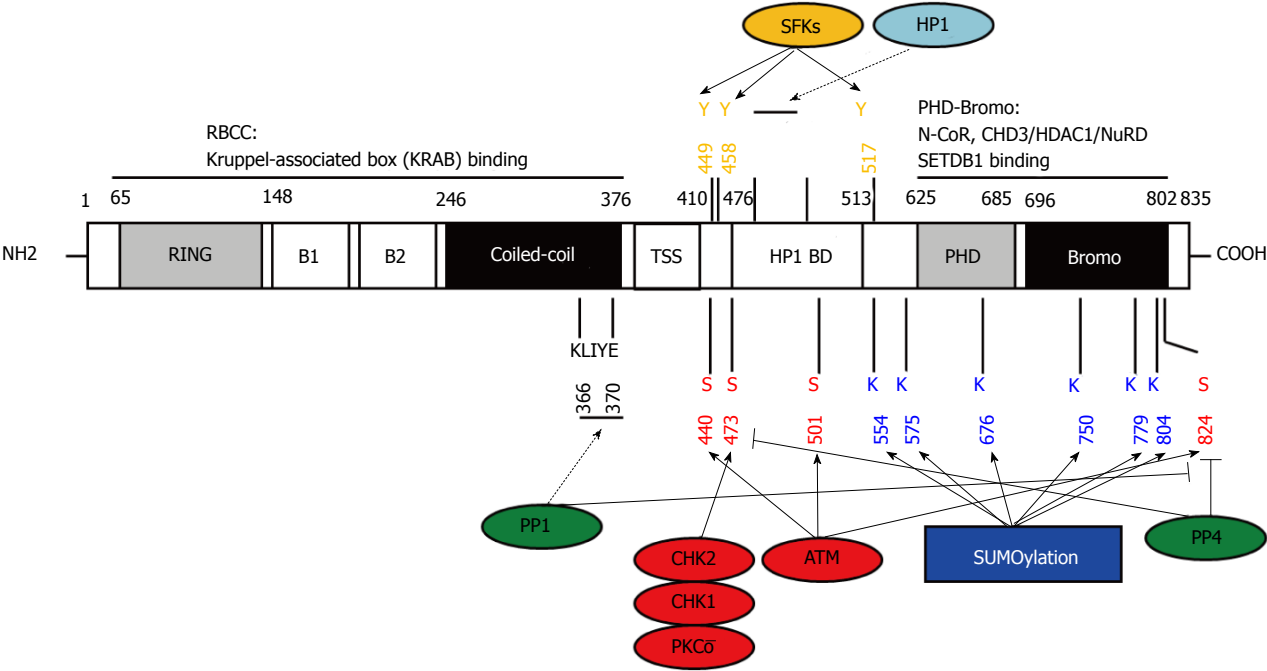
Figure 2 KAP1 structure, post-translational modifications and interacting proteins. KAP1 has multi-domains for protein-protein interaction and post-translational modification. RBCC: RING-B1-B2-coiled-coil; TSS: TIF signature sequence; HP1 BD: HP1 binding domain; PHD: Plant homeo domain. Numbers represent the sequence of amino acids; Blue: SUMOylation sites; Red: Serine phosphorylation sites targeted by the indicated kinases (shown in red) or antagonized by phosphatases (shown in green); Orange: Tyrosine phosphorylation sites targeted by the indicated kinase family; KLIYF: PP1 binding site; PxVxL: HP1 binding site; Dotted lines: Protein-protein interaction; SFKs: Src family kinases; HP1: Heterochromatin-associated protein 1; ATM: Ataxia-telangiectasia mutated; PP1: Protein phosphatase 1; CHD3/NuRD: Chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase; HDAC: Histone deacetylase.

Table 1 Chromatin-associated factors/chromatin-remodeling enzymes interacting with KAP1

Chromatin-associated factors/ chromatin-remodeling enzymes	Consequences of binding with KAP1	Ref.
HP1	HP1-KAP1 interaction leads to transcriptional repression and has an essential role in development and cell differentiation. Phosphorylation at Ser-473 or Tyr-449, 458, 517 of KAP1 inhibits its interaction with HP1.	[5,6,33,43,46,53,94, 97,105]
SETDB1	KAP1 binds to SETDB1 through SUMO:SIM interaction to methylate H3K9 at gene regulatory regions to achieve gene silencing.	[9,15,16,109]
N-CoR	N-CoR represses basal transcription by the recruitment of HDACs to deacetylate histones. KAP1 is involved in N-CoR-1 complex to mediate transcriptional repression.	[7]
CHD3 (Mi-2α)/NuRD	NuRD complex mediates chromatin remodelling and histone deacetylation *via* CHD3 (Mi-2α) and HDACs, respectively. KAP1 interacts with NuRD complex *via* PHD and bromodomain to alter the chromatin structure.	[8,21,37]
HDAC1	KAP1-HDAC1 complex interaction not only regulates histone modification but also non-histone protein deacetylation to exert a variety of different functions (also shown in Table 2).	[113]
SMARCAD1	SMARCAD1 mediates histone deacetylation and associates with KAP1-HDAC1 complex to regulate chromatin marks.	[58]
DNMT	KAP1 associates with DNMT to maintain DNA methylation at imprinting control region (also shown in Table 2).	[59,61]

HP1: Heterochromatin-associated protein 1; ATM: Ataxia-telangiectasia mutated; PP1: Protein phosphatase 1; CHD3/NuRD: Chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase; HDAC: Histone deacetylase; N-CoR: Nuclear co-repressor; SETDB1: Bifurcated 1; DNMT: DNA methyltransferase.

is required for nuclear retention of KAP1 and disrupting KAP1-HP1 interaction reactivates the imprinted gene expression by perturbing histone and DNA methylation levels *in vivo*[31,35]. However, the detailed mechanism underlying the KAP1 recruitment to HP1 on heterochromatin remains to be elucidated.

The C-terminal PHD and bromodomain of KAP1 (PB domain) recognize histone tail and are also required for the KAP1-mediated gene silencing by recruiting histone modifiers. Specifically, KAP1 interacts with CHD3/NuRD complex, and histone methyltransferase, SET domain, bifurcated 1 (SETDB1)[8,9]. These observations suggest a model for KAP1-dependent recruitment of histone modifiers for histone methylation and heterochromatin formation to achieve gene silencing[27].

Notably, the carboxyl-terminus of KAP1 is subjected

to multiple types of PTM. Several studies have revealed that KAP1 is SUMOylated and the SUMOylation of KAP1 is required for its repressive function[15,16,18]. Within or adjacent to the PB domain, six lysines of 554, 575, 676, 750, 779 and 804 have been validated as SUMOylation sites, and the distinct SUMOylation combinations differentially affect the interaction between the bromodomain with SETDB1 and CHD3[15,16,18,28]. KAP1 also undergoes auto-SUMOylation[15]. The magnitude of KAP1 SUMOylation is balanced by deSUMOylases, SENP1, sentrin specific peptidase (SENP7) and the phosphorylation status at serine-824 of KAP1[15,17,36,37].

The serine-824 of KAP1 is primarily phosphorylated by phosphoinositide 3 kinase-like protein kinases (PIKKs), including ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs)[38]. This specific serine 824-phosphorylation is crucial for DDR in different aspects, for examples, the ATM-mediated KAP1 serine-824 phosphorylation is responsible for activating DNA damage checkpoints and chromatin relaxation[17,20], whereas ATR or DNA-PKcs presumably compensates for ATM-deficiency during DDR[39]. Our laboratory has demonstrated that protein phosphatase 1 (PP1) interacts with KAP1 through the PP1-binding motif in the coiled-coil region of KAP1 to dephosphorylate KAP1 at serine-824[36]. In addition, protein phosphatase 4 (PP4) also mediates the KAP1 dephosphorylation at serine 824 and another phosphorylation site at serine-473 upon DNA damage[40,41].

Many DNA damage-inducing agents render both KAP1 serine-824 phosphorylation and serine-473 phosphorylation through ATM-Chk2 or ATR-Chk1 pathways[42]. KAP1 serine-473 phosphorylation is also involved in efficient DNA repair and cell survival upon DNA damage[42-44]. However, unlike the phosphorylation at serine-824, the KAP1 serine-473 phosphorylation is diffusely localized in the nucleus instead of accumulating at damage sites and forming foci[44,45]. It remains to be established whether different KAP1 phosphorylation sites play distinct roles in response to DNA damage. Interestingly, the KAP1 serine-473 phosphorylation regulates cell cycle progression. A study demonstrated that PKCδ phosphorylates KAP1 at serine-473 during S phase of cell cycle. This event dampens the KAP1 and HP1γ interaction and de-represses cyclin A2 to promote S phase progression[46]. Conceivably, KAP1 serine-473 phosphorylation perturbs its association with HP1γ, thereby rendering pan-nuclear distribution. This may be due to the structural alteration of PxVxL domain, which is the HP1 binding site of KAP1. KAP1 mutant that loses its binding ability with HP1 indeed translocates to cytoplasm[31]. This implies that KAP1 serine-473 phosphorylation could function beyond gene regulation in cytoplasm. Results from several studies also suggest that KAP1 serine-473 phosphorylation is associated with immune response. First, KAP1 is constitutively phosphorylated at serine-473 but not serine-824 upon T cell receptor activation in thymocytes[47,48]. Second, Kaposi's Sarcoma-

Associated Herpesvirus (KHSV) infection induces phosphorylation at KAP1 serine-473 in endothelial cells[49]. Many KAP1's partners, such as STAT1/3, NF-κB and IRF5/7[24,49-52], are involved in inflammation and immune response. To understand the role of KAP1 serine-473 phosphorylation in the immune responses induced by viral infection or other types of stress could be rewarding. More recently, Kubota *et al*[53] reported that tyrosines-449, 458 and 517 of KAP1 are phosphorylated by Src family kinases (SFKs). The phosphorylation of these tyrosine residues also interferes the interaction between KAP1 and HP1. As SFKs are involved in regulating a wide-range of oncogenic processes, including cell growth and differentiation, this finding implies that KAP1 plays a role in SFK-mediated oncogenic transformation.

KAP1 is also demonstrated to be acetylated and the level of KAP1 acetylation is downregulated by HDAC10[54]. Although HDAC10 regulates KAP1 transcriptional co-repressor activity, there is no direct evidence showing whether the acetylation affects KAP1-mediated transcriptional control. Studies on the regulation of KAP1 acetylation and whether KAP1 acetylation crosstalks with other types of KAP1 lysine-PTM are expected to provide additional insights into the roles of PTMs in governing KAP1 function.

TRANSCRIPTIONAL FUNCTIONS OF KAP1

KAP1 was originally identified to be associated with KRAB-ZFPs, suggesting a role of KAP1 in transcriptional control. In addition to the RBCC domain, there are also other domains allowing KAP1 to interact with a wide variety of proteins such as histone acetylases (HATs), HDACs, HMTs and DNA methyltransferases (DNMTs). Thus, KAP1 has the flexibility to regulate transcription through multiple mechanisms, possibly depending on its PTMs, location and specific binding partners (Figure 2, Tables 1 and 2).

Histone modification and chromatin remodeling

RBCC domain is required for the recruitment of KAP1 to KRAB-ZFPs, and KAP1-binding sites are enriched in the promoter regions of KRAB-ZFPs, suggesting an auto-regulation between KAP1 and KRAB-ZFPs[55]. However, a recent genome-wide study reports that KAP1 does not regulate the expression of those ZFPs and other genes that KAP1 is bound to their promoter regions. Instead, KAP1 regulates the expression of genes with KAP1 binding sites distant from their transcription start sites. Furthermore, deletion of the RBCC domain did not profoundly affect the binding of KAP1 to corresponding promoter regions[31]. Considering that KAP1 interacts with HP1 and other histone modifiers and is involved in long-range transcriptional repression[56], KAP1-mediated chromatin remodeling might contribute to the majority of the KAP1-mediated transcriptional repression.

In addition to the basal transcription, KAP1-mediated

Table 2 Transcription factors involved in KAP1-regulated gene expression

Transcription factor	KAP1 function	Ref.
Myc	KAP1 is involved in MM-1 and HDAC1 complex to suppress c-Myc transcription activity	[10,11]
ZNF160	KAP1 interacts with ZNF160 and recruits HDAC to downregulate TLR4 in intestinal epithelial cells	[79]
Oct3/4	KAP1 is potentially involved in regulating pluripotency of embryonic stem cells	[78]
E2F1	KAP1 suppresses E2F1 transcriptional activity and inhibits E2F1 acetylation in an HDAC1 dependent manner	[13]
p53	KAP1 is associated with MDM2-p53-HDAC1 complex and inhibits p53 acetylation and promotes p53 degradation	[75]
p53	MAGE proteins stabilize KAP1-p53 complex to decrease acetylation and promote degradation of p53	[80]
p53	KAP1 ubiquitinates p53 for proteasome degradation	[23]
IRF7	KAP1 sumolyates IRF7 and suppresses IRF7 transcriptional activity in IFN production	[24]
ZBRK1	KAP1 interacts with ZBRK1 to repress *Gadd45α* and *p21* gene expression	[16,17,69]
HIF-1α	VHLaK potentially recruits KAP1 to HIF-1α complex to suppress HIF-1α downstream gene expression	[72]
Nrf2	KAP1 interacts with Nrf2 and facilitates Nrf2 transactivation activity	[76]
STAT3	KAP1 interacts with STAT3 to suppress STAT3 transcriptional activity	[82]
STAT1	KAP1 interacts with STAT1 to suppress STAT1 transcriptional activity	[50]
ZNF689	ZNF689 potentially recruits KAP1 to suppress autophagy-gene-targeting miRNAs	[71]
FOXP3	KAP1 is recruited by FIK in FOXP3-FIK-KAP1 complex to suppress FOXP3-target genes	[74]
NFκB	KAP1 is associated with NFκB and negatively regulates it acetylation and transcriptional activity	[51]
SRY	KAP1 is recruited by KRAB-O to SRY binding sites for gene regulation	[68,73]
ZFP57	ZFP57 and KAP1 are associated with NP95-DNMT complex for maintaining DNA methylation at imprinting control region	[59,61,62]

VHLaK: VHL-associated KRAB-A domain-containing protein; HDAC: Histone deacetylase; DNMT: DNA methyltransferase.

chromatin remodeling also participates in the activated transcription. We have shown that the DNA damage-induced KAP1 serine-824 phosphorylation decreases SUMOylated KAP1 to reduce the H3K9 di- and tri-methylation, a mark enriched at repressed genes, and to increase H3K14 acetylation, a mark enriched at the active genes, on the promoter regions of KAP1-targeted genes, thereby relaxing the chromatin structure and activating the transcription of pro-arrest and pro-apoptotic genes, including *p21*, *Gadd45a*, *Bax*, *Noxa* and *Puma*[16,17,36]. Likewise, the phosphorylation of KAP1 serine-824 by the viral protein kinase induces chromatin remodeling to activate lytic genes to support Kaposi's sarcoma herpesvirus (KSHV) lytic replication[57]. Notably, KAP1 mediates the chromatin remodeling within the promoter regions, but not the distal regions of its targeted genes upon DNA damage and viral reactivation[16,57]. It would be interesting to investigate how KAP1 mediates chromatin remodeling at distinct gene regulatory regions during basal and activated transcription. Furthermore, a study showing that KAP1 interacts with a SWI/SNIF-like, ATP-dependent chromatin remodeling protein, SMARCAD1, demonstrates that the KAP1-SMARCAD1 complex regulates global H3K9 trimethylation and H3/H4 deacetylation to maintain the silenced loci during DNA replication[58]. Whether this complex is also responsible for KAP1-mediated transcriptional repression during other circumstances remains unknown.

DNA methylation

Emerging evidence suggests that KAP1-containing complex is not only associated with histone modification, but also regulates DNA methylation. KAP1-mediated DNA methylation is important for genomic imprinting and epigenetic reprogramming during embryogenesis[59-63]. Studies have demonstrated that ZFP57, a KRAB-ZFP important for maintaining DNA methylation at the imprinted loci, recruits KAP1 to imprinting control regions (ICRs) to interact with NP95, a protein responsible for recruiting DNMTs to hemimethylated DNA, resulting in the maintenance of genomic imprinting at ICRs[59,61]. Although these results implicate that KAP1 might indirectly recruit DNMT to specific loci, it remains elusive which domain of KAP1 is responsible for the DNMT recruitment. Histone modification and DNA methylation are interdependent, and histone lysine methylation is involved in maintaining genomic imprinting at ICRs[64,65]. Whether KAP1 plays a role in the crosstalk between these two epigenetic regulation pathways warrants further investigation.

Transcriptional co-regulator

Although it is known that KAP1 can regulate gene expression by chromatin remodeling and DNA methylation as discussed in the previous sections, the specificity of KAP1-regulated genes under certain conditions is still largely unknown. Here, we reviewed how KAP1 regulates transcription factors on specific gene loci for gene regulation (Table 2).

KAP1 has no DNA binding domain but is recruited by a variety of different KRAB-ZFPs *via* its RBCC domain[29,66,67]. Therefore, KAP1 is believed to exert its transcriptional repression by associating with KRAB-ZFPs to specific promoter region of its regulating gene[29]. Although KRAB-ZFP is a large family containing over 400 genes encoding more than 700 predicted proteins, only a few studies directly address the role of KAP1 in regulating the KRAB-ZFP-mediated transcriptional repression[68]. In 1996, KAP1 was for the first time demonstrated to interact with KRAB domain of a human ZFP KOX1/ZNF10 to exert transcriptional repression[4]. Later, ZBRK1 was identified as a sequence specific KRAB-ZFP to recruit KAP1 to suppress *Gadd45a* and *p21* expression[16,17,69]. Our laboratory has demonstrated that KAP1 SUMOylation status dictates the ZBRK1-

KAP1-mediated gene repressive function by enhancing histone methylation without altering KAP1-ZBRK1 interaction[16,17]. Another KRAB-ZFP, ZNF160 has been shown to suppress TLR4 through the binding with KAP1 in intestinal epithelial cells for immune tolerance to commensal bacteria in gut[70]. More recently, ZNF689 is identified as a potential KRAB-ZFP for recruiting KAP1 to suppress autophagy-gene-targeting microRNAs (miRNAs). The KAP1-mediated repression of these autophagy-gene-targeting miRNA promotes mitophagy, a specific type of autophagy targeting mitochondria, during erythrocyte maturation[71].

Interestingly, proteins with KRAB domain but not DNA binding domain are also reported to being capable of serving as a bridge for KAP1-mediated gene repression[68,72-74]. The KRAB Only (KRAB-O) protein is one example to recruit KAP1 to the sex determination transcription factor SRY for regulating SRY-targeted genes[68,73]. VHL-associated KRAB-A domain-containing protein (VHLaK) promotes the formation of HIF-1α-VHLaK-KAP1 complex and potentially suppresses HIF-1α signaling[72]. FOXP3-interacting KRAB domain-containing protein (FIK) is another bridge protein to recruit KAP1 to the FOXP3 binding sites for suppressing FOXP3-target genes[74]. Other transcription factors without KRAB domain including Myc, Oct3/4, E2F1, p53, IRF5/7, Nrf2, STAT1/3 and NF-κB can also interact with KAP1 for gene regulation[10,13,24,49-52,75-78]. More investigation is required to understand whether the formation of these KAP1-transcription factor complexes relies on other bridge proteins.

Several studies have reported that KAP1 is capable of modulating the acetylation status of histones or transcription factors. In general, KAP1-mediated HDAC recruitment negatively regulates gene expression. While mainly serving for histone deacetylation and for heterochromatin maintenance[79], the KAP1-HDAC complex is also postulated to negatively regulate the acetylation level of the transcription factors. For example, KAP1 stimulates E2F1-HDAC1 complex formation to deacetylate E2F1, which suppresses E2F1-mediated apoptotic gene expression in response to DNA damage[13]. KAP1 is also involved in MDM2-p53-HDAC1 complex and promotes deacetylation and MDM2-mediated degradation of p53[75]. More recently, melanoma antigen (MAGE) family proteins, which are highly expressed in many tumors, have been shown to enhance the formation of KAP1-p53 complex and to reduce p53 acetylation[80]. Interestingly, another study shows that MAGE proteins enhance the ubiquitin E3 ligase activity of KAP1 to ubiquitylate p53 for degradation[23]. These studies provide a rationale for developing compounds blocking KAP1-MAGE interaction for anti-cancer purpose[81].

In addition to regulating the activity of deacetylase complex, KAP1 is reported to disrupt the interaction of NF-κB and p300, an acetyltransferase. By this mechanism, the acetylation level of NF-κB is reduced and the transcriptional activity of NF-κB is dampened[51]. Besides, the physical interaction between KAP1 and STAT family members has been identified, and KAP1 negatively regulates STAT1 and STAT3 signaling[50,82]. However, the molecular mechanism underlying this event remains unclear. Whether KAP1 regulates the PTMs of STAT family still needs to be examined.

NON-TRANSCRIPTIONAL FUNCTIONS OF KAP1

While extensive efforts have been made in understanding how KAP1 regulates transcription, less is known about its non-transcriptional functions. In fact, KAP1 has a critical, transcription-independent role in DNA repair processes. Additionally, emerging evidence shows that KAP1 possesses enzymatic activity required for multiple cellular processes. In the following sections, we aim to discuss the role of KAP1 as a signaling scaffold protein in DNA repair and its novel roles as SUMO and ubiquitin E3 ligases.

Signaling scaffold protein in DNA damage response

White and colleagues identified that the PIKK family members, in response to DNA damage, phosphorylate KAP1 at serine-824. The serine-824 phosphorylated KAP1 co-localizes with several DNA repair factors, including γH2AX, 53BP1 and TopBP1, implicating a role for KAP1 in DNA repair processes[20,38]. It was further demonstrated that the KAP1 serine-824 phosphorylation is responsible for ATM-mediated chromatin relaxation, a crucial step for DNA double-strand break (DSB) repair[20]. Several lines of evidence suggest that the function of KAP1 in DSB repair is likely to be associated with the chromatin complexity and cell cycle status. Approximately 25% of DSBs that are located within heterochromatin require ATM signaling for repair, and knockdown of KAP1 bypasses the repair defects caused by ATM inhibition, suggesting that KAP1 is a direct downstream effector in ATM-mediated heterochromatin repair[83]. Although it has not been directly demonstrated, the SUMOylation of KAP1 seems to be important for heterochromatin maintenance because the interaction between KAP1 and the nucleosome remodeler, CHD3 requires KAP1 SUMOylation, and the chromatin retention of CHD3 is critical for chromatin plasticity during DDR[15,21,37]. It has been shown that the ATM-mediated KAP1 serine-824 phosphorylation perturbs the SUMO-dependent interaction of KAP1 and CHD3 at the carboxyl-terminus of KAP1, thereby resulting in the de-condensation of heterochromatin[21]. Another possibility would be the deSUMOylase, SENP7, negatively regulates the SUMOylation status of KAP1 to release CHD3 and promote chromatin relaxation[37]. However, how SENP7 is recruited to deSUMOylate KAP1 at the damage sites remains to be defined. It is also suggested that following the KAP1 phosphorylation-dependent chromatin relaxation, the KAP1-dependent heterochromatin reconstitution mediated by the release of ATM is a prerequisite for error-free homologous recombination (HR) repair[84]. It would be interesting to further delineate how KAP1 is involved in the heterochromatin reconstitution and whether the re-SUMOylation of

KAP1 is required for the reconstitution. In addition to its role in HR repair within heterochromatin, KAP1 has been shown to promote non-homologous end joining (NHEJ) repair, presumably within euchromatin[41]. Whether the chromatin localization of KAP1 determines its function in DSB repair is still unclear. Several studies have shown that HP1 is required for recruiting KAP1 to DNA damage sites for the repair within heterochromatin[44,85]. The disruption of HP1BD in KAP1 results in a defect in forming discrete serine-824 phosphorylated KAP1 foci, which have been considered as a critical signal for DSB repair[44]. How HP1 recruits KAP1 and whether KAP1 is responsible for HP1-mediated DSB repair warrant more detailed investigation.

Depletion of KAP1 is able to rescue the defects in NHEJ repair caused by ATM inhibition in G1 cells[83,86], whereas HR repair can be restored by knocking down KAP1 in ATM-inhibited G2 cells[87,88], indicating that the participation of KAP1 in specific DSB repair pathways is cell cycle-dependent. It is suggested that KAP1 serine-824 phosphorylation is enhanced by 53BP1 within heterochromatic regions and the concentrated phosphorylated KAP1 signal enables NHEJ repair[86]. On the other hand, a recent report shows that 53BP1 is required for enhancing KAP1 serine-824 phosphorylation and HR repair during G2-phase[89]. Collectively, these studies all indicate an important role of KAP1 in DSB repair. However, it is still unclear how cell cycle progression affects the role of KAP1 in selecting DSB repair pathway. Intriguingly, it has been observed that the retention of KAP1 on chromatin is largely reduced in G2-phase[87,90], suggesting that the association of KAP1 with chromatin is possibly regulated in a cell cycle-dependent manner and the chromatin retention of KAP1 may be a critical determinant that directs DSB pathway choice.

Taken together, chromatin remodeling could be one of the major functions served by KAP1 in DSB repair. Because KAP1 has multiple domains for protein-protein interaction, we speculate that KAP1 has additional roles, independent of chromatin remodeling, in DSB repair, such as the recruitment of members of DNA repair machinery.

Enzymatic activity

Recently, KAP1 has been found to possess SUMO E3 ligase activity *via* its PHD domain to recruit the SUMO-conjugating enzyme UBC9. This was first identified by demonstrating that KAP1 auto-SUMOylated its bromodomain to generate a repressive form of KAP1[15]. Later, another two proteins IRF7 and Vps34 have been identified as substrates of KAP1-mediated SUMOylation[24,25]. IRF7 is a transcription factor and master regulator of type I interferon-dependent immune responses. The SUMOylation of IRF7 by KAP1 RING finger domain reduces its transcription activity. Therefore, KAP1 could be a negative regulator of IRF7 and suppress IFN-based antiviral responses[24]. IRF7 is so far the only transcription factor identified as KAP1 target for SUMOylation. Several transcription factors are known to be SUMOylated

and growing evidence suggests SUMOylation negatively regulates transcription[91]. Whether KAP1 mediates SUMOylation-dependent suppression of other transcription factors deserves another look.

More interestingly, KAP1 not only SUMOylates nuclear proteins but also targets a cytoplasmic protein, vacuolar protein-sorting(Vps) 34, which is crucial for autophagosome formation and plays a central role during autophagy. The SUMOylation of Vps34 enhances its binding to Beclin 1 and triggers autophagosome formation in the presence of acetylated HSP70. This study also suggests that the accumulation of KAP1 in the cytoplasm of cells treated with a pan-HDAC inhibitor, panobinostat *via* an unknown mechanism[25].

In addition to SUMO E3 ligase, the RING finger domain of KAP1 has ubiquitin E3 ligase activity. Studies have demonstrated that KAP1, in the presence of MAGE, ubiquitinates p53 and ZNF382 to facilitate their degradation[23,80,92]. This could be an additional mechanism for KAP1 to regulate gene expression. Taken together, recent progresses suggest a non-canonical function of KAP1, dependent on its SUMO and ubiquitin E3 ligase activity. These novel functions of KAP1 are not exclusively confined in the nucleus and might impact cellular physiology in response to various stresses by mechanisms beyond transcriptional regulation.

IMPLICATIONS OF KAP1 IN CELLULAR PHYSIOLOGY

KAP1 is involved in many aspects of cellular physiology (Figure 1). First, KAP1 exerts critical function during embryonic development because global *Kap1*-knockout causes embryonic lethality in mice due to the inability to undergo gastrulation[19] (Table 3). The function of KAP1 in maintaining pluripotency of embryonic stem cells (ESCs) has been demonstrated[78,93]. In addition, KAP1 is also required for ESC differentiation[94-97]. Studies using conditional *Kap1*-knockout mice show that KAP1 plays pivotal roles in different cellular maturation processes such as spermatogenesis, erythropoiesis, and the development of T-cell and B-cell (Table 3)[71,94,98-101]. Beyond regulating T-cell and B-cell differentiation, KAP1 also functions in immune response by additional mechanisms. For example, KAP1 is present in FOXP3-containing complex and facilitates the suppressor activity of regulatory T cells[74]. Moreover, KAP1 is involved in immunoglobulin class switch recombination[102]. Recent studies demonstrate that KAP1 associates with STAT1 and STAT3, master regulators of immune response, to negatively regulate their downstream signaling. These results suggest the involvement of KAP1 in immune response[50,51,82].

Using a mouse model, Jakobsson *et al*[103] show that conditional deletion of *Kap1* in adult forebrain caused higher level of anxiety-like activity, suggesting that Kap1 is a regulator for behavioral stress response. Although the molecular mechanism remains elusive, this could be due to the function of Kap1 in regulating gene expression in hippocampus. It is worth noting that among

Table 3 Mouse models illustrating the physiological functions of Kap1

Animal model	Phenotype	Ref.
Kap1 knockout mice	Embryonic lethal prior to gastulation	[19]
Hemato-specific Kap1 knockout mice	Impaired erythropoiesis	[71,100]
T-cell-specific Kap1 knockout mice	Defective T-cell differentiation	[48,98]
B-cell-specific Kap1 knockout mice	Defective B-cell differentiation	[99]
Tamoxifen-inducible-germ cell-lineage-specific Kap1 depletion mice	Impaired spermatogenesis	[101]
Liver-specific Kap1 knockout mice	Male-predominant steatosis and hepatic adenoma	[120]
Kap1 knockout in mice forebrain	Anxiety-like-behavior and cognitive impairments	[103]

these dysregulated genes in hippocampus, some are imprinted genes[103]. We speculate that Kap1 is also required for the maintenance of a set of specific imprinted genes because many recent studies have demonstrated that KAP1 mediates DNA methylation by recruiting DNMTs to ICRs during early embryogenesis[59,61,62].

More than maintaining normal cellular physiological functions, KAP1 also regulates several pathways in response to different stresses. As mentioned earlier, several studies have focused on the role of KAP1 in DDR. KAP1 is known to be phosphorylated at serine-824 by ATM upon DNA damage and to transduce downstream signaling[20,38]. For instance, ATM-mediated KAP1 phosphorylation leads to de-repression of *p21*, *Gadd45a*, *Bax*, *Puma*, and *Noxa*, causing cell cycle arrest and apoptosis[16,17,36]. Additionally, KAP1 is an ATM downstream mediator during DSB-induced heterochromatin relaxation, which facilitates DNA repair[20,21,83]. KAP1 also cooperates with MDM2 to suppress p53 signaling by promoting p53 degradation[75,104]. Thus, PTMs of KAP1 might be important in regulating p53 activity as well.

In contrast to its co-repressor function, KAP1 can also be a co-activator in specific circumstances. It has been reported that mouse Kap1 interacts with Nrf2 to enhance Nrf2-meiated-cytoprotective function in NIH3T3 cells in response to oxidative stress[76]. However, whether KAP1 exerts similar co-activator activity in human cells is not clear.

Some studies demonstrate that KAP1 mediates viral gene expression and plays a role during viral latency. Specifically, KAP1 is associated with KHSV lytic gene promoter to suppress lytic gene expression and in turn maintains virus latency[57]. Similar observation has also been made in retroviruses such as Murine Leukemia Virus (MLV) and human T-cell lymphotropic virus-1 (HTLV-1), that KAP1 restricts pro-viral gene activation[105-108]. Interestingly, recent studies show that retroelements derived from endogenous retroviruses (ERVs), which are extensively present in mammalian genome, can also be silenced by KAP1 to protect genome integrity in embryonic stem cells[109-112]. In addition, KAP1-HDAC1 complex deacetylates and inhibits human immunodeficiency virus (HIV) integrase activity, thereby reducing HIV infectivity and its integration to host genome[113].

CLINICAL RELEVANCE FOCUSED ON CANCER BIOLOGY

The clinical relevance of KAP1 in diseases remains elusive. Most reports were based on studies in cancers. Higher expression of KAP1 has been linked to pro-metastatic cervical cancer[114]. Moreover, the up-regulation of KAP1 could be a potential marker in colorectal cancer patients and higher level of KAP1 is correlated with poorer overall survival in patients with gastric cancer and thyroid carcinoma[115-119]. These clinical studies suggest that higher KAP1 level is linked to a poor prognosis in certain cancers. However, opposite conclusions were also reported. KAP1 overexpression is associated with better overall survival in early-stage lung cancer[14]. Furthermore, a mouse model showed that *Kap1*-depletion in liver increases male-predominant hepatic adenoma[120] (Table 2).

Based on the studies focusing on transcriptional regulation, KAP1 suppresses p53 transcription activity and has been proposed to be a target for anti-cancer therapy[75,104]. Nonetheless, KAP1 also suppresses activity of oncogenic transcription factors HIF-1α and STAT3[72,82]. *In vitro* studies using cancer cell lines also show divergent results. KAP1 has been shown to restrain cell growth in breast and lung cancer cells whereas it promotes melanoma cell growth and enhanced KAP1 activity mediates cervical cancer invasion[14,80,114]. Taken together, it is still inconclusive how KAP1 regulates tumorigenesis. Because KAP1 is a multi-faceted protein, it might have tissue-specific function. More study is definitely required to understand how KAP1 contributes to tumorigenesis in particular tissue and whether KAP1 could be a target for cancer therapy.

CONCLUSION

The functions of KAP1 in diverse cellular physiology have been studied for 18 years. However, the mechanisms of KAP1 in regulating these different cellular processes are still largely unclear. The best-characterized role of KAP1 is its co-repressor function in regulating KRAB-ZFP-mediated or other transcription factor-associated gene silencing. In the future, the identification of more KAP1-interacting transcription factors may help to elucidate how KAP1 regulates gene expression under specific condition. Clearly, KAP1 is critical for maintaining genome integrity as KAP1 modulates the dynamics of hetero- and euchromatin maintenance. In addition, DNA damage induces a robust and transient KAP1 serine-824 phosphorylation and KAP1 is involved in DNA repair pathway choice. However, the molecular mechanism for KAP1-mediated DNA repair requires more rigorous in-

vestigation.

KAP1 is subjected to several types of PTM. The phosphorylation at serine-824 and SUMOylation at several lysine residues of KAP1 have been linked to gene regulation. Whether these lysine residues are subjected to additional PTMs and if yes, what is the associated functional consequences need to be further elucidated. Moreover, it would be interesting to look at other PTM sites, such as the phosphorylation of serine-473. Whether the serine-473 phosphorylation affects the transcriptional repressor activity of KAP1 is still under investigation. Another interesting question is how KAP1 shuttles among different cellular compartments by PTMs. The serine-824 phosphorylation has distinct nuclear localization pattern from the serine-473 phosphorylation, thus suggesting that KAP1 phosphorylation may affect its subcellular localization. Intriguingly, KAP1 is not only present in nucleus, and has been observed to translocate from nucleus to cytoplasm[25]. How PTM affects its nucleus-cytoplasmic shuttling and potential chromatin extraction of KAP1 deserve additional investigation. Finally, considering that KAP1 possesses SUMO and ubiquitin E3 activities, it might regulate the function or fate of its SUMO- and ubiquitin-targets. In the future, studying the non-transcriptional function of KAP1 might help to further decipher its unrecognized face.

REFERENCES

1 **Le Douarin B**, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, Chambon P. A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *EMBO J* 1996; **15**: 6701-6715 [PMID: 8978696]

2 **Friedman JR**, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev* 1996; **10**: 2067-2078 [PMID: 8769649 DOI: 10.1101/gad.10.16.2067]

3 **Kim SS**, Chen YM, O'Leary E, Witzgall R, Vidal M, Bonventre JV. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc Natl Acad Sci USA* 1996; **93**: 15299-15304 [PMID: 8986806 DOI: 10.1073/pnas.93.26.15299]

4 **Moosmann P**, Georgiev O, Le Douarin B, Bourquin JP, Schaffner W. Transcriptional repression by RING finger protein TIF1 beta that interacts with the KRAB repressor domain of KOX1. *Nucleic Acids Res* 1996; **24**: 4859-4867 [PMID: 9016654 DOI: 10.1093/nar/24.24.4859]

5 **Nielsen AL**, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, Chambon P, Losson R. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *EMBO J* 1999; **18**: 6385-6395 [PMID: 10562550 DOI: 10.1093/emboj/18.22.6385]

6 **Ryan RF**, Schultz DC, Ayyanathan K, Singh PB, Friedman JR, Fredericks WJ, Rauscher FJ. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Krüppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol Cell Biol* 1999; **19**: 4366-4378 [PMID: 10330177]

7 **Underhill C**, Qutob MS, Yee SP, Torchia J. A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. *J Biol Chem* 2000; **275**: 40463-40470 [PMID: 11013263 DOI: 10.1074/jbc.M007864200]

8 **Schultz DC**, Friedman JR, Rauscher FJ. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev* 2001; **15**: 428-443 [PMID: 11230151 DOI: 10.1101/gad.869501]

9 **Schultz DC**, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 2002; **16**: 919-932 [PMID: 11959841 DOI: 10.1101/gad.973302]

10 **Satou A**, Taira T, Iguchi-Ariga SM, Ariga H. A novel transrepression pathway of c-Myc. Recruitment of a transcriptional corepressor complex to c-Myc by MM-1, a c-Myc-binding protein. *J Biol Chem* 2001; **276**: 46562-46567 [PMID: 11585818 DOI: 10.1074/jbc.M104937200]

11 **Satou A**, Hagio Y, Taira T, Iguchi-Ariga SM, Ariga H. Repression of the c-fms gene in fibroblast cells by c-Myc-MM-1-TIF1beta complex. *FEBS Lett* 2004; **572**: 211-215 [PMID: 15304350 DOI: 10.1016/j.febslet.2004.07.034]

12 **Hagio Y**, Kimura Y, Taira T, Fujioka Y, Iguchi-Ariga SM, Ariga H. Distinct localizations and repression activities of MM-1 isoforms toward c-Myc. *J Cell Biochem* 2006; **97**: 145-155 [PMID: 16173081 DOI: 10.1002/jcb.20619]

13 **Wang C**, Rauscher FJ, Cress WD, Chen J. Regulation of E2F1 function by the nuclear corepressor KAP1. *J Biol Chem* 2007; **282**: 29902-29909 [PMID: 17704056 DOI: 10.1074/jbc.M704757200]

14 **Chen L**, Chen DT, Kurtyka C, Rawal B, Fulp WJ, Haura EB, Cress WD. Tripartite motif containing 28 (Trim28) can regulate cell proliferation by bridging HDAC1/E2F interactions. *J Biol Chem* 2012; **287**: 40106-40118 [PMID: 23060449 DOI: 10.1074/jbc.M112.380865]

15 **Ivanov AV**, Peng H, Yurchenko V, Yap KL, Negorev DG, Schultz DC, Psulkowski E, Fredericks WJ, White DE, Maul GG, Sadofsky MJ, Zhou MM, Rauscher FJ. PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. *Mol Cell* 2007; **28**: 823-837 [PMID: 18082607 DOI: 10.1016/j.molcel.2007.11.012]

16 **Lee YK**, Thomas SN, Yang AJ, Ann DK. Doxorubicin downregulates Kruppel-associated box domain-associated protein 1 sumoylation that relieves its transcription repression on p21WAF1/CIP1 in breast cancer MCF-7 cells. *J Biol Chem* 2007; **282**: 1595-1606 [PMID: 17079232 DOI: 10.1074/jbc.M606306200]

17 **Li X**, Lee YK, Jeng JC, Yen Y, Schultz DC, Shih HM, Ann DK. Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. *J Biol Chem* 2007; **282**: 36177-36189 [PMID: 17942393 DOI: 10.1074/jbc.M706912200]

18 **Mascle XH**, Germain-Desprez D, Huynh P, Estephan P, Aubry M. Sumoylation of the transcriptional intermediary factor 1beta (TIF1beta), the Co-repressor of the KRAB Multifinger proteins, is required for its transcriptional activity and is modulated by the KRAB domain. *J Biol Chem* 2007; **282**: 10190-10202 [PMID: 17298944 DOI: 10.1074/jbc.M611429200]

19 **Cammas F**, Mark M, Dollé P, Dierich A, Chambon P, Losson R. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. *Development* 2000; **127**: 2955-2963 [PMID: 10851139 DOI: 10.1016/j.neuron.2008.09.036]

20 **Ziv Y**, Bielopolski D, Galanty Y, Lukas C, Taya Y, Schultz DC, Lukas J, Bekker-Jensen S, Bartek J, Shiloh Y. Chromatin relaxation in response to DNA double-strand breaks is

modulated by a novel ATM- and KAP-1 dependent pathway. *Nat Cell Biol* 2006; **8**: 870-876 [PMID: 16862143 DOI: 10.1038/ncb1446]

21 **Goodarzi AA**, Kurka T, Jeggo PA. KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. *Nat Struct Mol Biol* 2011; **18**: 831-839 [PMID: 21642969 DOI: 10.1038/nsmb.2077]

22 **Liu B**, Wang Z, Ghosh S, Zhou Z. Defective ATM-Kap-1-mediated chromatin remodeling impairs DNA repair and accelerates senescence in progeria mouse model. *Aging Cell* 2013; **12**: 316-318 [PMID: 23173799 DOI: 10.1111/acel.12035]

23 **Doyle JM**, Gao J, Wang J, Yang M, Potts PR. MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. *Mol Cell* 2010; **39**: 963-974 [PMID: 20864041 DOI: 10.1016/j.molcel.2010.08.029]

24 **Liang Q**, Deng H, Li X, Wu X, Tang Q, Chang TH, Peng H, Rauscher FJ, Ozato K, Zhu F. Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *J Immunol* 2011; **187**: 4754-4763 [PMID: 21940674 DOI: 10.4049/jimmunol.1101704]

25 **Yang Y**, Fiskus W, Yong B, Atadja P, Takahashi Y, Pandita TK, Wang HG, Bhalla KN. Acetylated hsp70 and KAP1-mediated Vps34 SUMOylation is required for autophagosome creation in autophagy. *Proc Natl Acad Sci USA* 2013; **110**: 6841-6846 [PMID: 23569248 DOI: 10.1073/pnas.1217692110]

26 **Matsuda E**, Agata Y, Sugai M, Katakai T, Gonda H, Shimizu A. Targeting of Krüppel-associated box-containing zinc finger proteins to centromeric heterochromatin. Implication for the gene silencing mechanisms. *J Biol Chem* 2001; **276**: 14222-14229 [PMID: 11278721 DOI: 10.1074/jbc.M010663200]

27 **Iyengar S**, Farnham PJ. KAP1 protein: an enigmatic master regulator of the genome. *J Biol Chem* 2011; **286**: 26267-26276 [PMID: 21652716 DOI: 10.1074/jbc.R111.252569]

28 **Peng H**, Gibson LC, Capili AD, Borden KL, Osborne MJ, Harper SL, Speicher DW, Zhao K, Marmorstein R, Rock TA, Rauscher FJ. The structurally disordered KRAB repression domain is incorporated into a protease resistant core upon binding to KAP-1-RBCC domain. *J Mol Biol* 2007; **370**: 269-289 [PMID: 17512541 DOI: 10.1016/j.jmb.2007.03.047]

29 **Urrutia R**. KRAB-containing zinc-finger repressor proteins. *Genome Biol* 2003; **4**: 231 [PMID: 14519192 DOI: 10.1186/gb-2003-4-10-231]

30 **Peng H**, Begg GE, Schultz DC, Friedman JR, Jensen DE, Speicher DW, Rauscher FJ. Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. *J Mol Biol* 2000; **295**: 1139-1162 [PMID: 10653693 DOI: 10.1006/jmbi.1999.3402]

31 **Iyengar S**, Ivanov AV, Jin VX, Rauscher FJ, Farnham PJ. Functional analysis of KAP1 genomic recruitment. *Mol Cell Biol* 2011; **31**: 1833-1847 [PMID: 21343339 DOI: 10.1128/MCB.01331-10]

32 **Venturini L**, You J, Stadler M, Galien R, Lallemand V, Koken MH, Mattei MG, Ganser A, Chambon P, Losson R, de Thé H. TIF1gamma, a novel member of the transcriptional intermediary factor 1 family. *Oncogene* 1999; **18**: 1209-1217 [PMID: 10022127 DOI: 10.1038/onc.2012.501]

33 **Lechner MS**, Begg GE, Speicher DW, Rauscher FJ. Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. *Mol Cell Biol* 2000; **20**: 6449-6465 [PMID: 10938122 DOI: 10.1128/mcb.20.17.6449-6465.2000]

34 **Sripathy SP**, Stevens J, Schultz DC. The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. *Mol Cell Biol* 2006; **26**: 8623-8638 [PMID: 16954381

DOI: 10.1128/MCB.00487-06]

35 **Riclet R**, Chendeb M, Vonesch JL, Koczan D, Thiesen HJ, Losson R, Cammas F. Disruption of the interaction between transcriptional intermediary factor 1{beta} and heterochromatin protein 1 leads to a switch from DNA hyper- to hypomethylation and H3K9 to H3K27 trimethylation on the MEST promoter correlating with gene reactivation. *Mol Biol Cell* 2009; **20**: 296-305 [PMID: 18923144 DOI: 10.1091/mbc.E08-05-0510]

36 **Li X**, Lin HH, Chen H, Xu X, Shih HM, Ann DK. SUMOylation of the transcriptional co-repressor KAP1 is regulated by the serine and threonine phosphatase PP1. *Sci Signal* 2010; **3**: ra32 [PMID: 20424263 DOI: 10.1126/scisignal.2000781]

37 **Garvin AJ**, Densham RM, Blair-Reid SA, Pratt KM, Stone HR, Weekes D, Lawrence KJ, Morris JR. The deSUMOylase SENP7 promotes chromatin relaxation for homologous recombination DNA repair. *EMBO Rep* 2013; **14**: 975-983 [PMID: 24018422 DOI: 10.1038/embor.2013.141]

38 **White DE**, Negorev D, Peng H, Ivanov AV, Maul GG, Rauscher FJ. KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions. *Cancer Res* 2006; **66**: 11594-11599 [PMID: 17178852 DOI: 10.1158/0008-5472.CAN-06-4138]

39 **Tomimatsu N**, Mukherjee B, Burma S. Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells. *EMBO Rep* 2009; **10**: 629-635 [PMID: 19444312 DOI: 10.1038/embor.2009.60]

40 **Lee DH**, Goodarzi AA, Adelmant GO, Pan Y, Jeggo PA, Marto JA, Chowdhury D. Phosphoproteomic analysis reveals that PP4 dephosphorylates KAP-1 impacting the DNA damage response. *EMBO J* 2012; **31**: 2403-2415 [PMID: 22491012 DOI: 10.1038/emboj.2012.86]

41 **Liu J**, Xu L, Zhong J, Liao J, Li J, Xu X. Protein phosphatase PP4 is involved in NHEJ-mediated repair of DNA double-strand breaks. *Cell Cycle* 2012; **11**: 2643-2649 [PMID: 22732494 DOI: 10.4161/Cc.20957]

42 **Hu C**, Zhang S, Gao X, Gao X, Xu X, Lv Y, Zhang Y, Zhu Z, Zhang C, Li Q, Wong J, Cui Y, Zhang W, Ma L, Wang C. Roles of Kruppel-associated Box (KRAB)-associated Co-repressor KAP1 Ser-473 Phosphorylation in DNA Damage Response. *J Biol Chem* 2012; **287**: 18937-18952 [PMID: 22496453 DOI: 10.1074/jbc.M111.313262]

43 **Bolderson E**, Savage KI, Mahen R, Pisupati V, Graham ME, Richard DJ, Robinson PJ, Venkitaraman AR, Khanna KK. Kruppel-associated Box (KRAB)-associated co-repressor (KAP-1) Ser-473 phosphorylation regulates heterochromatin protein 1β (HP1-β) mobilization and DNA repair in heterochromatin. *J Biol Chem* 2012; **287**: 28122-28131 [PMID: 22715096 DOI: 10.1074/jbc.M112.368381]

44 **White D**, Rafalska-Metcalf IU, Ivanov AV, Corsinotti A, Peng H, Lee SC, Trono D, Janicki SM, Rauscher FJ. The ATM substrate KAP1 controls DNA repair in heterochromatin: regulation by HP1 proteins and serine 473/824 phosphorylation. *Mol Cancer Res* 2012; **10**: 401-414 [PMID: 22205726 DOI: 10.1158/1541-7786.MCR-11-0134]

45 **Blasius M**, Forment JV, Thakkar N, Wagner SA, Choudhary C, Jackson SP. A phospho-proteomic screen identifies substrates of the checkpoint kinase Chk1. *Genome Biol* 2011; **12**: R78 [PMID: 21851590 DOI: 10.1186/gb-2011-12-8-r78]

46 **Chang CW**, Chou HY, Lin YS, Huang KH, Chang CJ, Hsu TC, Lee SC. Phosphorylation at Ser473 regulates heterochromatin protein 1 binding and corepressor function of TIF1beta/KAP1. *BMC Mol Biol* 2008; **9**: 61 [PMID: 18590578 DOI: 10.1186/1471-2199-9-61]

47 **Zhou XF**, Yu J, Chang M, Zhang M, Zhou D, Cammas F, Sun SC. TRIM28 mediates chromatin modifications at the TCRα enhancer and regulates the development of T and natural killer T cells. *Proc Natl Acad Sci USA* 2012; **109**: 20083-20088 [PMID: 23169648 DOI: 10.1073/pnas.1214704109]

48   **Chikuma S**, Suita N, Okazaki IM, Shibayama S, Honjo T. TRIM28 prevents autoinflammatory T cell development in vivo. *Nat Immunol* 2012; **13**: 596-603 [PMID: 22544392 DOI: 10.1038/ni.2293]

49   **King CA**. Kaposi's sarcoma-associated herpesvirus kaposin B induces unique monophosphorylation of STAT3 at serine 727 and MK2-mediated inactivation of the STAT3 transcriptional repressor TRIM28. *J Virol* 2013; **87**: 8779-8791 [PMID: 23740979 DOI: 10.1128/JVI.02976-12]

50   **Kamitani S**, Ohbayashi N, Ikeda O, Togi S, Muromoto R, Sekine Y, Ohta K, Ishiyama H, Matsuda T. KAP1 regulates type I interferon/STAT1-mediated IRF-1 gene expression. *Biochem Biophys Res Commun* 2008; **370**: 366-370 [PMID: 18381204 DOI: 10.1016/j.bbrc.2008.03.104]

51   **Kamitani S**, Togi S, Ikeda O, Nakasuji M, Sakauchi A, Sekine Y, Muromoto R, Oritani K, Matsuda T. Krüppel-associated box-associated protein 1 negatively regulates TNF-α-induced NF-κB transcriptional activity by influencing the interactions among STAT3, p300, and NF-κB/p65. *J Immunol* 2011; **187**: 2476-2483 [PMID: 21810609 DOI: 10.4049/jimmunol.1003243]

52   **Eames HL**, Saliba DG, Krausgruber T, Lanfrancotti A, Ryzhakov G, Udalova IA. KAP1/TRIM28: an inhibitor of IRF5 function in inflammatory macrophages. *Immunobiology* 2012; **217**: 1315-1324 [PMID: 22995936 DOI: 10.1016/j.imbio.2012.07.026]

53   **Kubota S**, Fukumoto Y, Aoyama K, Ishibashi K, Yuki R, Morinaga T, Honda T, Yamaguchi N, Kuga T, Tomonaga T, Yamaguchi N. Phosphorylation of KRAB-associated protein 1 (KAP1) at Tyr-449, Tyr-458, and Tyr-517 by nuclear tyrosine kinases inhibits the association of KAP1 and heterochromatin protein 1α (HP1α) with heterochromatin. *J Biol Chem* 2013; **288**: 17871-17883 [PMID: 23645696 DOI: 10.1074/jbc.M112.437756]

54   **Lai IL**, Lin TP, Yao YL, Lin CY, Hsieh MJ, Yang WM. Histone deacetylase 10 relieves repression on the melanogenic program by maintaining the deacetylation status of repressors. *J Biol Chem* 2010; **285**: 7187-7196 [PMID: 20032463 DOI: 10.1074/jbc.M109.061861]

55   **O'Geen H**, Squazzo SL, Iyengar S, Blahnik K, Rinn JL, Chang HY, Green R, Farnham PJ. Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. *PLoS Genet* 2007; **3**: e89 [PMID: 17542650 DOI: 10.1371/journal.pgen.0030089]

56   **Groner AC**, Meylan S, Ciuffi A, Zangger N, Ambrosini G, Dénervaud N, Bucher P, Trono D. KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS Genet* 2010; **6**: e1000869 [PMID: 20221260 DOI: 10.1371/journal.pgen.1000869]

57   **Chang PC**, Fitzgerald LD, Van Geelen A, Izumiya Y, Ellison TJ, Wang DH, Ann DK, Luciw PA, Kung HJ. Kruppel-associated box domain-associated protein-1 as a latency regulator for Kaposi's sarcoma-associated herpesvirus and its modulation by the viral protein kinase. *Cancer Res* 2009; **69**: 5681-5689 [PMID: 19584288 DOI: 10.1158/0008-5472.CAN-08-4570]

58   **Rowbotham SP**, Barki L, Neves-Costa A, Santos F, Dean W, Hawkes N, Choudhary P, Will WR, Webster J, Oxley D, Green CM, Varga-Weisz P, Mermoud JE. Maintenance of silent chromatin through replication requires SWI/SNF-like chromatin remodeler SMARCAD1. *Mol Cell* 2011; **42**: 285-296 [PMID: 21549307 DOI: 10.1016/j.molcel.2011.02.036]

59   **Quenneville S**, Verde G, Corsinotti A, Kapopoulou A, Jakobsson J, Offner S, Baglivo I, Pedone PV, Grimaldi G, Riccio A, Trono D. In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Mol Cell* 2011; **44**: 361-372 [PMID: 22055183 DOI: 10.1016/j.molcel.2011.08.032]

60   **Quenneville S**, Turelli P, Bojkowska K, Raclot C, Offner S, Kapopoulou A, Trono D. The KRAB-ZFP/KAP1 system contributes to the early embryonic establishment of site-specific DNA methylation patterns maintained during development. *Cell Rep* 2012; **2**: 766-773 [PMID: 23041315 DOI: 10.1016/j.celrep.2012.08.043]

61   **Zuo X**, Sheng J, Lau HT, McDonald CM, Andrade M, Cullen DE, Bell FT, Iacovino M, Kyba M, Xu G, Li X. Zinc finger protein ZFP57 requires its co-factor to recruit DNA methyltransferases and maintains DNA methylation imprint in embryonic stem cells via its transcriptional repression domain. *J Biol Chem* 2012; **287**: 2107-2118 [PMID: 22144682 DOI: 10.1074/jbc.M111.322644]

62   **Takikawa S**, Wang X, Ray C, Vakulenko M, Bell FT, Li X. Human and mouse ZFP57 proteins are functionally interchangeable in maintaining genomic imprinting at multiple imprinted regions in mouse ES cells. *Epigenetics* 2013; **8**: 1268-1279 [PMID: 24135613]

63   **Messerschmidt DM**, de Vries W, Ito M, Solter D, Ferguson-Smith A, Knowles BB. Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. *Science* 2012; **335**: 1499-1502 [PMID: 22442485 DOI: 10.1126/science.1216154]

64   **Cedar H**, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009; **10**: 295-304 [PMID: 19308066 DOI: 10.1038/nrg2540]

65   **Kacem S**, Feil R. Chromatin mechanisms in genomic imprinting. *Mamm Genome* 2009; **20**: 544-556 [PMID: 19760321 DOI: 10.1007/s00335-009-9223-4]

66   **Itokawa Y**, Yanagawa T, Yamakawa H, Watanabe N, Koga H, Nagase T. KAP1-independent transcriptional repression of SCAN-KRAB-containing zinc finger proteins. *Biochem Biophys Res Commun* 2009; **388**: 689-694 [PMID: 19695231 DOI: 10.1016/j.bbrc.2009.08.065]

67   **Meylan S**, Groner AC, Ambrosini G, Malani N, Quenneville S, Zangger N, Kapopoulou A, Kauzlaric A, Rougemont J, Ciuffi A, Bushman FD, Bucher P, Trono D. A gene-rich, transcriptionally active environment and the pre-deposition of repressive marks are predictive of susceptibility to KRAB/KAP1-mediated silencing. *BMC Genomics* 2011; **12**: 378 [PMID: 21791101 DOI: 10.1186/1471-2164-12-378]

68   **Jin VX**, O'Geen H, Iyengar S, Green R, Farnham PJ. Identification of an OCT4 and SRY regulatory module using integrated computational and experimental genomics approaches. *Genome Res* 2007; **17**: 807-817 [PMID: 17567999 DOI: 10.1101/gr.6006107]

69   **Zheng L**, Pan H, Li S, Flesken-Nikitin A, Chen PL, Boyer TG, Lee WH. Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1. *Mol Cell* 2000; **6**: 757-768 [PMID: 11090615 DOI: 10.1016/S1097-2765(00)00075-7]

70   **Takahashi K**, Sugi Y, Hosono A, and Kaminogawa S. Epigenetic Regulation of TLR4 Gene Expression in Intestinal Epithelial Cells for the Maintenance of Intestinal Homeostasis. *J Immunol* 2009; **183**: 6522-6529 [DOI: 10.4049/jimmunol.0901271]

71   **Barde I**, Rauwel B, Marin-Florez RM, Corsinotti A, Laurenti E, Verp S, Offner S, Marquis J, Kapopoulou A, Vanicek J, Trono D. A KRAB/KAP1-miRNA cascade regulates erythropoiesis through stage-specific control of mitophagy. *Science* 2013; **340**: 350-353 [PMID: 23493425 DOI: 10.1126/science.1232398]

72   **Li Z**, Wang D, Na X, Schoen SR, Messing EM, Wu G. The VHL protein recruits a novel KRAB-A domain protein to repress HIF-1alpha transcriptional activity. *EMBO J* 2003; **22**: 1857-1867 [PMID: 12682018 DOI: 10.1093/Emboj/Cdg173]

73   **Peng H**, Ivanov AV, Oh HJ, Lau YF, Rauscher FJ. Epigenetic gene silencing by the SRY protein is mediated by a KRAB-O protein that recruits the KAP1 co-repressor machinery. *J Biol Chem* 2009; **284**: 35670-35680 [PMID: 19850934 DOI: 10.1074/jbc.M109.032086]

74   **Huang C**, Martin S, Pfleger C, Du J, Buckner JH, Bluestone JA, Riley JL, Ziegler SF. Cutting Edge: a novel, human-specific interacting protein couples FOXP3 to a chromatin-remodeling complex that contains KAP1/TRIM28. *J Immunol* 2013; **190**: 4470-4473 [PMID: 23543754 DOI: 10.4049/jimmunol.1203561]

75   **Wang C**, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ, Chen J. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J* 2005; **24**: 3279-3290 [PMID: 16107876 DOI: 10.1038/sj.emboj.7600791]

76   **Maruyama A**, Nishikawa K, Kawatani Y, Mimura J, Hosoya T, Harada N, Yamamato M, Itoh K. The novel Nrf2-interacting factor KAP1 regulates susceptibility to oxidative stress by promoting the Nrf2-mediated cytoprotective response. *Biochem J* 2011; **436**: 387-397 [PMID: 21382013 DOI: 10.1042/BJ20101748]

77   **Tian C**, Xing G, Xie P, Lu K, Nie J, Wang J, Li L, Gao M, Zhang L, He F. KRAB-type zinc-finger protein Apak specifically regulates p53-dependent apoptosis. *Nat Cell Biol* 2009; **11**: 580-591 [PMID: 19377469 DOI: 10.1038/ncb1864]

78   **Seki Y**, Kurisaki A, Watanabe-Susaki K, Nakajima Y, Nakanishi M, Arai Y, Shiota K, Sugino H, Asashima M. TIF-1beta regulates the pluripotency of embryonic stem cells in a phosphorylation-dependent manner. *Proc Natl Acad Sci USA* 2010; **107**: 10926-10931 [PMID: 20508149 DOI: 10.1073/pnas.0907601107]

79   **Takahashi K**, Sugi Y, Hosono A, Kaminogawa S. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. *J Immunol* 2009; **183**: 6522-6529 [PMID: 19846881 DOI: 10.4049/jimmunol.0901271]

80   **Yang B**, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ, Longley BJ. MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 2007; **67**: 9954-9962 [PMID: 17942928 DOI: 10.1158/0008-5472.CAN-07-1478]

81   **Bhatia N**, Yang B, Xiao TZ, Peters N, Hoffmann MF, Longley BJ. Identification of novel small molecules that inhibit protein-protein interactions between MAGE and KAP-1. *Arch Biochem Biophys* 2011; **508**: 217-221 [PMID: 21277283 DOI: 10.1016/j.abb.2011.01.007]

82   **Tsuruma R**, Ohbayashi N, Kamitani S, Ikeda O, Sato N, Muromoto R, Sekine Y, Oritani K, Matsuda T. Physical and functional interactions between STAT3 and KAP1. *Oncogene* 2008; **27**: 3054-3059 [PMID: 18037959 DOI: 10.1038/sj.onc.1210952]

83   **Goodarzi AA**, Noon AT, Deckbar D, Ziv Y, Shiloh Y, Löbrich M, Jeggo PA. ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol Cell* 2008; **31**: 167-177 [PMID: 18657500 DOI: 10.1016/j.molcel.2008.05.017]

84   **Geuting V**, Reul C, Löbrich M. ATM release at resected double-strand breaks provides heterochromatin reconstitution to facilitate homologous recombination. *PLoS Genet* 2013; **9**: e1003667 [PMID: 23935532 DOI: 10.1371/journal.pgen.1003667]

85   **Baldeyron C**, Soria G, Roche D, Cook AJ, Almouzni G. HP1alpha recruitment to DNA damage by p150CAF-1 promotes homologous recombination repair. *J Cell Biol* 2011; **193**: 81-95 [PMID: 21464229 DOI: 10.1083/jcb.201101030]

86   **Noon AT**, Shibata A, Rief N, Löbrich M, Stewart GS, Jeggo PA, Goodarzi AA. 53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair. *Nat Cell Biol* 2010; **12**: 177-184 [PMID: 20081839 DOI: 10.1038/Ncb2017]

87   **Beucher A**, Birraux J, Tchouandong L, Barton O, Shibata A, Conrad S, Goodarzi AA, Krempler A, Jeggo PA, Löbrich M. ATM and Artemis promote homologous recombination of

radiation-induced DNA double-strand breaks in G2. *EMBO J* 2009; **28**: 3413-3427 [PMID: 19779458 DOI: 10.1038/emboj.2009.276]

88   **Shibata A**, Conrad S, Birraux J, Geuting V, Barton O, Ismail A, Kakarougkas A, Meek K, Taucher-Scholz G, Löbrich M, Jeggo PA. Factors determining DNA double-strand break repair pathway choice in G2 phase. *EMBO J* 2011; **30**: 1079-1092 [PMID: 21317870 DOI: 10.1038/emboj.2011.27]

89   **Kakarougkas A**, Ismail A, Klement K, Goodarzi AA, Conrad S, Freire R, Shibata A, Lobrich M, Jeggo PA. Opposing roles for 53BP1 during homologous recombination. *Nucleic Acids Res* 2013; **41**: 9719-9731 [PMID: 23969417 DOI: 10.1093/nar/gkt729]

90   **Goodarzi AA**, Noon AT, Jeggo PA. The impact of heterochromatin on DSB repair. *Biochem Soc Trans* 2009; **37**: 569-576 [PMID: 19442252 DOI: 10.1042/BST0370569]

91   **Verger A**, Perdomo J, Crossley M. Modification with SUMO. A role in transcriptional regulation. *EMBO Rep* 2003; **4**: 137-142 [PMID: 12612601 DOI: 10.1038/sj.embor.embor738]

92   **Xiao TZ**, Bhatia N, Urrutia R, Lomberk GA, Simpson A, Longley BJ. MAGE I transcription factors regulate KAP1 and KRAB domain zinc finger transcription factor mediated gene repression. *PLoS One* 2011; **6**: e23747 [PMID: 21876767 DOI: 10.1371/journal.pone.0023747]

93   **Hu G**, Kim J, Xu Q, Leng Y, Orkin SH, Elledge SJ. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. *Genes Dev* 2009; **23**: 837-848 [PMID: 19339689 DOI: 10.1101/gad.1769609]

94   **Cammas F**, Oulad-Abdelghani M, Vonesch JL, Huss-Garcia Y, Chambon P, Losson R. Cell differentiation induces TIF-1beta association with centromeric heterochromatin via an HP1 interaction. *J Cell Sci* 2002; **115**: 3439-3448 [PMID: 12154074]

95   **Herzog M**, Wendling O, Guillou F, Chambon P, Mark M, Losson R, Cammas F. TIF1β association with HP1 is essential for post-gastrulation development, but not for Sertoli cell functions during spermatogenesis. *Dev Biol* 2011; **350**: 548-558 [PMID: 21163256 DOI: 10.1016/j.ydbio.2010.12.014]

96   **Cammas F**, Herzog M, Lerouge T, Chambon P, Losson R. Association of the transcriptional corepressor TIF1beta with heterochromatin protein 1 (HP1): an essential role for progression through differentiation. *Genes Dev* 2004; **18**: 2147-2160 [PMID: 15342492 DOI: 10.1101/gad.302904]

97   **Cammas F,** Janoshazi A, Lerouge T, and Losson R. Dynamic and selective interactions of the transcriptional corepressor TIF1 beta with the heterochromatin protein HP1 isotypes during cell differentiation. *Differentiation* 2007; **75**: 627-637 [DOI: 10.1111/j.1432-0436.2007.00166.x]

98   **Santoni de Sio FR**, Barde I, Offner S, Kapopoulou A, Corsinotti A, Bojkowska K, Genolet R, Thomas JH, Luescher IF, Pinschewer D, Harris N, Trono D. KAP1 regulates gene networks controlling T-cell development and responsiveness. *FASEB J* 2012; **26**: 4561-4575 [PMID: 22872677 DOI: 10.1096/fj.12-206177]

99   **Santoni de Sio FR**, Massacand J, Barde I, Offner S, Corsinotti A, Kapopoulou A, Bojkowska K, Dagklis A, Fernandez M, Ghia P, Thomas JH, Pinschewer D, Harris N, Trono D. KAP1 regulates gene networks controlling mouse B-lymphoid cell differentiation and function. *Blood* 2012; **119**: 4675-4685 [PMID: 22452978 DOI: 10.1182/blood-2011-12-401117]

100   **Hosoya T**, Clifford M, Losson R, Tanabe O, Engel JD. TRIM28 is essential for erythroblast differentiation in the mouse. *Blood* 2013; **122**: 3798-3807 [PMID: 24092935 DOI: 10.1182/blood-2013-04-496166]

101   **Weber P**, Cammas F, Gerard C, Metzger D, Chambon P, Losson R, Mark M. Germ cell expression of the transcriptional co-repressor TIF1beta is required for the maintenance of spermatogenesis in the mouse. *Development* 2002; **129**: 2329-2337 [PMID: 11973266]

102   **Jeevan-Raj BP**, Robert I, Heyer V, Page A, Wang JH, Cammas F, Alt FW, Losson R, Reina-San-Martin B. Epigenetic tethering of AID to the donor switch region during immunoglobulin class switch recombination. *J Exp Med* 2011; **208**: 1649-1660 [PMID: 21746811 DOI: 10.1084/jem.20110118]

103   **Jakobsson J**, Cordero MI, Bisaz R, Groner AC, Busskamp V, Bensadoun JC, Cammas F, Losson R, Mansuy IM, Sandi C, Trono D. KAP1-mediated epigenetic repression in the forebrain modulates behavioral vulnerability to stress. *Neuron* 2008; **60**: 818-831 [PMID: 19081377]

104   **Okamoto K**, Kitabayashi I, Taya Y. KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. *Biochem Biophys Res Commun* 2006; **351**: 216-222 [PMID: 17056014 DOI: 10.1016/j.bbrc.2006.10.022]

105   **Wolf D**, Cammas F, Losson R, Goff SP. Primer binding site-dependent restriction of murine leukemia virus requires HP1 binding by TRIM28. *J Virol* 2008; **82**: 4675-4679 [PMID: 18287239 DOI: 10.1128/JVI.02445-07]

106   **Wolf D**, Goff SP. TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. *Cell* 2007; **131**: 46-57 [PMID: 17923087 DOI: 10.1016/j.cell.2007.07.026]

107   **Wolf D**, Goff SP. Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* 2009; **458**: 1201-1204 [PMID: 19270682 DOI: 10.1038/Nature07844]

108   **Wolf D**, Hug K, Goff SP. TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells. *Proc Natl Acad Sci USA* 2008; **105**: 12521-12526 [PMID: 18713861 DOI: 10.1073/pnas.0805540105]

109   **Matsui T**, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, Tachibana M, Lorincz MC, Shinkai Y. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010; **464**: 927-931 [PMID: 20164836 DOI: 10.1038/nature08858]

110   **Schlesinger S**, Lee AH, Wang GZ, Green L, Goff SP. Proviral silencing in embryonic cells is regulated by Yin Yang 1. *Cell Rep* 2013; **4**: 50-58 [PMID: 23810560 DOI: 10.1016/j.celrep.2013.06.003]

111   **Rowe HM**, Jakobsson J, Mesnard D, Rougemont J, Reynard S, Aktas T, Maillard PV, Layard-Liesching H, Verp S, Marquis J, Spitz F, Constam DB, Trono D. KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* 2010; **463**: 237-240 [PMID: 20075919 DOI: 10.1038/nature08674]

112   **Tan X**, Xu X, Elkenani M, Smorag L, Zechner U, Nolte J, Engel W, Pantakani DV. Zfp819, a novel KRAB-zinc finger protein, interacts with KAP1 and functions in genomic integrity maintenance of mouse embryonic stem cells. *Stem Cell Res* 2013; **11**: 1045-1059 [PMID: 23954693 DOI: 10.1016/j.scr.2013.07.006]

113   **Allouch A**, Di Primio C, Alpi E, Lusic M, Arosio D, Giacca M, Cereseto A. The TRIM family protein KAP1 inhibits HIV-1 integration. *Cell Host Microbe* 2011; **9**: 484-495 [PMID: 21669397 DOI: 10.1016/j.chom.2011.05.004]

114   **Lin LF**, Li CF, Wang WJ, Yang WM, Wang DD, Chang WC, Lee WH, Wang JM. Loss of ZBRK1 contributes to the increase of KAP1 and promotes KAP1-mediated metastasis and invasion in cervical cancer. *PLoS One* 2013; **8**: e73033 [PMID: 23991171 DOI: 10.1371/journal.pone.0073033]

115   **Yokoe T**, Toiyama Y, Okugawa Y, Tanaka K, Ohi M, Inoue Y, Mohri Y, Miki C, and Kusunoki M. KAP1 Is Associated With Peritoneal Carcinomatosis in Gastric Cancer. *Ann Surg Oncol* 2009; **17**: 821-828

116   **Hector S**, Chen H, Kijanka G, Murray F, Prehn JH. A reverse-ELISA for the detection of TRIM28/KAP1 serum autoantibodies in colorectal cancer patients. *Acta Oncol* 2012; **51**: 394-396 [PMID: 22268577 DOI: 10.3109/0284186X.2011.652742]

117   **Wang YY**, Li L, Zhao ZS, Wang HJ. Clinical utility of measuring expression levels of KAP1, TIMP1 and STC2 in peripheral blood of patients with gastric cancer. *World J Surg Oncol* 2013; **11**: 81 [PMID: 23548070 DOI: 10.1186/1477-7819-11-81]

118   **Martins MB**, Marcello MA, Morari EC, Cunha LL, Soares FA, Vassallo J, Ward LS. Clinical utility of KAP-1 expression in thyroid lesions. *Endocr Pathol* 2013; **24**: 77-82 [PMID: 23645532 DOI: 10.1007/s12022-013-9245-z]

119   **Kijanka G**, Hector S, Kay EW, Murray F, Cummins R, Murphy D, MacCraith BD, Prehn JH, Kenny D. Human IgG antibody profiles differentiate between symptomatic patients with and without colorectal cancer. *Gut* 2010; **59**: 69-78 [PMID: 19828471 DOI: 10.1136/gut.2009.178574]

120   **Bojkowska K**, Aloisio F, Cassano M, Kapopoulou A, Santoni de Sio F, Zangger N, Offner S, Cartoni C, Thomas C, Quenneville S, Johnsson K, Trono D. Liver-specific ablation of Krüppel-associated box-associated protein 1 in mice leads to male-predominant hepatosteatosis and development of liver adenoma. *Hepatology* 2012; **56**: 1279-1290 [PMID: 22684873 DOI: 10.1002/hep.25767]

Mnk kinase pathway: Cellular functions and biological outcomes

Sonali Joshi, Leonidas C Platanias

Sonali Joshi, Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

Leonidas C Platanias, Robert H Lurie Comprehensive Cancer Center and Division of Hematology-Oncology, Northwestern University Medical School, Jesse Brown VA Medical Center, Chicago, IL 60611, United States

Author contributions: Joshi S wrote the draft manuscript; Platanias LC revised and finalized the manuscript.

Correspondence to: Sonali Joshi, PhD, Department of Molecular and Cellular Oncology, University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit Number: 108, Houston, TX 77030, United States. ssjoshi@mdanderson.org

Telephone: + 1-713-7941233   Fax: +1-713-7924454

Received: November 26, 2013   Revised: March 10, 2014

Accepted: May 31, 2014

Published online: August 26, 2014

Abstract

The mitogen-activated protein kinase (MAPK) interacting protein kinases 1 and 2 (Mnk1 and Mnk2) play important roles in controlling signals involved in mRNA translation. In addition to the MAPKs (p38 or Erk), multiple studies suggest that the Mnk kinases can be regulated by other known kinases such as Pak2 and/or other unidentified kinases by phosphorylation of residues distinct from the sites phosphorylated by the MAPKs. Several studies have established multiple Mnk protein targets, including PSF, heterogenous nuclear ribonucleoprotein A1, Sprouty 2 and have lead to the identification of distinct biological functions and substrate specificity for the Mnk kinases. In this review we discuss the pathways regulating the Mnk kinases, their known substrates as well as the functional consequences of engagement of pathways controlled by Mnk kinases. These kinases play an important role in mRNA translation *via* their regulation of eukaryotic initiation factor 4E (eIF4E) and their functions have important implications in tumor biology as well as the regulation of drug resistance to anti-oncogenic therapies. Other studies have identified a role for the Mnk kinases in cap-independent mRNA translation, suggesting that the Mnk kinases can exert important functional effects independently of the phosphorylation of eIF4E. The role of Mnk kinases in inflammation and inflammation-induced malignancies is also discussed.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Core tip: The Mnk kinases are important downstream targets of the Erk and p38 mitogen-activated protein kinase (MAPK) pathways and their activity can also be modulated by MAPK independent signals. The Mnk kinases play important roles in regulating mRNA translation and, because of this, are key mediators of oncogenic progression, drug resistance, production of pro-inflammatory cytokines and cytokine signaling. This review focuses on the pathways regulating the Mnk kinases, the substrates on the Mnk kinases as well as the biological functions of the Mnk kinases.

INTRODUCTION

The Erk (extracellular regulated kinase) and p38 (mitogen-activated protein kinase) MAPK pathways are known to play important roles in mediating multiple biological processes including development, apoptosis, autophagy, oncogenesis, inflammation, *etc*[1]. Kinases that can be

phosphorylated by multiple MAPKs such as the MAPK interacting protein kinases (Mnks) can exert multiple biological functions due to their ability to respond to a wide range of external stimuli such as mitogens as well as stress inducers[1]. The Mnk kinase family includes Mnk1 and Mnk2 which were originally discovered in two independent screens as substrates for Erk1[2] and Erk2[3]. It is now well established that the Mnk kinases can be activated by either Erk or p38 MAPKs in response to multiple extracellular stimuli and phosphorylate their major downstream effector, the cap binding eukaryotic initiation factor 4E (eIF4E)[4].

Mnk1 and Mnk2 are serine/threonine kinases with substantial similarity in their coding sequences and motifs present in their structures[3]. Both kinases contain a N-terminal basic amino acid rich region that can mediate their localization; a catalytic domain similar to the serine/threonine kinases such as the Rsks, Ca21/calmodulin (CaM)-dependent kinases, Mapkap kinase-2 and Mapkap kinase-3 containing conserved MAPK phosphorylation sites; and an MAPK binding domain in their carboxyl terminus[3]. Mnk1 is activated in response to treatment with growth factors, ultraviolet (UV) radiation, mitogens and stress inducing agents such as anisomycin or sorbitol as well as by cytokines such as type Ⅰ and type Ⅱ interferons (IFNs), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, *etc*.[3-6]. In contrast Mnk2 exhibits high basal activity that is more resistant to the inhibition of Erk and p38 and these observations can be partially explained by differences in the C-terminal domains of the Mnk kinases[3,7].

A detailed look at the Mnk kinases has shown that both Mnk1 and Mnk2 undergo alternative splicing[8,9]. Mnk1 and Mnk2 transcripts are alternatively spliced giving rise to two distinct isoforms for each[8,9]. The b isoforms lack the MAPK binding C-terminal domain and therefore their activity is MAPK independent[8,10,11]. The b isoforms also lack a nuclear export sequence while still retaining the nuclear localization signal and therefore both Mnk1b and Mnk2b are preferentially localized to the nucleus and in PML bodies which also contain eIF4E[8,10]. While most of the studies on the Mnk kinases have focused on the Mnk1a and Mnk2a isoforms, evidence suggests that aberrant regulation of Mnk splicing can have important biological consequences. The splicing factor SF2/ASF which can function as a proto-oncogene in multiple human cancers can regulate Mnk2 splicing[12]. Overexpression of SF2/ASF was shown to result in the increased expression of the MAPK independent Mnk2b isoform, while knockdown of SF2/ASF attenuated the expression of Mnk2b[12]. This study suggests the need for a better understanding of the factors that regulate Mnk splicing as the preferential expression of the MAPK independent Mnk isoforms can have important biological implications.

POST TRANSCRIPTIONAL REGULATION OF MNK KINASES

Mnk kinase activity is mainly regulated by the upstream p38 and Erk MAPK pathways. The p38 MAPK pathway is activated by a variety of stress inducers such as osmotic shock, UV radiation, as well as cytokine and chemokine stimulation[13]; while engagement of the Erk MAPK pathway is primarily mediated by pro-growth stimuli such as growth factors and phorbol esters[1]. Thus the Mnk kinases can play a dual role in mediating cellular responses to stress as well as responses to mitogens in a context-specific manner. MAPK phosphorylation of Mnk1 results in the phosphorylation of Thr 209 and Thr 214 located in the T loop activation domain, whereas mouse Mnk1 is phosphorylated on Thr 197 and Thr 202[14].

Phosphorylation of Mnk1 has been shown to activate its kinase activity as well as to enhance its binding to the eukaryotic initiation factor 4G (eIF4G) which functions as a scaffolding protein[14,15]. Additionally Mnk1 mediated phosphorylation of eIF4E regulates its release from eIF4G[14]. eIF4G contains binding sites for the cap binding eIF4E and the poly A tail protein (PABP) at the N-terminus[16,17] while the C-terminal domain contains docking sites for eIF3, eIF4A and Mnk1[15,17]. eIF4G along with its binding partners and the small ribosomal subunits are important components of the 48S initiation complex required for translation initiation[18]. Studies suggest that Mnk1 is unable to interact with eIF4E in the absence of eIF4G and a mutant eIF4E lacking the ability to bind eIF4G is not a good Mnk1 substrate[15]. Additionally Mnk1 can interact with the eIF4G related translational repressor p97[15]. p97 which functions as a cap dependent and cap independent translation repressor has a 28% homology to the C-terminal of eIF4G and can interact with translation initiation factors such as eIF3, eIF4A but is unable to interact with the mRNA recruiting eIF4E[19]. Thus p97 may be a potential negative regulator of Mnk1 mediated phosphorylation of eIF4E [15]. Also PKCα which was initially believed to be a Mnk1 kinase is known to phosphorylate eIF4G on Ser 1186 facilitating its binding to Mnk1[20] and may potentially play an important role in regulating Mnk1 activity by indirectly controlling the phosphorylation of eIF4E. Mnk2 has also been shown to interact with eIF4G and to function as an eIF4E kinase[21]. Thus, regulation of the Mnk-eIF4G interaction can play an important role in regulating Mnk activity.

Other studies have suggested that phosphorylation of Mnk1 by the p21 activated kinase 2 (Pak2/γ-Pak) can negatively regulate its kinase activity[22]. Pak2 belongs to a family of serine/threonine kinases and is activated in response to stress inducing stimuli such as UV and ionizing radiation induced DNA damage, serum starvation, by the binding of the GTP bound small G protein cdc24 as well as by caspase 3 mediated cleavage[23]. Caspase 3 activated Pak2 mediated engagement of Mnk1 results in the phosphorylation of Thr 22 and Ser 27, residues that lie in the N-terminal domain of Mnk1 that can interact with eIF4G and thereby attenuates the affinity of Mnk1 towards eIF4G[22]. Additionally Pak2 mediated engagement of Mnk1 also attenuated Mnk1 mediated phosphorylation of eIF4G[22]. As the experiments conducted in this study were for the most part performed *in vitro*,

proteins is summarized in Figure 1.
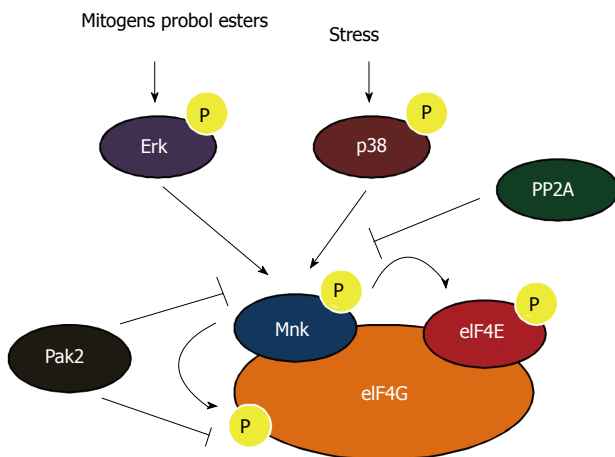
Figure 1 Regulation of Mnk kinases. The Mnk kinases are phosphorylated on Thr 197/202 by the p38 and Erk1/2 mitogen-activated protein kinases (MAPKs). They can associate with eIF4G and this interaction is essential for the efficient phosphorylation of their target eIF4E. The Mnk kinases are also known to phosphorylate eukaryotic initiation factor 4G (eIF4G) but its functional consequences remain to be determined. Pak2 can phosphorylate Mnk1 on Thr22/Ser27 resulting in decreased affinity for eIF4G and potentially interferes with Mnk1 mediated phosphorylation of eIF4E. Additionally Pak2 also phosphorylates eIF4G inhibiting its interaction with eIF4E. Protein phosphatase 2A (PP2A) is a phosphatase for Mnk1 and thereby negatively regulates Mnk kinase activity.

Pak2 mediated phosphorylation of Mnk1 did not affect Mnk1 mediated phosphorylation of eIF4E. Additionally Pak2 can also phosphorylate eIF4G at the eIF4E binding domain and compete with eIF4E to bind eIF4G, thereby exerting suppressive effects on cap dependent translation[24]. These results suggest that Mnk activity may be modulated independently of the MAPK pathway and may account for the observation that all stimuli that result in phosphorylation of Mnk1 do not result in activation of eIF4E on serine 209.

Mnk kinase activity can be negatively regulated by the protein phosphatase 2A (PP2A)[25]. Small interfering RNA mediated knockdown of PP2A or pharmacological inhibition of PP2A was found to result in increased phosphorylation of its direct target Mnk1 and subsequently increased phosphorylation of eIF4E[25]. Phosphorylation of eIF4E in response to PP2A inhibition leads to increased cap dependent translation of growth promoting mRNAs such as c-myc and Mcl-1[25].

Multiple studies have shown that Mnk2 has high basal activity that is mostly unresponsive to external stimuli. A study by Stead *et al*[26] showed that treatment of cells with rapamycin, the classic inhibitor of the mammalian target of rapamycin (mTOR), resulted in enhanced phosphorylation of eIF4E that was mediated by the enhanced activity of Mnk2 and not by Mnk1. The increase in Mnk2 activity was mediated by the decrease in phosphorylation of Mnk2 on Ser 437 by an unidentified mechanism[26]. These results suggest that Mnk2 activity may also be possibly modulated independently of the MAPK pathway. The regulation of Mnk kinases by upstream signaling

EFFECTORS OF THE MNK KINASES

The Mnk kinases function as serine/threonine kinases and are known to phosphorylate a number of downstream targets including eIF4E[3,21], hnRNPA1[27] and Sprouty2[28]. Additionally Mnk1 and Mnk2 can also exhibit substrate specificity[29], resulting in substrates that are unique to Mnk1 and Mnk2, respectively. Recent studies have surprising uncovered a kinase independent function for Mnk2 in negatively regulating eIF4G and p70S6K phosphorylations[30]. The proteins that regulate signaling downstream of the Mnk kinases are discussed in detail and are summarized in Figure 2.

eIF4E

A major and well characterized target of the Mnk kinases is the cap binding protein eIF4E. eIF4E is phosphorylated on Ser 209[31] by the Mnk kinases[3] but its role in regulating mRNA translation remains undetermined. Multiple biochemical studies have shown that phosphorylation of eIF4E reduces its affinity for the 5' m7G cap[32,33]. Based on X-ray crystallography data, Scheper *et al*[34] have speculated that the phosphate group on Ser 209 may negatively interact with the phosphate groups on the RNA backbone as well as the mRNA cap. They have put forth a model in which Mnk mediated phosphorylation of eIF4E after the formation of the pre-initiation translation complex leads to the release of eIF4E and thereby enables it to be available for another round of initiation of mRNA translation[34].

Studies based on the targeted deletion of Mnk1 and Mnk2 in mice have suggested that the expression of Mnk1 and/or Mnk2 and the phosphorylation of its target eIF4E is dispensable for survival[35]. Mice with a targeted deletion of Mnk1 and/or Mnk2 do not exhibit any developmental or reproductive defects[35]. Additionally the mouse studies also confirmed previous reports that Mnk1 is more sensitive to external stimuli as mitogen mediated eIF4E phosphorylation was defective in the Mnk1-/- cells, while basal eIF4E phosphorylation was attenuated in Mnk2-/- cells[35]. Mouse embryonic fibroblasts and adult tissues from mice lacking both Mnk1 and Mnk2 did not exhibit any basal or inducible eIF4E phosphorylation indicating that the Mnk kinases are key regulators of eIF4E phosphorylation[35]. Interestingly, cells from Mnk1 and Mnk2 deficient mice did not exhibit any defects in cap dependent translation or general protein synthesis, indicating that Mnk mediated phosphorylation of eIF4E is not critical under basal conditions but may be important during their activation with external stimuli[35].

Similarly knock-in mice expressing a mutant eIF4E (eIF4E S209A) which cannot be phosphorylated do not exhibit any developmental or viability defects[36]. These results suggest that while phosphorylation of eIF4E may not be critical for general mRNA translation, it may be
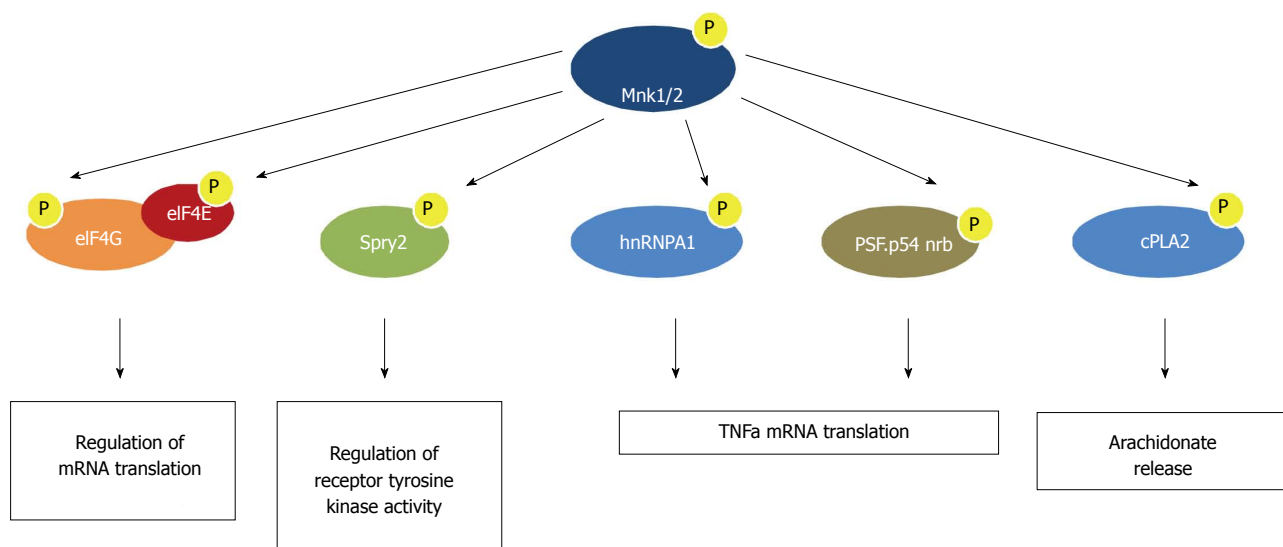
Figure 2 Effectors of the Mnk kinases. The Mnk kinases can regulate multiple biological processes by phosphorylating multiple substrates. Mnk mediated phosphorylation of eIF4E and eIF4G can play an important role in mediating cap dependent translation. The Mnk substrates hnRNPA1 and PSF play an important role in mediating the translation of AU rich elements containing mRNAs such as the TNF-α mRNA. The Mnk kinases also phosphorylate cPLA2 which plays an important role in arachidonate release from platelets. TNF-α: Tumor necrosis factor-α; eIF4G: Eukaryotic initiation factor 4G; hnRNPA1: Heterogenous nuclear ribonucleoprotein A1; cPLA2: Cytosolic phospholipase A2.

important for the translation of specific mRNAs, induced by specific stimuli. Phosphorylation of eIF4E is important for the translation of mRNAs containing 5' untranslated terminal regions (UTRs) with extensive secondary structure[37].

Besides its ability to bind capped mRNA, nuclear eIF4E can interact with a 100 nt eIF4E-sensitive element (4E-SE) region in the 3'UTRs of mRNAs and promote the nuclear export of the bound mRNA[38]. The phosphorylation of eIF4E on Ser 209 is required for its mRNA export activity, as well as transformation[39]. Mnk-mediated phosphorylation of eIF4E can facilitate the nuclear export of mRNAs such as HDM2[40], Cyclin D1[38] and other growth regulatory mRNAs[41].

Sprouty 2

Sprouty2 (Spry2) belongs family of proteins homologous to the Drosophila melanogaster Spry[42] that acts as a negative regulator of multiple receptor tyrosine kinase pathways[43,44] by negatively controlling the Erk MAPK pathway[45]. A study by DaSilva *et al*[28] showed that Mnk1 can phosphorylate Spry2 on Ser 112 and Ser 121 leading to increased stability of Spry2. Inhibition of Mnk activity resulted in increased tyrosine phosphorylation of Spry2 leading to increased binding of c-Cbl and promoting the polyubiquitination of Spry2; consequently resulting in a proteosome mediated decrease in Spry2 expression[28]. Additionally a mutant Spry2 (S112A and S121A) that cannot be phosphorylated by Mnk1 also increased proteosomal degradation of Spry2[28]. Mnk1 mediated stabilization of Spry2 was found to be functionally important for the antagonism of fibroblast growth factor (FGF) signaling by Spry2[28].

Another study showed that Mnk2 can regulate the phosphorylation of Spry2 on Ser 112 and Ser 121[46]. This study established that Mnk2-mediated phosphorylation of Spry2 increased its interaction with the E3 ubiquitin ligase NEDD4 and lead to increased proteosomal targeting of Spry2[46]. Additionally, small interfering RNA mediated silencing of Mnk2 attenuated Spry2 NEDD4 interactions and enhanced the ability of Spry2 to inhibit FGF signaling[46]. The results of the studies by DaSilva *et al*[28] and Edwin *et al*[46] are conflicting, but it is important to note that the studies were conducted in distinct biological cell lines. It is possible that Mnk kinases negatively or positively regulate Spry2 expression in a cell-specific manner, depending on the presence of additional regulatory cellular signals. More work focusing on the relevance of Mnk mediated phosphorylation of Spry2 is required to get a better understanding of the consequences of Spry2 phosphorylation by Mnk kinases.

Studies in our laboratory have previously shown that the Mnk kinases are activated by both type I and type II interferons (IFNs)[5,6]. IFNs are potent antiviral agents that also generate antiproliferative and antitumor responses[47,48]. Both type I and type II IFN mediated engagement of the Mnk kinases is important for regulating the inhibitory effects of IFNs on normal hematopoiesis by regulating the translation of specific IFN stimulated genes[5,6]. Importantly, engagement of Mnk kinases also play a critical role in mediating the anti-neoplastic effects of IFNs on primitive myeloproliferative neoplasm (MPN) precursors from patients with polycythemia vera[49]. Other work in our laboratory has shown that type I IFNs can upregulate the expression of both Spry1 and Spry2 in a Mnk1 and Mnk2 dependent manner[50]. Data from mouse embryonic fibroblasts (MEFs) derived from mice with a targeted deletion of Spry1, Spry2 and Spry4 suggests

lack of Spry expression promotes IFN mediated antiviral responses[50]. The Spry 1, 2, 3 triple knockout MEFs exhibit enhanced activation of the p38 MAPK pathway in response to IFN treatment and, consequently, enhanced transcriptional activity and expression of the IFN stimulated gene ISG15[50]. Additionally knockdown of either Spry1, Sry2 or Spry4 was found to result in enhanced anti-leukemic effects of type I IFNs[50]. Thus, Mnk mediated phosphorylation of Spry proteins can have important biological consequences, but more work is required to elucidate the role of Mnk mediated phosphorylation of Spry proteins and its biological relevance in response to tyrosine kinase signaling.

hnRNPA1

The Mnk pathway plays an important role in production of TNF-α *via* its effector hnRNPA1. TNF-α is mainly secreted by activated macrophages and T lymphocytes and plays important roles in regulating inflammation[51]. Enhanced secretion of TNF-α is implicated in diseases such as rheumatoid arthritis, and inflammatory bowel disease[52], as well as in superantigen-induced septic shock[53]. Thus, the mechanisms regulating its expression have important clinical-translational and therapeutic relevance. The TNF-α mRNA is tightly regulated by the AU rich elements (AREs) present in the 3'UTR that regulate its nuclear cytoplasmic export[54], mRNA stability[55] as well as its mRNA translation[56]. TNF-α production in activated macrophages, as well as T cells, is regulated by the p38 and Erk MAPK pathways[57,58], consistent with the concept that their common downstream effectors, Mnk kinases may play an important role in TNF-α production.

Buxade *et al*[27] showed that inhibition of Mnk1 activity/expression results in attenuated production of TNF-α in T cells. In that study, overexpression of Mnk1 resulted in increased expression of a reporter construct tagged with the TNF-α 3'UTR suggesting that Mnk1 regulation of TNF-α may be mediated by the AREs[27]. Mnk1 was found to phosphorylated the TNF-α ARE binding protein hnRNPA1 on Ser 192 and Ser 310/311/312 resulting in the disassociation of hnRNPA1 from the TNF-α 3'UTR[27]. Thus, during T cell activation, activation of the MAPK cascade leads to the engagement of Mnk1 and the phosphorylation of its target hnRNPA1 and its disassociation from the TNF-α ARE, consequently promoting the translation of the TNF-α mRNA[27]. ARE elements have also been identified in mRNA encoding cytokines (GM-CSF, IL-3, IFNγ, *etc.*), proto-oncogenes (bcl, c-myc etc.) as well as in nuclear transcription factors (c-fos, c-jun, junB, *etc.*)[59] suggesting that the Mnk kinases can mediate the translation of multiple mRNAs independently of translation initiation complex.

Guil *et al*[60] showed that stress induced engagement of the Mnk kinases results in the phosphorylation of hnRNPA1 leading to its accumulation in stress granules. Depletion of hnRNPA1 or the Mnk kinases attenuates cell recovery following osmotic stress, suggesting that Mnk-mediated recruitment of hnNPA1 to stress granules

plays an important role in regulating cell physiology possibly by controlling the expression of stress responsive mRNAs[60]. Many stress inducing stimuli can lead to senescence and this pathway[61,62] may potentially be mediated by the Mnk kinases due to their engagement by the stress activated p38 MAPK pathway. Mnk1 phosphorylation and expression is enhanced in senescent diploid human fibroblasts as compared to young fibroblasts[63]. In senescent cells, Mnk1 can phosphorylate hnRNPA1 leading to the cytoplasmic accumulation of hnRNPA1. Depletion of hnRNPA1 results in induction of senescence[64], suggesting that Mnk kinases may potentially regulate cellular senescence by regulating the cellular distribution of hnRNPA1.

PSF

Buxade *et al*[29] sought to identify novel substrates for the Mnk kinases. Using a proteomic approach, the researchers examined the ability of the Mnk kinases to phosphorylate proteins that could bind to a 5' cap resin[29]. They identified PSF [the PTB (polypyrimidine tract-binding protein)-associated splicing factor] as a potential Mnk substrate[29]. *In vitro* studies showed that the Mnk kinases could phosphorylate PSF on Ser 8 and Ser 283[29]. Remarkably, phosphorylation of PSF on Ser 8 was preferentially mediated by Mnk2 suggesting that Mnk1 and Mnk2 exhibit distinct substrate specificities[29]. PSF along with its partner p54 (nrb) was found to bind mRNAs containing AREs in their 3'UTR, and Mnk mediated phosphorylation of PSF was found to enhance its binding to the TNF-α mRNA containing AREs[29]. Notably, Mnk mediated phosphorylation of PSF did not affect the stability or the nuclear cytoplasmic localization of PSF or the bound TNF-α mRNA, but its effects on TNF-α mRNA translation were undetermined[29]. Thus another Mnk substrate can bind ARE elements in the 3'UTR of mRNAs again underscoring the role of Mnk kinases in mediating mRNA physiology independently of the cap translation initiation complex.

Cytosolic phospholipase A2

Cytosolic phospholipase A2 (cPLA2) is an enzyme activated by increased cytosolic calcium and catalyzes the release of arachidonate acid from glycerophospholipids to provide the precursor of the eicosanoids[65]. Eicosanoids are important secondary messenger molecules that play an important role in inflammation, immunity as well as regulation of the central nervous system[66]. Mnk1 was found to phosphorylate cPLA2 on Ser 727 resulting in the enhancement of its enzymatic activity[65]. Thrombin mediated platelet activation was found to result in Mnk1 mediated engagement of cPLA2 and arachidonate release[65]. Thus the Mnk kinases can play a role in regulating arachidonate acid release and thereby mediate eicosanoid signaling. Although no follow-up studies on the regulatory effects of the Mnk pathway on cPLA2 have been reported, further studies in that direction may provide important insights regarding the role of Mnk kinases

in various cellular and biological contexts.

Mnk2 specific interactions

The Mnk2 kinase was initially identified in a yeast two hybrid screen attempting to identify proteins that can interact with the ligand binding domain of the estrogen receptor β (ERβ)[9]. Only the nuclear Mnk2b isoform and not Mnk2a or Mnk1 was found to specifically interact with ERβ and not ERα[9]. Interestingly esterdiol treatment was found to augment Mnk2b binding to ERβ[9], but whether this interaction leads to the phosphorylation of ERβ or alters ERβ mediated transcription remains to be determined. Another study has reported that ERβ can be phosphorylated on Ser 105 by estradiol-mediated Erk1/2 activation or osmotic stress induced p38 MAPK activation and this phosphorylation was found to inhibit breast cancer migration and invasion[67]. These observations suggest that ERβ may be a potential substrate for the Mnk kinases.

Mnk2 has also been shown to phosphorylate plectin on Ser 4642[68]. Plectin is an ubiquitously expressed protein that can interact with microtubules, intermediate filaments and the actin microfilaments; and thereby plays an important role in regulating cellular responses to mechanical stress[69]. Mnk2 mediated plectin phosphorylation was found to attenuate plectin interactions with the intermediate filaments and reduced plectin phosphorylation was observed at sites of cell substrate contact that require a network of intermediate filaments[68]. These results suggest a potential role for Mnk kinases in mediating cytoskeletal integrity.

A study by Hu *et al*[30] showed that Mnk2 expression is augmented during muscle atrophy. Overexpression of Mnk2, but not Mnk1, was found to attenuate eIF4G phosphorylation on Ser 1108 and reduced basal p70 S6 kinase (p70S6K) phosphorylation at Thr 389 and Ser 371 in a kinase independent manner[30]. The serine-arginine rich protein kinase family members SRPK1, SRPK2 and SRPK3 were identified as the kinases that mediate eIF4G phosphorylation on Ser 1108[30]. Results from *in vivo* studies showed that dexamethasone treatment or starvation of Mnk2 knockout mice resulted in enhanced phosphorylation eIF4G Ser 1108 as compared to the wild type mice[30]. Mnk2 was found to selectively interact with the mammalian target of rapamycin complex 1 (mTORC1) , in a kinase independent manner and this interaction was essential to regulate Mnk2 mediated decreased phosphorylation of p70S6K[30]. As phosphorylation of eIF4G Ser 1108 and p70S6K Thr 389 and Ser 371 is associated with enhanced mRNA translation, these observations suggest Mnk2 may play an important role in negatively regulating protein synthesis during muscle atrophy[30]. These observations are consistent with other findings showing that overexpression of Mnk kinases can negatively regulate cap dependent translation[70] and suggest that Mnk mediated regulation of mRNA translation may be context dependent. Altogether, the available evidence indicates that Mnk1 and Mnk2 exhibit differing substrate specificities

and, possibly, distinct biological functions. The functional differences between Mnk1 and Mnk2 need to further explored in future studies using both *in vitro* and *in vivo* approaches.

BIOLOGICAL FUNCTIONS OF THE MNK KINASES

There is extensive and definitive evidence that Mnk kinases regulate the phosphorylation and/or activity of proteins involved in diverse cellular functions. As a result of such effects, the Mnk kinases play important roles in cancer biology, development of drug resistance to cancer therapeutics, cap independent translation, as well in mediating pro-inflammatory cytokine production and cytokine signaling (Figure 3).

Role of Mnk kinases in tumorigenesis

eIF4E is known to be upregulated in a variety of human cancers and is linked to poor prognosis[71]. Additionally, overexpression of eIF4E in NIH-3T3 and rat 2 fibroblasts results in their oncogenic transformation[72]. As eIF4E is modulated by phosphorylation by Mnk kinases, Mnk kinases and phosphorylated eIF4E may have important roles in cancer biology (reviewed in[73]). Studies with mouse models using a rapid adoptive transfer strategy suggest that a constitutively active Mnk1 leads to increased eIF4E phosphorylation and promotes lymphomagenesis by preventing apoptosis and/or by upregulating mRNA translation of the anti-apoptotic Mcl-1[74].

Mouse embryonic fibroblasts derived from mice with a targeted deletion of both Mnk1 and Mnk2 are resistant to Ras mediated transformation[75]. Deletion of both Mnk1 and Mnk2 in a T-cell specific Pten null lymphoma model resulted in delayed tumorigenesis and lymphomas with an absence of eIF4E phosphorylation[75]. Additionally knock-in mice expressing a mutant eIF4E (S209A) that cannot be phosphorylated are resistant to oncogenic transformation by both c-myc and a constitutively active Ras[36]. Additionally the knock-in mice are resistant to Pten loss-induced prostate cancer and exhibit decreased expression of proteins involved in tumorigenesis such as vascular endothelial growth factor (VEGF) and matrix metalloprotease 3 (MMP3)[36]. Moreover, phosphorylated eIF4E positively correlates with progression to human prostate carcinoma[36]. Other studies have shown that inhibition of Mnk activity and the consequent decrease in the phosphorylation of eIF4E strongly attenuates the polysomal recruitment of terminal oligopyrimidine messenger RNAs (TOP mRNAs) and results in decreased expression of mRNAs involved in proliferation in prostate cancer[76].

The Mnk kinases are overexpressed in glioblastoma and inhibition of the Mnk kinases results in attenuated cell growth and increased sensitivity to rapamycin[77]. Additionally, inhibition of Mnk activity was found to attenuate mRNA translation of a subset of genes involved
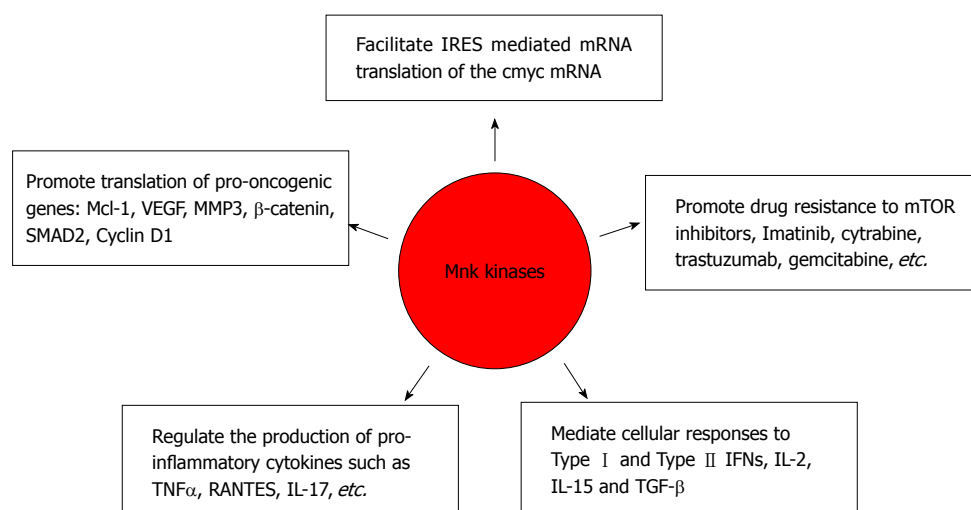
Figure 3 Biological functions of Mnk kinases. The Mnk kinases play an important role in multiple biological processes. Mnk1/2 can regulate tumor biology by mediating the translation of multiple genes that promote tumor growth and resistance to apoptosis. They also mediate resistance to chemotherapy as well as targeted therapy agents such as trastuzumab, imatinib, gemcitabine, *etc*. Mnk kinases are also implicated in regulating cap dependent translation of oncogenes as well as viral mRNA. Additionally the Mnk kinases play an important role in mediating the production of multiple pro-inflammatory cytokines such as TNF-α, RANTES and IL-17 and also mediate cellular responses to multiple cytokines such as Type Ⅰ and Type Ⅱ IFNs, IL-2, IL-15 and TGF-β. TNF-α: Tumor necrosis factor-α; IL: Interleukin; RANTES: Regulated upon activation normal T cell expressed and presumably secreted; TGF: Transforming growth factor; MMP3: Matrix metalloprotease 3; IRES: Internal ribosome entry sites; IFNs: Interferons.

in transforming growth factor β (TGFβ) signaling and regulation of signal transduction and induced cell cycle arrest[77]. A microarray analysis of polysomal mRNA revealed an important role for Mnk kinases in mediating the mRNA translation of SMAD2[77]. Importantly, SMAD2 expression positively correlated with Mnk1 expression in human glioblastoma patients and Mnk1 was found to play an important role in mediating TGFβ induced cell motility[77].

The phosphorylation of Mnk1 and Mnk2 is elevated in Her-2 over-expressing breast cancers and inhibition of Mnk activity can attenuate growth in soft agar[78]. Inhibition of Mnk activity in breast cancer cell lines exerts a cytostatic effect by downregulating the expression of Cyclin D1, one of the targets of phosphorylated eIF4E[79]. In breast cancer cell lines, the integrin α6β4 interaction leads to the engagement of the Mnk kinases in a p38 and Erk dependent manner and enhances VEGF mRNA translation[80].

The Mnk kinases are also known to play a role in hematological malignancies. Acute myeloid leukemia (AML) is often characterized by expression of different fusion proteins that account for leukemic transformation[81]. A microarray study demonstrated that MNK1 is post-translationally stabilized by PML-RAR alpha[82]. Notably, inhibition of Mnk1 activity/expression was found to enhance ATRA (all-trans retinoic acid) induced myeloid differentiation[82]. Another recent study has shown that chronic myeloid leukemia (CML) patients exhibiting blast crisis are characterized by enhanced Mnk-eIF4E phosphorylation consequently leading to augmented β-catenin protein synthesis as well as its nuclear translocation and activation[83]. These results suggest that inhibition of the Mnk kinases may have potential anti-leukemic properties.

Thus the Mnk kinases can play an important role in tumor progression and the development of Mnk inhibitors will have an important clinical applications.

Role of Mnk kinases in drug resistance

Mnk kinases can modulate multiple aspects of tumor biology and data from multiple studies suggest that they may also be involved in drug resistance by multiple mechanisms. Inhibition of mTOR by drugs such as temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus (AP-23573) has shown promising results in preclinical studies and are under investigation in cancer clinical trials[84]. Numerous studies in our laboratory as well as others have shown that rapamycin treatment of cancer cells results in the phosphorylation of the Mnk kinases as well as its target eIF4E[26,85] in a phosphoinositide 3-kinase (PI-3K) dependent manner[86]. In malignant hematopoietic cells, rapamycin treatment leads to a phosphorylation of Mnk1 and its target eIF4E, while simultaneous inhibition of both mTOR and Mnk kinases enhances the anti-leukemic effects of rapamycin[85]. Additionally Mnk1 inhibition has been shown to augment the anti-tumor effects of rapamycin in multiple human lung cancer cell lines[86]. In prostate cancer cells, inhibition of mTOR or the Mnk kinases results in distinct changes in translation initiation and the simultaneous inhibition both kinases exerts additive negative effects in the recruitment of TOP mRNAs and strong suppressive effects on cell cycle progression[76].

CML is characterized by the t(9; 22) translocation resulting in the constitutively active fusion oncogene bcr-abl, and its inhibition by imatinib mesylate (imatinib) results in a potent patient responses[87]. However, patients with late stage disease often develop resistance to imatinib resulting in decreased drug efficacy[88,89]. A study by Zhang *et al*[90] showed that simultaneous inhibition of the Mnk

kinases and imatinib treatment resulted in a synergistic enhancemet of the anti-leukemic effects of imatinib by augmenting its anti-proliferative and apoptotic effects. Inhibition of the Mnk kinases was found to attenuate polysomal mRNA recruitment by enhancing imatinib mediating inhibition of the pre-initiation complex eIF4F and by independently inhibiting the phosphorylation of the pre-initiation complex associated ribosomal protein S6 (rpS6)[90]. Additionally inhibition of the Mnk kinases has been found to also enhance the anti-leukemic effects of the chemotherapeutic drug cytarabine, currently in clinical use for the treatment of acute myeloid leukemia (AML)[85].

Breast cancers with overexpression of the oncogenic Her-2 are clinically treated with trastuzumab (herceptin), a monoclonal antibody targeting the ectodomain of Her-2[91]. Breast cancer patients that respond to trastuzumab often develop resistance within a year of initiation of treatment[92], underscoring a need to uncover the mechanisms contributing to drug resistance. The oncogenic Y-box-binding protein-1 (YB-1) can be phosphorylated by the p90 ribosomal S6 kinase as well as Akt promoting its nuclear translocation, upregulating the expression of the epidermal growth factor (EGFR), MET, PIK3CA and CD44 ultimately conferring trastuzumab resistance[93]. Using an unbiased chromatin immune-precipitation sequencing approach to identify the transcriptional targets of YB-1, Astanehe *et al*[94] identified Mnk1 as a YB-1 transcriptional target. Mnk1 and Mnk2 were found to be overexpressed in trastuzumab resistant cell lines and depletion of Mnk1 was found to augment trastuzumab sensitivity[94]. Consistently, overexpression of Mnk1 was sufficient to confer trastuzumab resistance[94] suggesting a causative role for Mnk1 in the process.

Pancreatic ductal adenocarcinoma (PDAC) is clinically treated with the chemotherapeutic drug gemcitabine which results in marginal benefits when used as a single agent[95]. A study by Adesso *et al*[96], showed that eIF4E phosphorylation positively correlates with PDAC tumor grade and predicts a ppor prognosis. *In vitro* studies showed that gemcitabine treatment can induce eIF4E phosphorylation in a Mnk2 dependent and Mnk1 independent manner[96]. Gemcitabine was found to induce the expression of the oncogenic splicing factor serine/arginine rich splicing factor (SRSF1) which preferentially promoted the expression of the MAPK independent Mnk2b isoform with high basal activity[96]. Interestingly, inhibition of Mnk activity synergistically enhanced the anti-oncogenic effects of gemcitabine by promoting apoptosis suggesting an important role for Mnk2 and SRSF1 in mediating gemcitabine resistance[96].

Thus the Mnk kinases can regulate resistance to chemotherapy as well as targeted therapy in multiple cancer types. The clinical development of Mnk inhibitors may therefore play an important role in enhancing the efficacy of cancer therapeutics.

Role of Mnk kinases in cap independent translation
The role of Mnk kinases in cap dependent translation

had been the subject of extensive work, but more recent evidence suggests that the Mnk kinases may also play an important role in mediating cap independent translation. Cap independent translation is mediated by the internal ribosome entry sites (IRES) in the 5'UTR of the target mRNAs[97,98]. The IRES elements possess complex secondary and tertiary structures that facilitate the interaction with the 40S ribosome in the absence of eIF4E and other translation initiation factors[99]. IRES elements can thereby facilitate mRNA translation when cap dependent translation is impaired in virus infected cells[100] or in malignant cells treated with drugs inhibiting cap dependent translation[101].

Cap dependent translation is often dis-regulated in malignant cells and drugs inhibiting cap dependent translation are in common clinical use. Studies in multiple neoplastic cell types have suggested that cancer sensitivity to rapalogs is decreased by induction of the Akt pathway[102,103] subsequently resulting in IRES mediated translation of oncogenes such as VEGF[104], cyclin D1 and c-myc[105]. Interestingly, the IRES mediated translation of oncogenes is also regulated by the p38 and Erk MAPK pathways[105] suggesting a role for the Mnk kinases in controlling cap independent translation. A recent study by Shi *et al*[106] demonstrated that mTOR inhibition by rapamycin in multiple myeloma cells results in the activation of Mnk1. Inhibition of Mnk activity or expression was found to attenuate rapamycin induced upregulation of c-myc IRES activity[106]. Combination treatment of malignant cells with rapamycin and a Mnk inhibitor was found to abolish c-myc expression and enhanced the anti-oncogenic activity of rapamycin[106].

Additional evidence from viral studies also supports a role for the Mnk kinases in the regulation of IRES mediated translation. A study by Goetz *et al*[107] showed that replication and cytotoxicity of the prototype oncolytic poliovirus PVSRIPO in glioblastoma multiforme (GBM) results in the engagement of Mnk1 subsequently resulting in the enhanced cap independent translation of the viral RNA[107]. Taken together, these results suggest that Mnk kinases play important roles in regulating cap independent translation and more studies along this line are required to gain mechanistic insight into such effects.

Role of Mnk kinases in inflammation
MAPK pathways such as Erk and p38 have been shown to play important roles in modulating immune responses by mediating the production of cytokines that control the initiation of innate immunity; the activation of adaptive immunity; and by regulating cellular responses to cytokines involved in immune responses[108]. As Mnk kinases are effectors of MAPK pathways, these observations suggest that they may play important roles in mediating cytokine production at the translational level. Indeed pharmacological blockade of Mnk kinases was found to attenuate the production of pro-inflammatory cytokines such TNF-α, IL-6, and monocyte chemo-attractant protein-1 and enhanced the production of the anti-inflam-

matory cytokine IL-10 in macrophages stimulated with multiple Toll like receptor (TLR) agonists[109]. Also, data from multiple studies have shown that Mnk kinases play important roles in mediating the production of multiple pro-inflammatory cytokines such as TNF-α, RANTES and IL-17 and in mediating the cellular responses to Type Ⅰ and Type Ⅱ IFNs, IL-2, IL-15 and TGF-β (reviewed in[110]).

Most of the studies focusing on the role of the Mnk kinases in inflammation have utilized small interfering RNA mediated Mnk knockdown or pharmacological inhibitors of the Mnk kinases. Recently a study by Gorentla *et al*[111], examined the role of Mnk kinases in T cell development in mice with a targeted deletion of Mnk1 and Mnk2. This study showed that in mice lacking Mnk1 and Mnk2, T-cell receptor mediated Ser 209 phosphorylation of eIF4E in T cells was completely abolished[111]. Lack of of Mnk1 and Mnk2 expression in T cells had no influence on the development of conventional αβ T cells, regulatory T cells, or NKT (natural killer T cells)[111]. The Mnk1/2 double knockout mice also did not exhibit any deficiencies in CD8 T cell response to bacterial or viral infection[111]. Interestingly, while lack of the Mnk kinases does not inhibit Th1 and Th17 differentiation *in vitro*, immunization of mice with myelin oligodendrocyte glycoprotein peptide in complete Freund's adjuvant, an experimental model of autoimmune encephalomyelitis, resulted in attenuated production of IFNγ and IL-17 by CD4 T cells and attenuated differentiation of Th1 and Th17 cells[111]. Collectively, these results suggest that while the Mnk kinases are dispensable for normal T cell development and function, they may play important roles in regulating the cytokines required for T cell differentiation or antigen presenting cell (APC) activation pathways, and thereby modulate Th cell differentiation in an T cell extrinsic manner[111].

Another recent study focused on the role of the Mnk kinases in the generation of neutrophil responses. Neutrophils are involved in acute inflammatory response and secrete proinflammatory cytokines such as TNF-α, IL-1β, IL-8, IFNγ, IL-4, IL-10, *etc*.[112]. Mnk1 is phosphorylated in human neutrophils upon treatment with LPS or TNF-α[113]. Inhibition of the Mnk kinases in LPS or TNF-α stimulated human neutrophils was found to attenuate the secretion of CXCL8, CCL-3 and CCL4 while the mRNA levels of the cytokines were unaffected, Mnk inhibition also attenuated the anti-apoptotic effects of LPS and TNF-α[113]. Overexpression of a kinase active Mnk1 and not a kinase dead Mnk1 mutant was found to enhance LPS- and TNF-α- induced cytokine secretion[113]. Similarly the Mnk kinases play important roles in pro-inflamatory cytokine production in macrophages[109]. These studies further support the observation that the Mnk kinases are attractive targets for diseases associated with inflammation.

While pro-inflammatory cytokines play an important role in mediating an effective immune response to pathogens, their persistent enhanced expression is associated with multiple disorders such as auto-immune diseases[114],

allergies[115], neurological disorders[116], sepsis[117], cardiovascular diseases[118], obesity[119] and cancer[120]. As the Mnk kinases represent a central node in regulating proinflammatory cytokine production, development of Mnk inhibitors will have important broad spectrum translational implications.

CONCLUSION

The Mnk kinases are regulated by the p38 and Erk MAPK pathways and their activity can also be modulated by other MAPK independent mechanisms. Multiple proteins such as those involved in mRNA translation (eIF4E, eIF4G), in TNF-α mRNA expression (hnRNPA1, PSF), in platelet activity (cPLA2) and in regulation of receptor tyrosine kinase activity (Spry2) are regulated by the Mnk kinases. As a result, the Mnk kinases can play important roles in controlling cap-dependent and -independent translation, participate in the pathophysiology of several malignant and inflammatory diseases and diminish responses to cancer therapeutics (Figure 3).

The above observations suggest that development of Mnk inhibitors can have broad spectrum clinical applications. Most of the studies discussed in this review used the Mnk inhibitor CGP57380 a low weight molecular compound identified from the Novartis Pharma compound collection that can inhibit both Mnk1 and Mnk2 activity[121]. The IC$_{50}$ of CGP57380 against Mnk1 is seen at a concentration of 2.2 μmol/L[70], the concentration at which it can also inhibit the activity of other kinases such as casein kinase, MAP2K1 and BR serine/threonine-protein kinase 2[122]. As a result this compound cannot be used for *in vivo* studies and research mainly utilizing CGP57380 should be interpreted with caution. The antifungal agent cercosporamide is also reported to inhibit Mnk activity, although it exhibits higher specificity for Mnk2 as compared to Mnk1[123]. Importantly cercosporamide has been shown to exhibit anti-tumor effects in both *in vitro* and *in vivo* studies[123, 124]. More research efforts are needed to develop Mnk inhibitors that can be tested in clinical settings.

REFERENCES

1 **Roux PP**, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004; **68**: 320-344 [PMID: 15187187 DOI: 10.1128/MMBR.68.2.320-344.2004]
2 **Fukunaga R**, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J* 1997; **16**: 1921-1933 [PMID: 9155018 DOI: 10.1093/emboj/16.8.1921]
3 **Waskiewicz AJ**, Flynn A, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* 1997; **16**: 1909-1920 [PMID: 9155017 DOI: 10.1093/emboj/16.8.1909]
4 **Wang X**, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA, Proud CG. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP

kinase pathways. *J Biol Chem* 1998; **273**: 9373-9377 [PMID: 9545260]

5   **Joshi S**, Sharma B, Kaur S, Majchrzak B, Ueda T, Fukunaga R, Verma AK, Fish EN, Platanias LC. Essential role for Mnk kinases in type II interferon (IFNgamma) signaling and its suppressive effects on normal hematopoiesis. *J Biol Chem* 2011; **286**: 6017-6026 [PMID: 21149447 DOI: 10.1074/jbc. M110.197921]

6   **Joshi S**, Kaur S, Redig AJ, Goldsborough K, David K, Ueda T, Watanabe-Fukunaga R, Baker DP, Fish EN, Fukunaga R, Platanias LC. Type I interferon (IFN)-dependent activation of Mnk1 and its role in the generation of growth inhibitory responses. *Proc Natl Acad Sci USA* 2009; **106**: 12097-12102 [PMID: 19574459 DOI: 10.1073/pnas.0900562106]

7   **Parra JL**, Buxadé M, Proud CG. Features of the catalytic domains and C termini of the MAPK signal-integrating kinases Mnk1 and Mnk2 determine their differing activities and regulatory properties. *J Biol Chem* 2005; **280**: 37623-37633 [PMID: 16162500]

8   **O'Loghlen A**, González VM, Piñeiro D, Pérez-Morgado MI, Salinas M, Martín ME. Identification and molecular characterization of Mnk1b, a splice variant of human MAP kinase-interacting kinase Mnk1. *Exp Cell Res* 2004; **299**: 343-355 [PMID: 15350534]

9   **Slentz-Kesler K**, Moore JT, Lombard M, Zhang J, Hollingsworth R, Weiner MP. Identification of the human Mnk2 gene (MKNK2) through protein interaction with estrogen receptor beta. *Genomics* 2000; **69**: 63-71 [PMID: 11013076]

10   **Scheper GC**, Parra JL, Wilson M, Van Kollenburg B, Vertegaal AC, Han ZG, Proud CG. The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization. *Mol Cell Biol* 2003; **23**: 5692-5705 [PMID: 12897141 DOI: 10.1128/MCB.23.16.5692-5705.2003]

11   **Goto S**, Yao Z, Proud CG. The C-terminal domain of Mnk1a plays a dual role in tightly regulating its activity. *Biochem J* 2009; **423**: 279-290 [PMID: 19650764]

12   **Karni R**, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol* 2007; **14**: 185-193 [PMID: 17310252]

13   **Zarubin T**, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 2005; **15**: 11-18 [PMID: 15686620]

14   **Shveygert M**, Kaiser C, Bradrick SS, Gromeier M. Regulation of eukaryotic initiation factor 4E (eIF4E) phosphorylation by mitogen-activated protein kinase occurs through modulation of Mnk1-eIF4G interaction. *Mol Cell Biol* 2010; **30**: 5160-5167 [PMID: 20823271 DOI: 10.1128/MCB.00448-10]

15   **Pyronnet S**, Imataka H, Gingras AC, Fukunaga R, Hunter T, Sonenberg N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *EMBO J* 1999; **18**: 270-279 [PMID: 9878069 DOI: 10.1093/emboj/18.1.270]

16   **Imataka H**, Gradi A, Sonenberg N. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J* 1998; **17**: 7480-7489 [PMID: 9857202 DOI: 10.1093/emboj/17.24.7480]

17   **Lamphear BJ**, Kirchweger R, Skern T, Rhoads RE. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J Biol Chem* 1995; **270**: 21975-21983 [PMID: 7665619]

18   **Rhoads RE**, Joshi B, Minich WB. Participation of initiation factors in the recruitment of mRNA to ribosomes. *Biochimie* 1994; **76**: 831-838 [PMID: 7880899]

19   **Imataka H**, Olsen HS, Sonenberg N. A new translational regulator with homology to eukaryotic translation initiation factor 4G. *EMBO J* 1997; **16**: 817-825 [PMID: 9049310 DOI: 10.1093/emboj/16.4.817]

20   **Dobrikov M**, Dobrikova E, Shveygert M, Gromeier M. Phosphorylation of eukaryotic translation initiation factor 4G1 (eIF4G1) by protein kinase C{alpha} regulates eIF4G1 binding to Mnk1. *Mol Cell Biol* 2011; **31**: 2947-2959 [PMID: 21576361 DOI: 10.1128/MCB.05589-11]

21   **Scheper GC**, Morrice NA, Kleijn M, Proud CG. The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol Cell Biol* 2001; **21**: 743-754 [PMID: 11154262 DOI: 10.1128/MCB.21.3.743-754.2001]

22   **Orton KC**, Ling J, Waskiewicz AJ, Cooper JA, Merrick WC, Korneeva NL, Rhoads RE, Sonenberg N, Traugh JA. Phosphorylation of Mnk1 by caspase-activated Pak2/gamma-PAK inhibits phosphorylation and interaction of eIF4G with Mnk. *J Biol Chem* 2004; **279**: 38649-38657 [PMID: 15234964]

23   **Roig J**, Traugh JA. Cytostatic p21 G protein-activated protein kinase gamma-PAK. *Vitam Horm* 2001; **62**: 167-198 [PMID: 11345898]

24   **Ling J**, Morley SJ, Traugh JA. Inhibition of cap-dependent translation via phosphorylation of eIF4G by protein kinase Pak2. *EMBO J* 2005; **24**: 4094-4105 [PMID: 16281055]

25   **Li Y**, Yue P, Deng X, Ueda T, Fukunaga R, Khuri FR, Sun SY. Protein phosphatase 2A negatively regulates eukaryotic initiation factor 4E phosphorylation and eIF4F assembly through direct dephosphorylation of Mnk and eIF4E. *Neoplasia* 2010; **12**: 848-855 [PMID: 20927323]

26   **Stead RL**, Proud CG. Rapamycin enhances eIF4E phosphorylation by activating MAP kinase-interacting kinase 2a (Mnk2a). *FEBS Lett* 2013; **587**: 2623-2628 [PMID: 23831578]

27   **Buxadé M**, Parra JL, Rousseau S, Shpiro N, Marquez R, Morrice N, Bain J, Espel E, Proud CG. The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. *Immunity* 2005; **23**: 177-189 [PMID: 16111636]

28   **DaSilva J**, Xu L, Kim HJ, Miller WT, Bar-Sagi D. Regulation of sprouty stability by Mnk1-dependent phosphorylation. *Mol Cell Biol* 2006; **26**: 1898-1907 [PMID: 16479008 DOI: 10.1128/MCB.26.5.1898-1907.2006]

29   **Buxadé M**, Morrice N, Krebs DL, Proud CG. The PSF. p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. *J Biol Chem* 2008; **283**: 57-65 [PMID: 17965020]

30   **Hu SI**, Katz M, Chin S, Qi X, Cruz J, Ibebunjo C, Zhao S, Chen A, Glass DJ. MNK2 inhibits eIF4G activation through a pathway involving serine-arginine-rich protein kinase in skeletal muscle. *Sci Signal* 2012; **5**: ra14 [PMID: 22337810]

31   **Flynn A**, Proud CG. Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. *J Biol Chem* 1995; **270**: 21684-21688 [PMID: 7665584]

32   **Scheper GC**, van Kollenburg B, Hu J, Luo Y, Goss DJ, Proud CG. Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. *J Biol Chem* 2002; **277**: 3303-3309 [PMID: 11723111]

33   **von Der Haar T**, Ball PD, McCarthy JE. Stabilization of eukaryotic initiation factor 4E binding to the mRNA 5'-Cap by domains of eIF4G. *J Biol Chem* 2000; **275**: 30551-30555 [PMID: 10887196]

34   **Scheper GC**, Proud CG. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? *Eur J Biochem* 2002; **269**: 5350-5359 [PMID: 12423333]

35   **Ueda T**, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R. Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. *Mol Cell Biol* 2004; **24**: 6539-6549 [PMID: 15254222 DOI: 10.1128/MCB.24.15.6539-6549.2004]

36   **Furic L**, Rong L, Larsson O, Koumakpayi IH, Yoshida

K, Brueschke A, Petroulakis E, Robichaud N, Pollak M, Gaboury LA, Pandolfi PP, Saad F, Sonenberg N. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proc Natl Acad Sci USA* 2010; **107**: 14134-14139 [PMID: 20679199 DOI: 10.1073/pnas.1005320107]

37 **Koromilas AE**, Lazaris-Karatzas A, Sonenberg N. mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J* 1992; **11**: 4153-4158 [PMID: 1396596]

38 **Culjkovic B**, Topisirovic I, Skrabanek L, Ruiz-Gutierrez M, Borden KL. eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3'UTR. *J Cell Biol* 2005; **169**: 245-256 [PMID: 15837800 DOI: 10.1083/jcb.200501019]

39 **Topisirovic I**, Ruiz-Gutierrez M, Borden KL. Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities. *Cancer Res* 2004; **64**: 8639-8642 [PMID: 15574771]

40 **Phillips A**, Blaydes JP. MNK1 and EIF4E are downstream effectors of MEKs in the regulation of the nuclear export of HDM2 mRNA. *Oncogene* 2008; **27**: 1645-1649 [PMID: 17828301]

41 **Strudwick S**, Borden KL. The emerging roles of translation factor eIF4E in the nucleus. *Differentiation* 2002; **70**: 10-22 [PMID: 11963652]

42 **Hacohen N**, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* 1998; **92**: 253-263 [PMID: 9458049]

43 **Mason JM**, Morrison DJ, Basson MA, Licht JD. Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol* 2006; **16**: 45-54 [PMID: 16337795]

44 **Kim HJ**, Bar-Sagi D. Modulation of signalling by Sprouty: a developing story. *Nat Rev Mol Cell Biol* 2004; **5**: 441-450 [PMID: 15173823]

45 **Cabrita MA**, Christofori G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* 2008; **11**: 53-62 [PMID: 18219583]

46 **Edwin F**, Anderson K, Patel TB. HECT domain-containing E3 ubiquitin ligase Nedd4 interacts with and ubiquitinates Sprouty2. *J Biol Chem* 2010; **285**: 255-264 [PMID: 19864419 DOI: 10.1074/jbc.M109.030882]

47 **Borden EC**, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* 2007; **6**: 975-990 [PMID: 18049472]

48 **Platanias LC**. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 2005; **5**: 375-386 [PMID: 15864272]

49 **Mehrotra S**, Sharma B, Joshi S, Kroczynska B, Majchrzak B, Stein BL, McMahon B, Altman JK, Licht JD, Baker DP, Eklund EA, Wickrema A, Verma A, Fish EN, Platanias LC. Essential role for the Mnk pathway in the inhibitory effects of type I interferons on myeloproliferative neoplasm (MPN) precursors. *J Biol Chem* 2013; **288**: 23814-23822 [PMID: 23814052 DOI: 10.1074/jbc.M113.476192]

50 **Sharma B**, Joshi S, Sassano A, Majchrzak B, Kaur S, Aggarwal P, Nabet B, Bulic M, Stein BL, McMahon B, Baker DP, Fukunaga R, Altman JK, Licht JD, Fish EN, Platanias LC. Sprouty proteins are negative regulators of interferon (IFN) signaling and IFN-inducible biological responses. *J Biol Chem* 2012; **287**: 42352-42360 [PMID: 23074222 DOI: 10.1074/jbc.M112.400721]

51 **Locksley RM**, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; **104**: 487-501 [PMID: 11239407]

52 **Feldmann M**, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996; **14**: 397-440

[PMID: 8717520]

53 **Miethke T**, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J Exp Med* 1992; **175**: 91-98 [PMID: 1730929]

54 **Dumitru CD**, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 2000; **103**: 1071-1083 [PMID: 11163183]

55 **Kontoyiannis D**, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999; **10**: 387-398 [PMID: 10204494]

56 **Han J**, Brown T, Beutler B. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. *J Exp Med* 1990; **171**: 465-475 [PMID: 2303781]

57 **Buxadé M**, Ramírez-Alvarado M, Fernández-Troy N, MacKenzie S, Casaroli-Marano RP, Vilella R, Espel E. Integrating signals from T-cell receptor and serum by T cells enhance translation of tumour necrosis factor-alpha. *Immunology* 2001; **102**: 416-425 [PMID: 11328375 DOI: 10.1046/j.1365-2567.2001.01206.x]

58 **Hoffmeyer A**, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S. Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. *J Biol Chem* 1999; **274**: 4319-4327 [PMID: 9933633]

59 **Chen CY**, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* 1995; **20**: 465-470 [PMID: 8578590]

60 **Guil S**, Long JC, Cáceres JF. hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Mol Cell Biol* 2006; **26**: 5744-5758 [PMID: 16847328 DOI: 10.1128/MCB.00224-06]

61 **Marusyk A**, Wheeler LJ, Mathews CK, DeGregori J. p53 mediates senescence-like arrest induced by chronic replicational stress. *Mol Cell Biol* 2007; **27**: 5336-5351 [PMID: 17515610 DOI: 10.1128/MCB.01316-06]

62 **Toussaint O**, Remacle J, Dierick JF, Pascal T, Frippiat C, Royer V, Magalhaes JP, Zdanov S, Chainiaux F. Stress-induced premature senescence: from biomarkers to likeliness of in vivo occurrence. *Biogerontology* 2002; **3**: 13-17 [PMID: 12014832]

63 **Ziaei S**, Shimada N, Kucharavy H, Hubbard K. MNK1 expression increases during cellular senescence and modulates the subcellular localization of hnRNP A1. *Exp Cell Res* 2012; **318**: 500-508 [PMID: 22227431 DOI: 10.1016/j.yexcr.2011.12.015]

64 **Shimada N**, Rios I, Moran H, Sayers B, Hubbard K. p38 MAP kinase-dependent regulation of the expression level and subcellular distribution of heterogeneous nuclear ribonucleoprotein A1 and its involvement in cellular senescence in normal human fibroblasts. *RNA Biol* 2009; **6**: 293-304 [PMID: 19430204]

65 **Hefner Y**, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson SP, Gelb MH. Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *J Biol Chem* 2000; **275**: 37542-37551 [PMID: 10978317]

66 **Funk CD**. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001; **294**: 1871-1875 [PMID: 11729303]

67 **Lam HM**, Suresh Babu CV, Wang J, Yuan Y, Lam YW, Ho SM, Leung YK. Phosphorylation of human estrogen recep-

tor-beta at serine 105 inhibits breast cancer cell migration and invasion. *Mol Cell Endocrinol* 2012; **358**: 27-35 [PMID: 22370157 DOI: 10.1016/j.mce.2012.02.012]

68   **Bouameur JE**, Schneider Y, Begré N, Hobbs RP, Lingasamy P, Fontao L, Green KJ, Favre B, Borradori L. Phosphorylation of serine 4,642 in the C-terminus of plectin by MNK2 and PKA modulates its interaction with intermediate filaments. *J Cell Sci* 2013; **126**: 4195-4207 [PMID: 23843618 DOI: 10.1242/jcs.127779]

69   **Svitkina TM**, Verkhovsky AB, Borisy GG. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J Cell Biol* 1996; **135**: 991-1007 [PMID: 8922382]

70   **Knauf U**, Tschopp C, Gram H. Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. *Mol Cell Biol* 2001; **21**: 5500-5511 [PMID: 11463832 DOI: 10.1128/MCB.21.16.5500-5511.2001]

71   **De Benedetti A**, Harris AL. eIF4E expression in tumors: its possible role in progression of malignancies. *Int J Biochem Cell Biol* 1999; **31**: 59-72 [PMID: 10216944]

72   **Lazaris-Karatzas A**, Montine KS, Sonenberg N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* 1990; **345**: 544-547 [PMID: 2348862]

73   **Hou J**, Lam F, Proud C, Wang S. Targeting Mnks for cancer therapy. *Oncotarget* 2012; **3**: 118-131 [PMID: 22392765]

74   **Wendel HG**, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Teruya-Feldstein J, Pelletier J, Lowe SW. Dissecting eIF4E action in tumorigenesis. *Genes Dev* 2007; **21**: 3232-3237 [PMID: 18055695 DOI: 10.1101/gad.1604407]

75   **Ueda T**, Sasaki M, Elia AJ, Chio II, Hamada K, Fukunaga R, Mak TW. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. *Proc Natl Acad Sci USA* 2010; **107**: 13984-13990 [PMID: 20679220 DOI: 10.1073/pnas.1008136107]

76   **Bianchini A**, Loiarro M, Bielli P, Busà R, Paronetto MP, Loreni F, Geremia R, Sette C. Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. *Carcinogenesis* 2008; **29**: 2279-2288 [PMID: 18809972]

77   **Grzmil M**, Morin P, Lino MM, Merlo A, Frank S, Wang Y, Moncayo G, Hemmings BA. MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-β signaling pathway in human glioblastoma. *Cancer Res* 2011; **71**: 2392-2402 [PMID: 21406405]

78   **Chrestensen CA**, Shuman JK, Eschenroeder A, Worthington M, Gram H, Sturgill TW. MNK1 and MNK2 regulation in HER2-overexpressing breast cancer lines. *J Biol Chem* 2007; **282**: 4243-4252 [PMID: 17130135]

79   **Wheater MJ**, Johnson PW, Blaydes JP. The role of MNK proteins and eIF4E phosphorylation in breast cancer cell proliferation and survival. *Cancer Biol Ther* 2010; **10**: 728-735 [PMID: 20686366 DOI: 10.4161/cbt.10.7.12965]

80   **Korneeva NL**, Soung YH, Kim HI, Giordano A, Rhoads RE, Gram H, Chung J. Mnk mediates integrin α6β4-dependent eIF4E phosphorylation and translation of VEGF mRNA. *Mol Cancer Res* 2010; **8**: 1571-1578 [PMID: 21047768 DOI: 10.1158/1541-7786.MCR-10-0091]

81   **Müller-Tidow C**, Steffen B, Cauvet T, Tickenbrock L, Ji P, Diederichs S, Sargin B, Köhler G, Stelljes M, Puccetti E, Ruthardt M, deVos S, Hiebert SW, Koeffler HP, Berdel WE, Serve H. Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. *Mol Cell Biol* 2004; **24**: 2890-2904 [PMID: 15024077 DOI: 10.1128/MCB.24.7.2890-2904.2004]

82   **Worch J**, Tickenbrock L, Schwäble J, Steffen B, Cauvet T, Mlody B, Buerger H, Koeffler HP, Berdel WE, Serve H, Müller-Tidow C. The serine-threonine kinase MNK1 is post-translationally stabilized by PML-RARalpha and regulates differentiation of hematopoietic cells. *Oncogene* 2004; **23**: 9162-9172 [PMID: 15516979]

83   **Lim S**, Saw TY, Zhang M, Janes MR, Nacro K, Hill J, Lim AQ, Chang CT, Fruman DA, Rizzieri DA, Tan SY, Fan H, Chuah CT, Ong ST. Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proc Natl Acad Sci USA* 2013; **110**: E2298-E2307 [PMID: 23737503 DOI: 10.1073/pnas.1301838110]

84   **Brachmann S**, Fritsch C, Maira SM, García-Echeverría C. PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. *Curr Opin Cell Biol* 2009; **21**: 194-198 [PMID: 19201591 DOI: 10.1016/j.ceb.2008.12.011]

85   **Altman JK**, Glaser H, Sassano A, Joshi S, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Tallman MS, Platanias LC. Negative regulatory effects of Mnk kinases in the generation of chemotherapy-induced antileukemic responses. *Mol Pharmacol* 2010; **78**: 778-784 [PMID: 20664001 DOI: 10.1124/mol.110.064642]

86   **Wang X**, Yue P, Chan CB, Ye K, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Fu H, Khuri FR, Sun SY. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. *Mol Cell Biol* 2007; **27**: 7405-7413 [PMID: 17724079 DOI: 10.1128/MCB.00760-07]

87   **Simonsson B**, Grp IS. Beneficial effects of cytogenetic and molecular response on long-term outcome in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib (IM): Update from the IRIS study. *Blood* 2005; **106**: 52a-52a

88   **Gorre ME**, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001; **293**: 876-880 [PMID: 11423618 DOI: 10.1126/science.1062538]

89   **Shah NP**, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002; **2**: 117-125 [PMID: 12204532 DOI: 10.1016/S1535-6108(02)00096-X]

90   **Zhang M**, Fu W, Prabhu S, Moore JC, Ko J, Kim JW, Druker BJ, Trapp V, Fruehauf J, Gram H, Fan HY, Ong ST. Inhibition of polysome assembly enhances imatinib activity against chronic myelogenous leukemia and overcomes imatinib resistance. *Mol Cell Biol* 2008; **28**: 6496-6509 [PMID: 18694961 DOI: 10.1128/MCB.00477-08]

91   **Carter P**, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992; **89**: 4285-4289 [PMID: 1350088]

92   **Nahta R**, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res* 2006; **8**: 215 [PMID: 17096862 DOI: 10.1186/bcr1612]

93   **Dhillon J**, Astanehe A, Lee C, Fotovati A, Hu K, Dunn SE. The expression of activated Y-box binding protein-1 serine 102 mediates trastuzumab resistance in breast cancer cells by increasing CD44+ cells. *Oncogene* 2010; **29**: 6294-6300 [PMID: 20802512]

94   **Astanehe A**, Finkbeiner MR, Krzywinski M, Fotovati A, Dhillon J, Berquin IM, Mills GB, Marra MA, Dunn SE. MKNK1 is a YB-1 target gene responsible for imparting trastuzumab resistance and can be blocked by RSK inhibition. *Oncogene* 2012; **31**: 4434-4446 [PMID: 22249268]

95   **Kern SE**, Shi C, Hruban RH. The complexity of pancreatic ductal cancers and multidimensional strategies for therapeutic targeting. *J Pathol* 2011; **223**: 295-306 [PMID: 21125682 DOI: 10.1002/path.2813]

96   **Adesso L**, Calabretta S, Barbagallo F, Capurso G, Pilozzi

E, Geremia R, Delle Fave G, Sette C. Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway. *Oncogene* 2013; **32**: 2848-2857 [PMID: 22797067 DOI: 10.1038/onc.2012.306]

97   **Pelletier J,** Sonenberg N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* 1988; **334**: 320-325 [PMID: 2839775 DOI: 10.1038/334320a0]

98   **Jang SK**, Kräusslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* 1988; **62**: 2636-2643 [PMID: 2839690]

99   **Komar AA**, Hatzoglou M. Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell Cycle* 2011; **10**: 229-240 [PMID: 21220943 DOI: 10.4161/cc.10.2.14472]

100   **Hanson PJ**, Zhang HM, Hemida MG, Ye X, Qiu Y, Yang D. IRES-Dependent Translational Control during Virus-Induced Endoplasmic Reticulum Stress and Apoptosis. *Front Microbiol* 2012; **3**: 92 [PMID: 22461781 DOI: 10.3389/fmicb.2012.00092]

101   **Holcík M.** Targeting translation for treatment of cancer--a novel role for IRES? *Curr Cancer Drug Targets* 2004; **4**: 299-311 [PMID: 15134536 DOI: 10.2174/1568009043333005]

102   **Wan X,** Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 2007; **26**: 1932-1940 [PMID: 17001314 DOI: 10.1038/sj.onc.1209990]

103   **Sun SY**, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 2005; **65**: 7052-7058 [PMID: 16103051 DOI: 10.1158/0008-5472.CAN-05-0917]

104   **Frost P,** Shi Y, Hoang B, Lichtenstein A. AKT activity regulates the ability of mTOR inhibitors to prevent angiogenesis and VEGF expression in multiple myeloma cells. *Oncogene* 2007; **26**: 2255-2262 [PMID: 17016437 DOI: 10.1038/sj.onc.1210019]

105   **Shi Y**, Sharma A, Wu H, Lichtenstein A, Gera J. Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. *J Biol Chem* 2005; **280**: 10964-10973 [PMID: 15634685]

106   **Shi Y**, Frost P, Hoang B, Yang Y, Fukunaga R, Gera J, Lichtenstein A. MNK kinases facilitate c-myc IRES activity in rapamycin-treated multiple myeloma cells. *Oncogene* 2013; **32**: 190-197 [PMID: 22370634 DOI: 10.1038/onc.2012.43]

107   **Goetz C**, Everson RG, Zhang LC, Gromeier M. MAPK signal-integrating kinase controls cap-independent translation and cell type-specific cytotoxicity of an oncolytic poliovirus. *Mol Ther* 2010; **18**: 1937-1946 [PMID: 20648000 DOI: 10.1038/mt.2010.145]

108   **Dong C,** Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol* 2002; **20**: 55-72 [PMID: 11861597 DOI: 10.1146/annurev.immunol.20.091301.131133]

109   **Rowlett RM,** Chrestensen CA, Nyce M, Harp MG, Pelo JW, Cominelli F, Ernst PB, Pizarro TT, Sturgill TW, Worthington MT. MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G452-G459 [PMID: 18032482 DOI: 10.1152/ajpgi.00077.2007]

110   **Joshi S**, Platanias LC. Mnk Kinases in Cytokine Signaling and Regulation of Cytokine Responses. *Biomol Concepts* 2012; **3**: 127-139 [PMID: 23710261 DOI: 10.1515/bmc-2011-0057]

111   **Gorentla BK**, Krishna S, Shin J, Inoue M, Shinohara ML, Grayson JM, Fukunaga R, Zhong XP. Mnk1 and 2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* 2013; **190**: 1026-1037 [PMID: 23269249 DOI: 10.4049/jimmunol.1200026]

112   **Kasama T,** Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy* 2005; **4**: 273-279 [PMID: 16101533 DOI: 10.2174/1568010054022114]

113   **Fortin CF,** Mayer TZ, Cloutier A, McDonald PP. Translational control of human neutrophil responses by MNK1. *J Leukoc Biol* 2013; **94**: 693-703 [PMID: 23401599 DOI: 10.1189/jlb.0113012]

114   **Brennan FM,** Feldmann M. Cytokines in autoimmunity. *Curr Opin Immunol* 1992; **4**: 754-759 [PMID: 1466799 DOI: 10.1016/0952-7915(92)90057-L]

115   **Bachert C,** Hauser U, Prem B, Rudack C, Ganzer U. Proinflammatory cytokines in allergic rhinitis. *Eur Arch Otorhinolaryngol* 1995; **252** Suppl 1: S44-S49 [PMID: 7537566 DOI: 10.1007/BF02484434]

116   **Merrill JE**. Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome. *J Immunother* (1991) 1992; **12**: 167-170 [PMID: 1445805]

117   **Netea MG,** van der Meer JW, van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol* 2003; **24**: 254-258 [PMID: 12738419 DOI: 10.1016/S1471-4906(03)00079-6]

118   **Dinarello CA,** Pomerantz BJ. Proinflammatory cytokines in heart disease. *Blood Purif* 2001; **19**: 314-321 [PMID: 11244192 DOI: 10.1159/000046960]

119   **Stienstra R,** Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. *Cell Metab* 2012; **15**: 10-18 [PMID: 22225872 DOI: 10.1016/j.cmet.2011.10.011]

120   **Arya M,** Patel HR, Williamson M. Chemokines: key players in cancer. *Curr Med Res Opin* 2003; **19**: 557-564 [PMID: 14594528 DOI: 10.1185/030079903125002216]

121   **Tschopp C,** Knauf U, Brauchle M, Zurini M, Ramage P, Glueck D, New L, Han J, Gram H. Phosphorylation of eIF-4E on Ser 209 in response to mitogenic and inflammatory stimuli is faithfully detected by specific antibodies. *Mol Cell Biol Res Commun* 2000; **3**: 205-211 [PMID: 10891393 DOI: 10.1006/mcbr.2000.0217]

122   **Bain J,** Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007; **408**: 297-315 [PMID: 17850214 DOI: 10.1042/BJ20070797]

123   **Konicek BW,** Stephens JR, McNulty AM, Robichaud N, Peery RB, Dumstorf CA, Dowless MS, Iversen PW, Parsons S, Ellis KE, McCann DJ, Pelletier J, Furic L, Yingling JM, Stancato LF, Sonenberg N, Graff JR. Therapeutic inhibition of MAP kinase interacting kinase blocks eukaryotic initiation factor 4E phosphorylation and suppresses outgrowth of experimental lung metastases. *Cancer Res* 2011; **71**: 1849-1857 [PMID: 21233335 DOI: 10.1158/0008-5472.CAN-10-3298]

124   **Altman JK**, Szilard A, Konicek BW, Iversen PW, Kroczynska B, Glaser H, Sassano A, Vakana E, Graff JR, Platanias LC. Inhibition of Mnk kinase activity by cercosporamide and suppressive effects on acute myeloid leukemia precursors. *Blood* 2013; **121**: 3675-3681 [PMID: 23509154 DOI: 10.1182/blood-2013-01-477216]

REVIEW

Critical role of bicarbonate and bicarbonate transporters in cardiac function

Hong-Sheng Wang, Yamei Chen, Kanimozhi Vairamani, Gary E Shull

Hong-Sheng Wang, Yamei Chen, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, United States
Kanimozhi Vairamani, Gary E Shull, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524, United States
Correspondence to: Gary E Shull, PhD, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0524, United States. shullge@ucmail.uc.edu
Telephone: +1-513-5580056 Fax: +1-513-5591885

Abstract

Bicarbonate is one of the major anions in mammalian tissues and extracellular fluids. Along with accompanying H^+, HCO_3^- is generated from CO_2 and H_2O, either spontaneously or *via* the catalytic activity of carbonic anhydrase. It serves as a component of the major buffer system, thereby playing a critical role in pH homeostasis. Bicarbonate can also be utilized by a variety of ion transporters, often working in coupled systems, to transport other ions and organic substrates across cell membranes. The functions of HCO_3^- and HCO_3^--transporters in epithelial tissues have been studied extensively, but their functions in heart are less well understood. Here we review studies of the identities and physiological functions of Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters of the *SLC4A* and *SLC26A* families in heart. We also present RNA Seq analysis of their cardiac

mRNA expression levels. These studies indicate that *slc4a*3 (AE3) is the major Cl^-/HCO_3^- exchanger and plays a protective role in heart failure, and that *Slc4a4* (NBCe1) is the major Na^+/HCO_3^- cotransporter and affects action potential duration. In addition, previous studies show that HCO_3^- has a positive inotropic effect in the perfused heart that is largely independent of effects on intracellular Ca^{2+}. The importance of HCO_3^- in the regulation of contractility is supported by experiments showing that isolated cardiomyocytes exhibit sharply enhanced contractility, with no change in Ca^{2+} transients, when switched from Hepes-buffered to HCO_3^--buffered solutions. These studies demonstrate that HCO_3^- and HCO_3^--handling proteins play important roles in the regulation of cardiac function.

Key words: SLC4; SLC26; Slc26a6; AE1; AE2; NBCn1

Core tip: Bicarbonate is one of the major anions in mammalian tissues and fluids. It plays a critical role in pH homeostasis and is utilized by various transporters to transport other ions and organic substrates across cell membranes. Here we review studies of the physiological functions of Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters in heart, present RNA Seq analysis of their cardiac mRNA expression levels, and show that bicarbonate is required for optimal contractility in isolated cardiac myocytes. These studies demonstrate that HCO_3^- and HCO_3^- handling proteins are abundant in heart and play important roles in the regulation of cardiac function.

INTRODUCTION

In mammalian tissues, bicarbonate/CO_2 is the major extrinsic buffer system of both extracellular and intracellular fluids. HCO_3^-/CO_2 are likely to play a particularly important role in cardiac muscle[1,2], which relies almost entirely on oxidative metabolism and continuously converts large quantities of O_2 to CO_2. *In vivo*, HCO_3^- is usually formed by carbonic anhydrase-mediated hydration of CO_2[2,3], in a reaction that also generates H^+ ($CO_2 + H_2O \rightarrow H^+ + HCO_3^-$). As might be expected, cardiac myocytes express a variety of ion transporters that mediate extrusion of H^+ and either extrusion or uptake of HCO_3^-. This allows fine control of intracellular pH (pH_i) and coupling of H^+ and HCO_3^- transport to the transport of other ions, thereby affecting not only pH_i, but also cell volume and both cellular and systemic ion homeostasis[4-6].

H^+ and HCO_3^- are, in effect, transient ions[2] that can be used to transport other ions and organic substrates across cell membranes, both directly and by secondary active transport. These ion transporters include Cl^-/HCO_3^- exchangers, Na^+/HCO_3^- cotransporters (NBCs), and Na^+/H^+ exchangers (NHEs). The functions of the various acid-base transporters have been studied most extensively in epithelial tissues; however, they exhibit a surprising abundance and diversity in cardiac tissues. In this paper we review studies describing the identities, membrane locations, and functions of the major HCO_3^- transporters in heart. In addition, we report relative mRNA expression levels in mouse heart for members of the *SLC4A* and *SLC26A* anion transporter families[7-9], which include all of the known Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters. Finally, we discuss previous studies of the effects of HCO_3^- on the isolated heart[10] and correlate those results with new data using isolated cardiac myocytes. The available evidence shows that a diverse group of transporters are responsible for movements of HCO_3^- into and out of the heart and demonstrate that the presence of HCO_3^- has a major stimulatory effect on contractility that is, at least in part, independent of changes in intracellular Ca^{2+}.

IDENTIFICATION AND LOCATIONS OF CARDIAC HCO₃⁻ TRANSPORTERS

Cloning and hybridization analyses have led to the identification of three Cl^-/HCO_3^- exchangers of the *SLC4A* family in heart. These anion exchangers are termed AE1, AE2, and AE3 (Anion Exchanger 1, 2, and 3; gene symbols, *Slc4a1-3*), and one Cl^-/HCO_3^- exchanger of the *SLC26A* family, termed Slc26a6 or PAT1 (putative anion transporter 1). Among the known Na^+/HCO_3^- cotransporters (NBCs), which are members of the *SLC4A* family, the electrogenic NBCe1 and electroneutral NBCn1 (gene symbols, *Slc4a4* and *Slc4a7*) have been identified in heart. Excellent reviews of the *SLC4A* and *SLC26A*[7-9,11,12] families of transporters have been published recently and provide detailed information about specific isoforms, including their ion transport specificities and their physiological functions in various tissues.

AE1 is the band 3 Cl^-/HCO_3^- exchanger that is expressed most prominently in red blood cells[13] and also includes a kidney variant[14] derived from an alternative promoter in intron 4 of the erythrocyte transcription unit[15]. Erythrocyte AE1 plays major roles in maintaining the stability of the cytoskeleton[16] and in gas exchange[17]. Cardiac AE1 mRNA identified in rat heart is smaller than that of the erythroid and kidney variants[18]. It encodes a truncated protein based on immunoblot analyses, although the exact identity of the cardiac AE1 protein in rat heart has not been determined[19]. Immunofluorescence studies of rat heart suggest that truncated cardiac AE1 protein is restricted to intercalated discs[20]. Expression of AE1 is sharply reduced in adult mouse heart compared with its levels in fetal heart[21], consistent with the RNA Seq data discussed below. AE2 is expressed at low levels in heart[18,21,22], and AE2a, one of 4 variants derived from the use of alternative promoters[23], was the only variant detected[22]. The membrane location of AE2 in heart has not been determined. AE3 mRNAs are expressed at very high levels in heart[18,24] and encode both a full-length variant (AE3fl) that is expressed in brain and other tissues and a much more abundant cardiac variant[25-27]. The cardiac AE3 (AE3c) mRNA is derived from an alternative promoter located in intron 6 of the longer transcription unit and has a unique 73-amino acid sequence that replaces the first 270 amino acids of AE3fl[25]. In fetal mouse heart, AE3fl is the predominant form; however, in adult heart, AE3fl is largely restricted to the atria, while AE3c is the predominant form in ventricles[27]. In cardiac myocytes, AE3 protein has been localized to t-tubules and the sarcolemma, with apparent foci of expression at costameres[28].

The *SLC26A* family transports a broad range of anions, including sulfate, chloride, iodide, bicarbonate, oxalate, and formate, and some isoforms can function as anion channels[9]. The first member of this family shown to function as a Cl^-/HCO_3^- exchanger was *Slc26a3*[29]; however, it is primarily an epithelial transporter and is expressed at only low levels in adult heart[21]. Slc26a6 can mediate Cl^-/HCO_3^- exchange[30-32], which appears to be its major function in apical membranes of the intestine[33]. In the renal proximal tubule, however, Slc26a6 functions primarily as a Cl^-/formate and Cl^-/oxalate exchanger[34,35]. Slc26a6 also mediates Cl^-/OH^- exchange and has been proposed to serve both as a Cl^-/HCO_3^- exchanger and as a Cl^-/OH^- exchanger in heart[21,36]. Slc26a6 protein has been localized to the t-tubules and sarcolemma[28].

Prior to the molecular cloning of Na^+/HCO_3^- cotransporter (NBC) isoforms, both electroneutral[37,38] and electrogenic[39,40] Na^+/HCO_3^- cotransport activities had been identified in cardiac muscle. NBCe1 (gene symbol, *Slc4a4*), the first NBC to be cloned[41,42], is electrogenic. In kidney, NBCe1 mediates outward transport of 1 Na^+ and 3 HCO_3^- across the basolateral membrane of proximal tubule epithelial cells[41,42]. In most other tissues, including
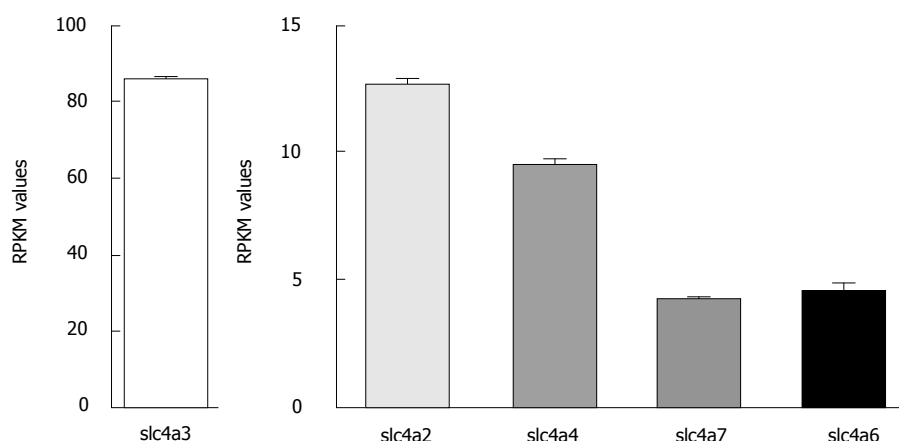
Figure 1 Relative expression levels of the major Cl⁻/HCO₃⁻ exchangers and Na⁺/HCO₃⁻ cotransporters in mouse heart. RPKM values ± SE as determined by RNA Seq analysis (see Table 1 legend) are shown for the most abundant known HCO₃⁻ transporters in wild-type FVB/N mouse hearts (n = 4). Note the difference in scale for AE3 and the other transporters.

heart, regulation *via* phosphorylation results in a stoichiometry of 1:2[43]. Cloning of the cardiac form of NBCe1 revealed that it has a different N-terminus than the kidney variant[44], which is derived from an alternative promoter and first exon. NBCn1 is electroneutral and transports Na⁺ and HCO₃⁻ in a 1:1 ratio. It was cloned from rat smooth muscle[45] and skeletal muscle[46] and shown to be expressed in heart. A study using an antibody to the N-terminal sequence of an NBCn1 variant indicated that expression in heart was restricted to endothelial and smooth muscle cells[47]. However, NBCn1 transcripts undergo extensive alternative splicing, including a relatively cardiac-specific exon[48], and the use of alternative promoters that yield alternative N-termini[49]. Both NBCe1 and NBCn1 have been shown by Western blots to be expressed in cardiac myocytes[50]. Immunolocalization studies demonstrated that both isoforms are localized to t-tubules, lateral sarcolemma, and intercalated discs[50].

EXPRESSION LEVELS OF HCO₃⁻ TRANSPORTERS IN HEART

As discussed above, the major HCO₃⁻ transporters in the mammalian heart include both Cl⁻/HCO₃⁻ exchangers and Na⁺/HCO₃⁻ cotransporters of the *SLC4A* gene family and at least one Cl⁻/HCO₃⁻ exchanger of the *SLC26A* family. Gene expression data for hearts of wild-type FVBN mice were determined by RNA Seq analysis[51], a powerful method for determining the expression levels of all known mRNAs in a tissue of interest[52,53]. Relative mRNA expression levels for the *SLC4A* and *SLC26A* families are shown in Table 1. Transcript levels are expressed as RPKM values (reads per kilobase of exon per million mapped reads), which normalizes expression to the length of the mRNA. Graphical representations of mRNA expression levels of the HCO₃⁻ transporters that have been identified in heart and appear to be expressed at physiologically relevant levels are shown in Figure 1.

Among the *SLC4A* transporters, the AE3 Cl⁻/HCO₃⁻ exchanger was expressed at very high levels (RPKM =

85.7 ± 0.6), suggesting that it serves as the major HCO₃⁻ efflux mechanism in mouse cardiac myocytes. AE2 (RPKM = 12.57 ± 0.28) was expressed at lower levels than AE3, but its levels of expression were still greater than some of the other transporters. Although NBC4 (*Slc4a5* or NBCe2) was reported in human heart[54], it is not expressed at significant levels in mouse heart or in rat heart[55]. Of the two major Na⁺/HCO₃⁻ cotransporters in mouse heart, NBCe1 (RPKM = 9.45 ± 0.25) was more abundant than NBCn1 (RPKM = 4.24 ± 0.05). By comparison, RPKM values for the NHE1 Na⁺/H⁺ exchanger[51], recognized as the major Na⁺-dependent acid extruder in heart, were 9.10 ± 0.20.

Among the SLC26A transporters, Slc26a2, Slc26a6, and Slc26a10 were expressed most abundantly. However, among these three *SLC26A* transporters, the only known HCO₃⁻ transporter is Slc26a6 (RPKM = 4.56 ± 0.26). As discussed above, it has been shown to function as a Cl⁻/HCO₃⁻ exchanger, but it also mediates Cl⁻/OH⁻, Cl⁻/formate, and Cl⁻/oxalate exchange[9]. Slc26a2 is a sulfate transporter and also transports Cl⁻ and oxalate[9]. The ion specificity of Slc26a10 has not been determined[9], but its high level of expression (RPKM = 31.66 ± 2.55) suggests that it plays an important role in mouse heart.

Some of the HCO₃⁻ transporters expressed at low levels (*e.g.*, the Slc4a8 Na⁺-dependent Cl⁻/HCO₃⁻ exchanger and Slc26a3 Cl⁻/HCO₃⁻ exchanger) could still play important roles in heart, particularly if they were restricted to specialized regions of the heart or were expressed primarily in earlier stages of development or in cell-types other than cardiac myocytes. For example, Na⁺-dependent Cl⁻/HCO₃⁻ exchange, which could be due to the activity of Slc4a8[56], has been identified in both chicken embryonic cardiomyocytes[57] and in vascular endothelial cells[58].

PHYSIOLOGICAL FUNCTIONS OF CARDIAC HCO₃⁻ TRANSPORTERS

Cl⁻/HCO₃⁻ Exchangers
Because of the high Cl⁻ concentrations of extracellular

Table 1 Relative mRNA levels for the Slc4a and Slc26a anion transporters in mouse heart

SLC4A family			*SLC26A* family		
Gene symbol	Transporter name and major function(s); alternate names	Average ± SE	Gene symbol	Transporter name and major function(s); alternate names	Average ± SE
Slc4a1	AE1 Cl$^-$/HCO$_3^-$ exchanger; Band 3	0.2 ± 0.05	*Slc26a1*	SAT1 sulfate/anion exchanger; Slc26a1	0.34 ± 0.07
Slc4a2	AE2 Cl$^-$/HCO$_3^-$ exchanger	12.57 ± 0.28	*Slc26a2*	DTDST sulfate/anion exchanger; Slc26a2	2.98 ± 0.17
Slc4a3	AE3 Cl$^-$/HCO$_3^-$ exchanger	85.7 ± 0.64	*Slc26a3*	DRA Cl$^-$/HCO3$^-$, exchanger; Slc26a3	0.64 ± 0.05
Slc4a4	NBCe1 Na$^+$/HCO$_3^-$ cotransporter; NBC1	9.45 ± 0.25	*Slc26a4*	Pendrin Cl$^-$/HCO$_3^-$ exchanger; Slc26a4	0.02 ± 0.01
Slc4a5	NBCe2 Na$^+$/HCO$_3^-$ cotransporter; NBC4	0.003 ± 0.002	*Slc26a6*	PAT1 Cl$^-$/HCO$_3^-$, Cl$^-$/formate exchanger; Slc26a6	4.56 ± 0.26
Slc4a7	NBCn1 Na$^+$/HCO$_3^-$ cotransporter; NBC2; NBC3	4.24 ± 0.05	*Slc26a7*	Slc26a7 Cl$^-$/HCO$_3^-$ exchanger, Cl$^-$ channel; TAT1	0.15 ± 0.04
Slc4a8	NDCBE Na$^+$-driven Cl$^-$/HCO$_3^-$ exchanger; Slc4a8	0.73 ± 0.04	*Slc26a9*	Slc26a9 Cl$^-$/HCO$_3^-$ exchanger, Cl$^-$ channel	0.01 ± 0.01
Slc4a9	AE4 Cl$^-$/HCO$_3^-$ exchanger	0.02 ± 0.01	*Slc26a10*	Slc26a10, transporter function unknown	31.66 ± 2.55
Slc4a10	NBCn2 Na$^+$/HCO$_3^-$ cotransporter	0.02 ± 0.003	*Slc26a11*	Slc26a11 anion exchanger, Cl$^-$ channel; KBAT	1.15 ± 0.14

Relative mRNA expression levels were determined using RNA from 4-month-old male FVB/N mouse hearts (n = 4) as described previously[51]. Values are RPKM (reads per kilobase per million mapped reads) ± SE and are a measure of the relative abundance of specific gene transcripts[53]. For some Slc26a transporters, ion transport specificities are more complex than indicated; see Alper and Sharma[9].

fluids, electroneutral Cl$^-$/HCO$_3^-$ exchangers mediate outward transport of HCO$_3^-$ and inward transport of Cl$^-$. The direct effect of this activity is to reduce pH$_i$, thereby contributing to pH$_i$ regulation[59], and to enhance Cl$^-$-loading, which could affect Cl$^-$ currents that in turn could affect action potentials or rhythmicity[60,61]. Also, when coupled with Na$^+$-dependent acid extrusion mechanisms, Cl$^-$/HCO$_3^-$ exchange facilitates Na$^+$-loading, which can affect contractility as discussed below and may contribute to cardiac hypertrophy[2]. AE1, AE2, and AE3 are electroneutral, but Cl$^-$/HCO$_3^-$ exchangers of the *SLC26A* family are reported to support both electroneutral or electrogenic anion exchange[9,12].

The physiological functions of AE1 in heart are unclear. An AE1 global knockout mouse has been shown to develop cardiac hypertrophy[28]. The investigators noted, however, that the levels of AE1 in the adult heart are relatively low (confirmed by the data in Table 1) and that null mutants exhibit severe hemolytic anemia and spherocytosis. They attributed the hypertrophy to the blood defect and concluded that one of the more abundant Cl$^-$/HCO$_3^-$ exchangers, possibly AE3, was more likely to provide the HCO$_3^-$ extrusion capability that has been proposed to balance Na$^+$-dependent acid extrusion *via* transporters such as the NHE1 Na$^+$/H$^+$ exchanger (discussed below). AE2 is a potential candidate for this activity as it is known to operate in concert with NHE1 on basolateral membranes of colonic epithelial cells[62]. AE2 is much less abundant than AE3 and its functions in heart have not been determined.

It has been suggested that one of the major functions of Cl$^-$/HCO$_3^-$ exchange in heart is to counter the alkalinizing effects of Na$^+$/H$^+$ exchange. This would allow increased Na$^+$/H$^+$ exchange activity[2,63], which in turn would lead to increased Na$^+$-loading and Ca^{2+}-loading *via* reverse activity of the Na$^+$/Ca^{2+} exchanger[64]. Inhibition of Na$^+$/H$^+$ exchange reduces cardiac hypertrophy[4,65] and overexpression of an activated NHE1 Na$^+$/H$^+$ exchang-

er induces hypertrophy and increases cytosolic Na$^+$, Ca^{2+} transients, and contractility[66]. Studies have shown that the reduction in hypertrophy in spontaneously hypertensive rats in response to angiotensin II blockade involves reductions in both Na$^+$/H$^+$ exchange and Cl$^-$/HCO$_3^-$ exchange[67]. Intracellular pH in NHE1-overexpressing myocytes was significantly higher when they were maintained in Hepes-buffered media than in CO$_2$/HCO$_3^-$-buffered media[66]. This is consistent with the view[68,69] that Cl$^-$/HCO$_3^-$ exchange balances the alkalinizing effects of Na$^+$/H$^+$ exchange, which would be expected to facilitate pH$_i$-neutral Na$^+$-loading *in vivo*.

The Cl$^-$/HCO$_3^-$ exchanger that has been most heavily studied in heart is AE3. Its mRNA is expressed at much higher levels in heart than those of the other HCO$_3^-$ transporters (see Table 1) and it has a cardiac specific variant[25-27], indicating that it serves a specialized function. Earlier studies showed that Cl$^-$/HCO$_3^-$ exchange and Na$^+$/H$^+$ exchange were increased in the hypertrophic heart of spontaneously hypertensive rats[67] and that AE3fl mRNA was upregulated[70]. Although this might suggest that AE3fl accounts for the increased anion exchange activity, the investigators cautioned against this interpretation as AE3fl is expressed at low levels in the adult rat and mouse heart[19,25,27]. Treatment of papillary muscles with an inhibitory anti-AE3 antibody led to an increase in the slow-force response to stretch, which is dependent on Na$^+$/H$^+$ exchange, and caused a substantial reduction in Cl$^-$/HCO$_3^-$ exchange, supporting the view that AE3 is the major Cl$^-$/HCO$_3^-$ exchanger in cardiac muscle[71]. Analysis of pH$_i$ in tissues treated with the anti-AE3 antibody indicated that AE3 is the major anion exchanger responsible for countering the alkalinizing effects of NHE1-mediated Na$^+$/H$^+$ exchange[71]. It has been suggested that activation of AE3 and NHE1 together might contribute to hypertrophy[72], but so far there is no direct proof of this hypothesis.

The initial studies of a gene-targeted AE3-null mouse

showed that the loss of AE3 did not impair cardiovascular performance *in vivo* under basal conditions or after β-adrenergic stimulation, and it also had no effect on ischemia-reperfusion injury using the Langendorff-perfused heart[6]. The latter finding was surprising as there is evidence that Cl^-/HCO_3^- exchange mediates some of the changes in pH_i and intracellular Cl^- that contribute to reperfusion injury[73]. Heart weight/body weight ratios were significantly reduced in null mutants relative to wild-type mice, consistent with the possibility that loss of AE3 activity might contribute to a reduction in hypertrophy. When AE3-null mice were crossed with an NKCC1 Na^+-K^+-$2Cl^-$ cotransporter-null mouse, which had normal contractility[74], the double mutant mice exhibited a contractility defect *in vivo* and in isolated myocytes, and Ca^{2+} extrusion mediated by the Na^+/Ca^{2+} exchanger was increased[6]. NKCC1 has been shown to provide substantial Na^+ influx in chick cardiac myocytes[75] and to affect Na^+/Ca^{2+} exchange in mouse astrocytes[76]. Therefore, it is possible that the additional loss of NKCC1 caused a reduction in Na^+-loading in AE3/NKCC1 double mutants, with subsequent effects on contractility.

The above studies were consistent with the possibility that loss of AE3 might protect against hypertrophy; however, they also showed that its absence can impair cardiac function under certain conditions. To test whether AE3-deficiency might protect against hypertrophy, AE3-null mice were crossed with a transgenic hypertrophic cardiomyopathy mouse model[77] carrying a Glu180Gly mutation in α-tropomyosin[78]. The additional loss of AE3 in the Glu180Gly mutant caused no decrease in the degree of hypertrophy and led to more rapid decompensation, heart failure, and death. Cardiac performance in response to β-adrenergic stimulation was severely impaired in double mutants. The double mutants exhibited more arrhythmic events as heart rates were increased by electrical pacing to assess force-frequency responses and Ca^{2+}-handling was also impaired. It was concluded that AE3 activity is needed for better preservation of cardiac function during heart failure and that it would not be an appropriate therapeutic target for cardiac arrhythmias or hypertrophy. In a more recent study[79] it was shown that hearts of AE3-null mice exhibit blunting of the force-frequency response when they are paced to higher heart rates *in vivo*. Phosphorylation of Akt, which plays a central role in mechanosensory signaling, was increased in paced AE3-null hearts and phosphorylation of adenosine 5'-monophosphate-activated protein kinase (AMPK) was reduced[79]. These data suggest that the increased susceptibility of AE3-null mice to decompensation in heart failure might be due impaired rate dependent inotropy, an insufficient response to biomechanical stress, and metabolic perturbations.

The functions of Slc26a6 in heart have not yet been determined. As discussed by Alper and Sharma[9], there is controversy about the electrogenicity of the Cl^-/HCO_3^- exchange activity of Slc26a6, which is the only known Cl^-/HCO_3^- exchanger of the *SLC26A* family expressed

at significant levels in heart[21]. Some studies[80,81] reported electrogenic Cl^-/HCO_3^- exchange for Slc26a6 with a stoichiometry of $1Cl^-/2HCO_3^-$ and others[82] reported electroneutral exchange with a 1:1 ratio. In the latter study[82], the investigators could detect electrogenic $Cl^-/oxalate$ transport mediated by Slc26a6 in oocytes but Cl^-/HCO_3^- and Cl^-/OH^- exchange appeared to be electroneutral. Cl^-/OH^- exchange in cardiac myocytes, which has been attributed to Slc26a6[21], is electroneutral[36], and Slc26a6-mediated $Cl^-/formate$ exchange is electroneutral[83]. Regardless of whether Slc26a6-mediated Cl^-/HCO_3^- exchange is electrogenic, during most of the excitation-relaxation cycle, when the membrane potential is negative, it would transport HCO_3^- out of the cell. However, it is possible that reversal of electrogenic Cl^-/HCO_3^- exchange might occur at positive membrane potentials. The potential functions of electrogenic Slc26a6-mediated Cl^-/HCO_3^- exchange in heart have not been studied. Furthermore, it is not clear which of the various transport functions of Slc26a6 is the most important in heart.

Na^+/HCO_3^- Cotransporters

As discussed above, there is evidence for both electroneutral and electrogenic NBC activities in cardiac myocytes[37-40,84,85]. NBCe1 and NBCn1, along with NHE1, have been immunolocalized in rat myocytes[50]. These three transporters are the major Na^+-dependent alkalinizing mechanisms in cardiac myocytes. In the isolated perfused ferret heart, NBC and NHE activities contributed equally to recovery of pH_i, both after an acid load[86] and also during reperfusion following ischemia[87]. In the latter study, it was suggested that NBC-mediated Na^+ influx might contribute to Ca^{2+} overload and injury after reperfusion. Later studies using an inhibitory antibody showed that inhibition of NBCe1 protected against ischemia-reperfusion injury in the isolated rat heart[88]. Similarly, in rat ventricular myocytes subjected to anoxic conditions, simultaneous inhibition of NBC and NHE1 activities prevented hypercontracture induced by Ca^{2+}-overload during reoxygenation, whereas inhibition of either activity alone was insufficient[89]. This suggests that NBCe1, like the NHE1 Na^+/H^+ exchanger, can be a significant source of Na^+-loading, although the magnitude of Na^+-loading *via* NBCe1 has been estimated to be lower than that of Na^+/H^+ exchange[64]. Also, NHE1 has cardioprotective effects that appear to be independent of effects on Na^+ and Ca^{2+} loading[51,90].

NBCe1 is localized to t-tubules[50], along with the L-type Ca channel (LTCC) and NCX1 Na^+/Ca^{2+} exchanger[91], whereas NHE1 is expressed at highest levels in intercalated discs[50,92]. Thus, NBCe1 appears to be well situated to affect excitation-contraction coupling[93], particularly since it is electrogenic. In fact, a substantial NBC-mediated HCO_3^- current has been demonstrated beginning at -50 millivolts[84], and electrogenic NBC activity causes a shortening of the action potential duration (APD) and affects the resting membrane potential[40,94]. By shortening the APD, NBCe1 could reduce the open time of the LTCC and

with its location in the t-tubule it could reduce intraluminal (extracellular) pH and increase pH_i, both of which reduce LTCC-mediated Ca^{2+} currents[95]. Thus, while NBCe1 activity may serve as a Na^+-loading mechanism that could, in principle, contribute to Ca^{2+}-loading *via* reverse mode activity of the Na^+/Ca^{2+} exchanger, its effects on the APD and on LTCC activity might counteract this tendency. NBCn1 is also in t-tubules of ventricular myocytes[50] and because it is electroneutral it could operate throughout the excitation-contraction cycle.

Both NBCe1 and NBCn1, along with NHE1, were induced in rat heart during pressure overload hypertrophy[55]. After an acid load, the rate of pH_i recovery *via* NBC and NHE activities were increased accordingly, and NBC activity in the physiological pH_i range was similar to that of NHE1[55]. Additional experiments[55] showed that when rats were subjected to pressure overload and then treated with losartan, an angiotensin Ⅱ AT_1 receptor antagonist, both hypertrophy and the induction of NBCe1 and NBCn1 were sharply reduced. The results suggest, but do not prove, that increased NBC activities contribute to the development of hypertrophy. NBCe1 mRNA and protein were also induced in human failing hearts[88] and in rat hearts following myocardial infarction[96]; however, in the latter study, treatment with an angiotensin Ⅱ AT_1 receptor antagonist had no effect on NBCe1 expression. The effects of angiotensin Ⅱ on NBC activity in cardiac myocytes are complex as some studies report activation of NBC activity[97,98] and others report inhibition[99]. A more recent study showed that cardiac expression of both NBCn1 and NBCe1 were induced in spontaneously hypertensive rats in which angiotensin II plays a major role[100]. However, NBCe1 activity was reduced due to a reduction in its protein expression in t-tubule and sarcolemmal membranes; nevertheless, total NBC activity increased due to an increase in NBCn1 activity. The authors noted that a reduction in NBCe1 activity leads to an increase in APD, which is a common occurrence during cardiac hypertrophy[101], and that this would likely cause an increase in Ca^{2+}-influx *via* LTCC[102]. Thus, NBCe1 activity, rather than inhibition of its activity, may be cardioprotective in some disease conditions.

EFFECTS OF HCO_3^- ON CONTRACTILITY AND Ca^{2+} IN ISOLATED HEARTS

Given the abundance and diversity of HCO_3^- transporters in heart and the fact that HCO_3^- is part of the major buffer system in biological systems, it is surprising that there has been little reported work on the specific effects of HCO_3^- on contractility and Ca^{2+}-handling. In an interesting and important study, Fülöp *et al*[10] analyzed Langendorff-perfused guinea pig hearts in the presence of both Krebs solution buffered with CO_2/HCO_3^- and Tyrode solution buffered with HEPES[10]. Contractility in isolated hearts was significantly greater in Krebs solution than in Tyrode solution. However, when Tyrode solution was supplemented with CO_2/HCO_3^-, in the continuing

presence of HEPES, contractility increased to the levels observed in Krebs solution. Changes in contractility were reversible as the buffers were switched between those containing CO_2/HCO_3^- or HEPES alone. Despite increased contractility, both the amplitude and duration of the Ca^{2+} transients were lower in solutions containing CO_2/HCO_3^- buffer, indicating that enhanced Ca^{2+} transients were not responsible for the enhanced contractility. Analyses of isolated trabeculae also revealed increased contractility in CO_2/HCO_3^- buffer, with faster times to peak tension and shorter relaxation times[10]. In purkinje fibers and papillary muscles, the duration of the action potential was reduced in the presence of CO_2/HCO_3^- buffer[10]. This finding is consistent with the proposed effects of NBCe1 activity on the action potential[103].

The reduced contractility in the isolated heart and isolated tissues in response to the absence of HCO_3^- may have been due to reduced pH_i or buffering power[10]. However, in wild-type myocytes used in a study of the effects of Na^+/H^+ exchange on Ca^{2+} and contractility, pH_i was the same in HEPES buffer as in buffer containing CO_2/HCO_3^-[66]. Also, in myocytes overexpressing an activated NHE1, the absence of CO_2/HCO_3^- led to an increase in pH_i[66]. These results suggest that a HCO_3^--dependent transport mechanisms, *i.e.,* Cl^-/HCO_3^- exchange, is needed to counter the alkalinizing effects of NHE1.

EFFECTS OF HCO_3^- ON CONTRACTILITY AND Ca^{2+} IN ISOLATED MYOCYTES

Although studies of cardiac myocyte mechanics and Ca^{2+} handling are commonly performed in HEPES buffered solution (Tyrode's solution), we are unaware of studies directly comparing the effects of the two buffer conditions. Therefore, we performed experiments to assess the effects of HEPES-buffered and HCO_3^--buffered solutions on both contraction of rat ventricular myocytes and Ca^{2+} transients. The concentrations of cations were identical for the two solutions, and their osmolarities were the same.

Switching from HEPES-buffered solution to HCO_3^--buffered solution had a bi-phasic effect on myocyte contraction, determined by measurements of cell shortening as described previously[104]. It first resulted in transient suppression of myocyte contraction, followed by reversal of suppression, and enhancement of contraction (Figure 2A). On average, the downward suppressive phase lasted about 50 s (Figure 2B), resulting in a peak suppression of cell shortening from 7.04% in control (*i.e.*, HEPES) to 4.35% (Figure 2C). This was followed by gradual stimulation of contraction; at steady-state, contraction was significantly increased to 11.39% (Figure 2C). Switching from HEPES-buffered Tyrode's solution to an isosmotic solution that contained both HEPES and HCO_3^- produced the same bi-phasic effect and reached the same steady-state increase in contractility. Thus, the stimulation of contraction by HCO_3^- in isolated cardiac myocytes was fully reversible, as observed previously in the isolated

A

B

C
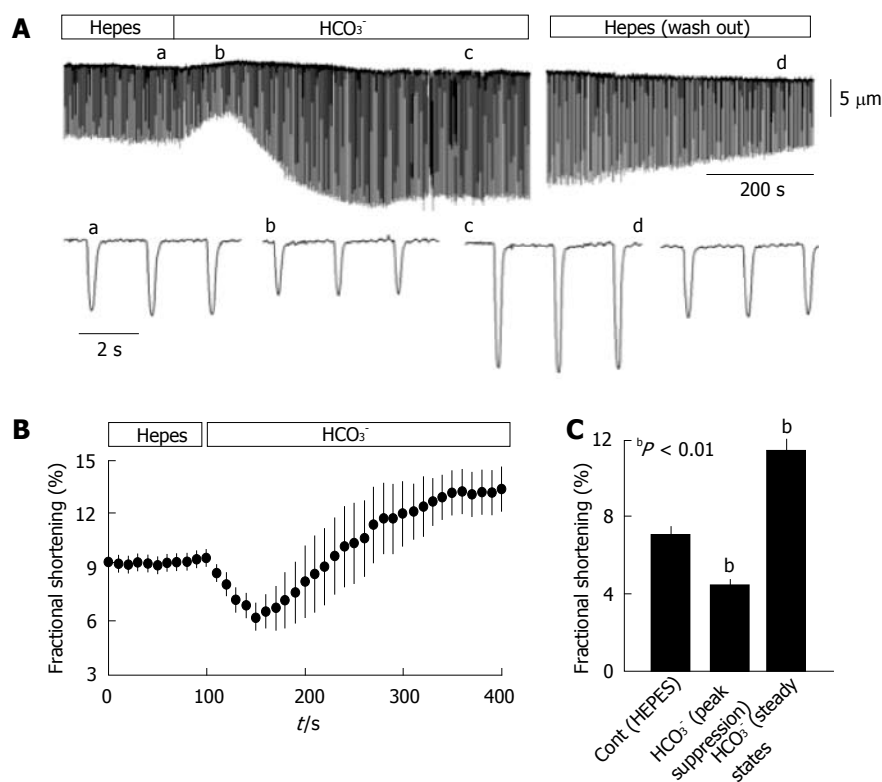
Figure 2 Isolated myocytes exhibit greater contractility in CO_2/HCO_3^- buffer than in HEPES buffer. Ventricular myocytes from rat hearts were enzymatically dissociated using Langendorff perfusion[104] and myocyte mechanics were analyzed at room temperature (24 ℃), with stimulation at 0.5 Hz as described previously[107]. Myocytes were switched between HEPES-buffered Tyrode's solution (in mmol/L: NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 10, and Na-HEPES 5; pH = 7.4; bubbled with 100% O_2) and HCO_3^--buffered Krebs solution (in mmol/L: NaCl 120, $NaHCO_3^-$ 25, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.8, and glucose 10; pH = 7.4 when bubbled with 95% O_2 and 5% CO_2). A: Representative contraction tracing of a myocyte bathed in HEPES buffer, then switched to HCO_3^- buffer, and then returned to HEPES buffer; the lower tracings show an expanded scale for the indicated (a-d) time points. The lower panels show the time course of fractional shortening (B) and average fractional shortening (C) of myocytes (*n* = 19) in HEPES buffer and then switched to HCO_3^--containing buffer. Experiments were performed using myocytes from 3 hearts and statistical analysis was conducted using a paired t-test. Values are means ± SE.

A

B
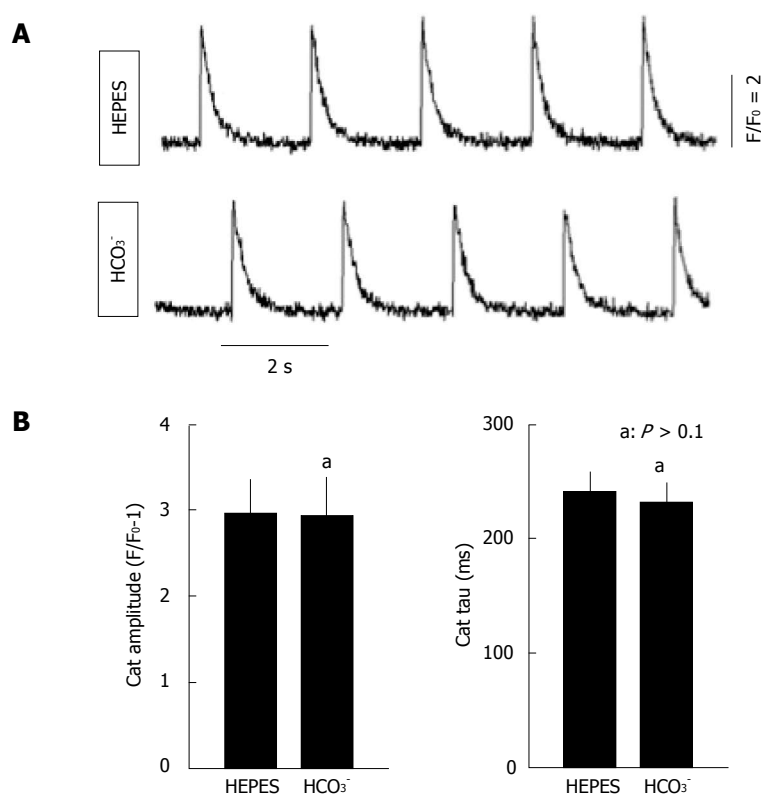
Figure 3 Ca^{2+} transient analysis in isolated rat myocytes bathed in CO_2/HCO_3^- buffer and in HEPES buffer. For recording of Ca^{2+} transients, isolated ventricular myocytes were loaded with fluo-4 acetoxymethyl ester (5 μmol/L, Molecular Probes, Eugene, OR) and activated with field stimulation at 0.5 Hz. Fluorescence signals were measured using a Nikon TE 2000 microscope and an InCyt Standard PM photometry system (Intracellular Imaging, Cincinnati, OH) as described previously[104]. A: Representative Ca^{2+} transients in HEPES and HCO_3^--containing buffers; B: Average Ca^{2+} transient (CaT) amplitudes and tau values, a measure of the rate of decay of the Ca^{2+} transient in HEPES and HCO_3^--containing buffers (*n* = 12). The same cells were imaged in both buffers. Myocytes were from the same preparations used in Figure 2, with statistical analyses performed using a paired *t*-test. No significant differences were observed.

heart[10].

In addition to affecting myocyte contractility, HCO_3^- also had a small but significant effect on myocyte length. Switching from HEPES to the HCO_3^--buffered solution transiently increased myocyte length by approximately 1% (data not shown). Such transient lengthening was independent of myocyte contraction, and was also observed in unpaced, quiescent myocytes. This suggests the possibility that cell volume was increased by the addition of HCO_3^-, which is reasonable given the role of Cl^-/HCO_3^- exchange in cell volume regulation[105,106].

Interestingly, the marked effect of HCO_3^- on myo-

cyte contraction was not accompanied by any detectable change in the Ca^{2+} transient. Figure 3A shows Ca^{2+} transients from the same myocyte in the presence of HEPES or HCO_3^- solutions. Switching from HEPES-buffered solutions to HCO_3^--buffered solutions altered neither the amplitude nor the time constant of the Ca^{2+} transient (Figure 3B). These data using isolated myocytes correlate well with the previous studies using isolated hearts[10]. Both sets of data show that HCO_3^- has a major effect on contractility, without any major effects on the amplitude of the Ca^{2+} transient.

CONCLUSION

The studies reviewed here show that the mammalian heart contains an abundance of HCO_3^- transporters, which include both Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters. Their most obvious function is regulation of pH_i, although it is possible that this is not their major function. This is particularly apparent in the case of the Cl^-/HCO_3^- exchangers because at the high frequencies occurring *in vivo*, cardiac myocytes generate a substantial acid load. Thus, there would appear to be little need to maintain a robust capacity for recovery from an alkaline load. Nevertheless, it is possible that the cardiac anion exchangers regulate the pH or electrolyte concentrations of sub-sarcolemmal or t-tubule microdomains. With regard to electrolyte homeostasis, coupling of Cl^-/HCO_3^- exchange and Na^+/HCO_3^- cotransport (or Na^+/H^+ exchange) can serve as a pH_i-neutral Na^+- and Cl^--loading mechanism, with Na^+ affecting Ca^{2+}-loading *via* Na^+/Ca^{2+} exchange. In addition to effects on Na^+-loading, electrogenic Na^+/HCO_3^- cotransport can affect the duration of the action potential[40,84,94] and, by affecting subsarcolemmal and t-tubular pH, it might also affect the activity of L-type Ca^{2+} channels[95]. Finally, the available data show that the presence of CO_2/HCO_3^- buffer has a major effect on contractility that cannot be readily explained by effects on Ca^{2+}-handling, thus suggesting that HCO_3^- homeostasis plays an important role in the regulation of cardiac contractility. The mechanism is not known, but it is conceivable that intracellular HCO_3^- concentrations affect myofibrillar function and that dynamic transporter-mediated HCO_3^- fluxes have a major effect on electrical and ionic properties of the myocyte. Further studies of the effects of HCO_3^- and the cardiac functions of each of the major HCO_3^- transporters will be needed to resolve these issues.

REFERENCES

1   **Vaughan-Jones RD**, Spitzer KW. Role of bicarbonate in the regulation of intracellular pH in the mammalian ventricular myocyte. *Biochem Cell Biol* 2002; **80**: 579-596 [PMID: 12440699]

2   **Casey JR**. Why bicarbonate? *Biochem Cell Biol* 2006; **84**: 930-939 [PMID: 17215880 DOI: 10.1139/o06-184]

3   **Spitzer KW**, Skolnick RL, Peercy BE, Keener JP, Vaughan-Jones RD. Facilitation of intracellular H(+) ion mobility by CO(2)/HCO(3)(-) in rabbit ventricular myocytes is regulat-ed by carbonic anhydrase. *J Physiol* 2002; **541**: 159-167 [PMID: 12015427 DOI: 10.1113/jphysiol.2001.013268]

4   **Karmazyn M**, Kilić A, Javadov S. The role of NHE-1 in myocardial hypertrophy and remodelling. *J Mol Cell Cardiol* 2008; **44**: 647-653 [PMID: 18329039 DOI: 10.1016/j.yjmcc.2008.01.005]

5   **Vaughan-Jones RD**, Spitzer KW, Swietach P. Intracellular pH regulation in heart. *J Mol Cell Cardiol* 2009; **46**: 318-331 [PMID: 19041875 DOI: 10.1016/j.yjmcc.2008.10.024]

6   **Prasad V**, Bodi I, Meyer JW, Wang Y, Ashraf M, Engle SJ, Doetschman T, Sisco K, Nieman ML, Miller ML, Lorenz JN, Shull GE. Impaired cardiac contractility in mice lacking both the AE3 Cl-/HCO3- exchanger and the NKCC1 Na+-K+-2Cl- cotransporter: effects on Ca2+ handling and protein phosphatases. *J Biol Chem* 2008; **283**: 31303-31314 [PMID: 18779325 DOI: 10.1074/jbc.M803706200]

7   **Alper SL**. Molecular physiology and genetics of Na+-independent SLC4 anion exchangers. *J Exp Biol* 2009; **212**: 1672-1683 [PMID: 19448077 DOI: 10.1242/jeb.029454]

8   **Romero MF**, Chen AP, Parker MD, Boron WF. The SLC4 family of bicarbonate (HCO_3^-) transporters. *Mol Aspects Med* 2013; **34**: 159-182 [PMID: 23506864 DOI: 10.1007/s00424-003-1180-2]

9   **Alper SL**, Sharma AK. The SLC26 gene family of anion transporters and channels. *Mol Aspects Med* 2013; **34**: 494-515 [PMID: 23506885 DOI: 10.1016/j.mam.2012.07.009]

10 **Fülöp L**, Szigeti G, Magyar J, Szentandrássy N, Ivanics T, Miklós Z, Ligeti L, Kovács A, Szénási G, Csernoch L, Nánási PP, Bányász T. Differences in electrophysiological and contractile properties of mammalian cardiac tissues bathed in bicarbonate - and HEPES-buffered solutions. *Acta Physiol Scand* 2003; **178**: 11-18 [PMID: 12713510 DOI: 10.1046/j.1365-201X.2003.01114.x]

11 **Soleimani M**. SLC26 Cl-/HCO3- exchangers in the kidney: roles in health and disease. *Kidney Int* 2013; **84**: 657-666 [PMID: 23636174 DOI: 10.1038/ki.2013.138]

12 **Bonar PT**, Casey JR. Plasma membrane Cl^-/HCO_3^- exchangers: structure, mechanism and physiology. *Channels* (Austin) 2008; **2**: 337-345 [PMID: 19066446 DOI: 10.4161/chan.2.5.6899]

13 **Kopito RR**, Lodish HF. Primary structure and transmembrane orientation of the murine anion exchange protein. *Nature* 1985; **316**: 234-238 [PMID: 2410791 DOI: 10.1038/316234a0]

14 **Kudrycki KE**, Shull GE. Primary structure of the rat kidney band 3 anion exchange protein deduced from a cDNA. *J Biol Chem* 1989; **264**: 8185-8192 [PMID: 2722777]

15 **Kudrycki KE**, Shull GE. Rat kidney band 3 Cl-/HCO3- exchanger mRNA is transcribed from an alternative promoter. *Am J Physiol* 1993; **264**: F540-F547 [PMID: 8456965]

16 **Peters LL**, Shivdasani RA, Liu SC, Hanspal M, John KM, Gonzalez JM, Brugnara C, Gwynn B, Mohandas N, Alper SL, Orkin SH, Lux SE. Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. *Cell* 1996; **86**: 917-927 [PMID: 8808627 DOI: 10.1016/S0092-8674(00)80167-1]

17 **Bruce LJ**, Beckmann R, Ribeiro ML, Peters LL, Chasis JA, Delaunay J, Mohandas N, Anstee DJ, Tanner MJ. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood* 2003; **101**: 4180-4188 [PMID: 12531814 DOI: 10.1182/blood-2002-09-2824]

18 **Kudrycki KE**, Newman PR, Shull GE. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl-/HCO3- exchanger. *J Biol Chem* 1990; **265**: 462-471 [PMID: 2294114]

19 **Richards SM**, Jaconi ME, Vassort G, Pucéat M. A spliced variant of AE1 gene encodes a truncated form of Band 3 in heart: the predominant anion exchanger in ventricular myocytes. *J Cell Sci* 1999; **112** (Pt 10): 1519-1528 [PMID: 10212146]

20 **Moura Lima PR**, Salles TS, Costa FF, Saad ST. alpha-cardiac actin (ACTC) binds to the band 3 (AE1) cardiac isoform.

J Cell Biochem 2003; **89**: 1215-1221 [PMID: 12898519 DOI: 10.1002/jcb.10561]

21　**Alvarez BV**, Kieller DM, Quon AL, Markovich D, Casey JR. Slc26a6: a cardiac chloride-hydroxyl exchanger and predominant chloride-bicarbonate exchanger of the mouse heart. *J Physiol* 2004; **561**: 721-734 [PMID: 15498800 DOI: 10.1113/jphysiol.2004.077339]

22　**Wang Z**, Schultheis PJ, Shull GE. Three N-terminal variants of the AE2 Cl-/HCO3- exchanger are encoded by mRNAs transcribed from alternative promoters. *J Biol Chem* 1996; **271**: 7835-7843 [PMID: 8631828]

23　**Medina JF**, Lecanda J, Acín A, Ciesielczyk P, Prieto J. Tissue-specific N-terminal isoforms from overlapping alternate promoters of the human AE2 anion exchanger gene. *Biochem Biophys Res Commun* 2000; **267**: 228-235 [PMID: 10623603 DOI: 10.1006/bbrc.1999.1951]

24　**Kopito RR**, Lee BS, Simmons DM, Lindsey AE, Morgans CW, Schneider K. Regulation of intracellular pH by a neuronal homolog of the erythrocyte anion exchanger. *Cell* 1989; **59**: 927-937 [PMID: 2686841 DOI: 10.1016/0092-8674(89)90615-6]

25　**Linn SC**, Kudrycki KE, Shull GE. The predicted translation product of a cardiac AE3 mRNA contains an N terminus distinct from that of the brain AE3 Cl-/HCO3- exchanger. Cloning of a cardiac AE3 cDNA, organization of the AE3 gene, and identification of an alternative transcription initiation site. *J Biol Chem* 1992; **267**: 7927-7935 [PMID: 1560021]

26　**Yannoukakos D**, Stuart-Tilley A, Fernandez HA, Fey P, Duyk G, Alper SL. Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart. *Circ Res* 1994; **75**: 603-614 [PMID: 7923606 DOI: 10.1161/01.RES.75.4.603]

27　**Linn SC**, Askew GR, Menon AG, Shull GE. Conservation of an AE3 Cl-/HCO3- exchanger cardiac-specific exon and promoter region and AE3 mRNA expression patterns in murine and human hearts. *Circ Res* 1995; **76**: 584-591 [PMID: 7895333 DOI: 10.1161/01.RES.76.4.584]

28　**Alvarez BV**, Kieller DM, Quon AL, Robertson M, Casey JR. Cardiac hypertrophy in anion exchanger 1-null mutant mice with severe hemolytic anemia. *Am J Physiol Heart Circ Physiol* 2007; **292**: H1301-H1312 [PMID: 17056673 DOI: 10.1152/ajpheart.00449.2006]

29　**Melvin JE**, Park K, Richardson L, Schultheis PJ, Shull GE. Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is up-regulated in colon of mice lacking the NHE3 Na(+)/H(+) exchanger. *J Biol Chem* 1999; **274**: 22855-22861 [PMID: 10428871 DOI: 10.1074/jbc.274.32.22855]

30　**Wang Z**, Petrovic S, Mann E, Soleimani M. Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G573-G579 [PMID: 11842009 DOI: 10.1152/ajpgi.00338.2001]

31　**Petrovic S**, Ma L, Wang Z, Soleimani M. Identification of an apical Cl-/HCO-3 exchanger in rat kidney proximal tubule. *Am J Physiol Cell Physiol* 2003; **285**: C608-C617 [PMID: 12736136 DOI: 10.1152/ajpcell.00084.2003]

32　**Jiang Z**, Grichtchenko II, Boron WF, Aronson PS. Specificity of anion exchange mediated by mouse Slc26a6. *J Biol Chem* 2002; **277**: 33963-33967 [PMID: 12119287 DOI: 10.1074/jbc.M202660200]

33　**Simpson JE**, Schweinfest CW, Shull GE, Gawenis LR, Walker NM, Boyle KT, Soleimani M, Clarke LL. PAT-1 (Slc26a6) is the predominant apical membrane Cl-/HCO3- exchanger in the upper villous epithelium of the murine duodenum. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G1079-G1088 [PMID: 17170027 DOI: 10.1152/ajpgi.00354.2006]

34　**Knauf F**, Yang CL, Thomson RB, Mentone SA, Giebisch G, Aronson PS. Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proxi-mal tubule cells. *Proc Natl Acad Sci USA* 2001; **98**: 9425-9430 [PMID: 11459928 DOI: 10.1073/pnas.141241098]

35　**Aronson PS**. Ion exchangers mediating Na+, HCO3 - and Cl- transport in the renal proximal tubule. *J Nephrol* 2006; **19** Suppl 9: S3-S10 [PMID: 16736438]

36　**Niederer SA**, Swietach P, Wilson DA, Smith NP, Vaughan-Jones RD. Measuring and modeling chloride-hydroxyl exchange in the Guinea-pig ventricular myocyte. *Biophys J* 2008; **94**: 2385-2403 [PMID: 18055536 DOI: 10.1529/biophysj.107.118885]

37　**Dart C**, Vaughan-Jones RD. Na(+)-HCO3- symport in the sheep cardiac Purkinje fibre. *J Physiol* 1992; **451**: 365-385 [PMID: 1403816]

38　**Lagadic-Gossmann D**, Buckler KJ, Vaughan-Jones RD. Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J Physiol* 1992; **458**: 361-384 [PMID: 1302269]

39　**Camilión de Hurtado MC**, Alvarez BV, Pérez NG, Cingolani HE. Role of an electrogenic Na(+)-HCO3- cotransport in determining myocardial pHi after an increase in heart rate. *Circ Res* 1996; **79**: 698-704 [PMID: 8831493 DOI: 10.1161/01.RES.79.4.698]

40　**Aiello EA**, Petroff MG, Mattiazzi AR, Cingolani HE. Evidence for an electrogenic Na+-HCO3- symport in rat cardiac myocytes. *J Physiol* 1998; **512** (Pt 1): 137-148 [PMID: 9729624 DOI: 10.1111/j.1469-7793.1998.137bf.x]

41　**Romero MF**, Hediger MA, Boulpaep EL, Boron WF. Expression cloning and characterization of a renal electrogenic Na+/HCO3- cotransporter. *Nature* 1997; **387**: 409-413 [PMID: 9163427 DOI: 10.1038/387409a0]

42　**Burnham CE**, Amlal H, Wang Z, Shull GE, Soleimani M. Cloning and functional expression of a human kidney Na+: HCO3- cotransporter. *J Biol Chem* 1997; **272**: 19111-19114 [PMID: 9235899 DOI: 10.1074/jbc.272.31.19111]

43　**Gross E**, Kurtz I. Structural determinants and significance of regulation of electrogenic Na(+)-HCO(3)(-) cotransporter stoichiometry. *Am J Physiol Renal Physiol* 2002; **283**: F876-F887 [PMID: 12372762]

44　**Choi I**, Romero MF, Khandoudi N, Bril A, Boron WF. Cloning and characterization of a human electrogenic Na+-HCO-3 cotransporter isoform (hhNBC). *Am J Physiol* 1999; **276**: C576-C584 [PMID: 10069984]

45　**Choi I**, Aalkjaer C, Boulpaep EL, Boron WF. An electroneutral sodium/bicarbonate cotransporter NBCn1 and associated sodium channel. *Nature* 2000; **405**: 571-575 [PMID: 10850716 DOI: 10.1038/35014615]

46　**Pushkin A**, Abuladze N, Lee I, Newman D, Hwang J, Kurtz I. Cloning, tissue distribution, genomic organization, and functional characterization of NBC3, a new member of the sodium bicarbonate cotransporter family. *J Biol Chem* 1999; **274**: 16569-16575 [PMID: 10347222 DOI: 10.1074/jbc.274.23.16569]

47　**Damkier HH**, Nielsen S, Praetorius J. An anti-NH2-terminal antibody localizes NBCn1 to heart endothelia and skeletal and vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2006; **290**: H172-H180 [PMID: 16126812 DOI: 10.1152/ajpheart.00713.2005]

48　**Cooper DS**, Lee HJ, Yang HS, Kippen J, Yun CC, Choi I. The electroneutral sodium/bicarbonate cotransporter containing an amino terminal 123-amino-acid cassette is expressed predominantly in the heart. *J Biomed Sci* 2006; **13**: 593-595 [PMID: 16547769 DOI: 10.1007/s11373-006-9078-6]

49　**Liu Y**, Qin X, Wang DK, Guo YM, Gill HS, Morris N, Parker MD, Chen LM, Boron WF. Effects of optional structural elements, including two alternative amino termini and a new splicing cassette IV, on the function of the sodium-bicarbonate cotransporter NBCn1 (SLC4A7). *J Physiol* 2013; **591**: 4983-5004 [PMID: 23959679 DOI: 10.1113/jphysi-

ol.2013.258673]

50 **Garciarena CD**, Ma YL, Swietach P, Huc L, Vaughan-Jones RD. Sarcolemmal localisation of Na+/H+ exchange and Na+-HCO3- co-transport influences the spatial regulation of intracellular pH in rat ventricular myocytes. *J Physiol* 2013; **591**: 2287-2306 [PMID: 23420656 DOI: 10.1113/jphysiol.2012.249664]

51 **Prasad V**, Lorenz JN, Miller ML, Vairamani K, Nieman ML, Wang Y, Shull GE. Loss of NHE1 activity leads to reduced oxidative stress in heart and mitigates high-fat diet-induced myocardial stress. *J Mol Cell Cardiol* 2013; **65**: 33-42 [PMID: 24080184 DOI: 10.1016/j.yjmcc.2013.09.013]

52 **Martin JA**, Wang Z. Next-generation transcriptome assembly. *Nat Rev Genet* 2011; **12**: 671-682 [PMID: 21897427 DOI: 10.1038/nrg3068]

53 **Malone JH**, Oliver B. Microarrays, deep sequencing and the true measure of the transcriptome. *BMC Biol* 2011; **9**: 34 [PMID: 21627854 DOI: 10.1186/1741-7007-9-34]

54 **Pushkin A**, Abuladze N, Newman D, Lee I, Xu G, Kurtz I. Cloning, characterization and chromosomal assignment of NBC4, a new member of the sodium bicarbonate cotransporter family. *Biochim Biophys Acta* 2000; **1493**: 215-218 [PMID: 10978526 DOI: 10.1016/S0167-4781(00)00149-4]

55 **Yamamoto T**, Shirayama T, Sakatani T, Takahashi T, Tanaka H, Takamatsu T, Spitzer KW, Matsubara H. Enhanced activity of ventricular Na+-HCO3- cotransport in pressure overload hypertrophy. *Am J Physiol Heart Circ Physiol* 2007; **293**: H1254-H1264 [PMID: 17416604 DOI: 10.1152/ajpheart.00964.2006]

56 **Grichtchenko II**, Choi I, Zhong X, Bray-Ward P, Russell JM, Boron WF. Cloning, characterization, and chromosomal mapping of a human electroneutral Na(+)-driven Cl-HCO3 exchanger. *J Biol Chem* 2001; **276**: 8358-8363 [PMID: 11133997 DOI: 10.1074/jbc.C000716200]

57 **Liu S**, Piwnica-Worms D, Lieberman M. Intracellular pH regulation in cultured embryonic chick heart cells. Na(+)-dependent Cl-/HCO3- exchange. *J Gen Physiol* 1990; **96**: 1247-1269 [PMID: 1962815]

58 **Sun B**, Vaughan-Jones RD, Kambayashi J. Two distinct HCO(-)(3)-dependent H(+) efflux pathways in human vascular endothelial cells. *Am J Physiol* 1999; **277**: H28-H32 [PMID: 10409178]

59 **Chiappe de Cingolani GE**, Ennis IL, Morgan PE, Alvarez BV, Casey JR, Camilión de Hurtado MC. Involvement of AE3 isoform of Na(+)-independent Cl(-)/HCO(3)(-) exchanger in myocardial pH(i) recovery from intracellular alkalization. *Life Sci* 2006; **78**: 3018-3026 [PMID: 16458934 DOI: 10.1016/j.lfs.2005.11.030]

60 **Hume JR**, Duan D, Collier ML, Yamazaki J, Horowitz B. Anion transport in heart. *Physiol Rev* 2000; **80**: 31-81 [PMID: 10617765]

61 **Vaughan-Jones RD**. Chloride activity and its control in skeletal and cardiac muscle. *Philos Trans R Soc Lond B Biol Sci* 1982; **299**: 537-548 [PMID: 6130545 DOI: 10.1098/rstb.1982.0150]

62 **Gawenis LR**, Bradford EM, Alper SL, Prasad V, Shull GE. AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G493-G503 [PMID: 20110461 DOI: 10.1152/ajpgi.00178.2009]

63 **Pérez NG**, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. pHi regulation in myocardium of the spontaneously hypertensive rat. Compensated enhanced activity of the Na(+)-H+ exchanger. *Circ Res* 1995; **77**: 1192-1200 [PMID: 7586232 DOI: 10.1161/01.RES.77.6.1192]

64 **Garciarena CD**, Youm JB, Swietach P, Vaughan-Jones RD. H⁺-activated Na⁺ influx in the ventricular myocyte couples Ca²⁺-signalling to intracellular pH. *J Mol Cell Cardiol* 2013;

61: 51-59 [PMID: 23602948 DOI: 10.1016/j.yjmcc.2013.04.008]

65 **Yoshida H**, Karmazyn M. Na(+)/H(+) exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. *Am J Physiol Heart Circ Physiol* 2000; **278**: H300-H304 [PMID: 10644613]

66 **Nakamura TY**, Iwata Y, Arai Y, Komamura K, Wakabayashi S. Activation of Na+/H+ exchanger 1 is sufficient to generate Ca2+ signals that induce cardiac hypertrophy and heart failure. *Circ Res* 2008; **103**: 891-899 [PMID: 18776042 DOI: 10.1161/CIRCRESAHA.108.175141]

67 **Ennis IL**, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. Enalapril induces regression of cardiac hypertrophy and normalization of pHi regulatory mechanisms. *Hypertension* 1998; **31**: 961-967 [PMID: 9535421 DOI: 10.1161/01.HYP.31.4.961]

68 **Camilión de Hurtado MC**, Alvarez BV, Pérez NG, Ennis IL, Cingolani HE. Angiotensin II activates Na+-independent Cl-HCO3- exchange in ventricular myocardium. *Circ Res* 1998; **82**: 473-481 [PMID: 9506708 DOI: 10.1161/01.RES.82.4.473]

69 **Alvarez BV**, Fujinaga J, Casey JR. Molecular basis for angiotensin II-induced increase of chloride/bicarbonate exchange in the myocardium. *Circ Res* 2001; **89**: 1246-1253 [PMID: 11739292 DOI: 10.1161/hh2401.101907]

70 **Chiappe de Cingolani G**, Morgan P, Mundiña-Weilenmann C, Casey J, Fujinaga J, Camilión de Hurtado M, Cingolani H. Hyperactivity and altered mRNA isoform expression of the Cl(-)/HCO(3)(-) anion-exchanger in the hypertrophied myocardium. *Cardiovasc Res* 2001; **51**: 71-79 [PMID: 11399249 DOI: 10.1016/S0008-6363(01)00276-0]

71 **Cingolani HE**, Chiappe GE, Ennis IL, Morgan PG, Alvarez BV, Casey JR, Dulce RA, Pérez NG, Camilión de Hurtado MC. Influence of Na+-independent Cl--HCO3- exchange on the slow force response to myocardial stretch. *Circ Res* 2003; **93**: 1082-1088 [PMID: 14576196 DOI: 10.1161/01.RES.0000102408.25664.01]

72 **Alvarez BV**, Johnson DE, Sowah D, Soliman D, Light PE, Xia Y, Karmazyn M, Casey JR. Carbonic anhydrase inhibition prevents and reverts cardiomyocyte hypertrophy. *J Physiol* 2007; **579**: 127-145 [PMID: 17124262 DOI: 10.1113/jphysiol.2006.123638]

73 **Lai ZF**, Nishi K. Intracellular chloride activity increases in guinea pig ventricular muscle during simulated ischemia. *Am J Physiol* 1998; **275**: H1613-H1619 [PMID: 9815068]

74 **Meyer JW**, Flagella M, Sutliff RL, Lorenz JN, Nieman ML, Weber CS, Paul RJ, Shull GE. Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na(+)-K(+)-2Cl(-) cotransporter. *Am J Physiol Heart Circ Physiol* 2002; **283**: H1846-H1855 [PMID: 12384462 DOI: 10.1152/ajpheart.00083.2002]

75 **Frelin C**, Chassande O, Lazdunski M. Biochemical characterization of the Na+/K+/Cl- co-transport in chick cardiac cells. *Biochem Biophys Res Commun* 1986; **134**: 326-331 [PMID: 3947328 DOI: 10.1016/0006-291X(86)90566-8]

76 **Lenart B**, Kintner DB, Shull GE, Sun D. Na-K-Cl cotransporter-mediated intracellular Na+ accumulation affects Ca2+ signaling in astrocytes in an in vitro ischemic model. *J Neurosci* 2004; **24**: 9585-9597 [PMID: 15509746 DOI: 10.1523/JNEUROSCI.2569-04.2004]

77 **Prabhakar R**, Boivin GP, Grupp IL, Hoit B, Arteaga G, Solaro RJ, Wieczorek DF. A familial hypertrophic cardiomyopathy alpha-tropomyosin mutation causes severe cardiac hypertrophy and death in mice. *J Mol Cell Cardiol* 2001; **33**: 1815-1828 [PMID: 11603924 DOI: 10.1006/jmcc.2001.1445]

78 **Al Moamen NJ**, Prasad V, Bodi I, Miller ML, Neiman ML, Lasko VM, Alper SL, Wieczorek DF, Lorenz JN, Shull GE. Loss of the AE3 anion exchanger in a hypertrophic cardiomyopathy model causes rapid decompensation and heart failure. *J Mol Cell Cardiol* 2011; **50**: 137-146 [PMID: 21056571

DOI: 10.1016/j.yjmcc.2010.10.028]

79   **Prasad V**, Lorenz JN, Lasko VM, Nieman ML, Al Moamen NJ, Shull GE. Loss of the AE3 Cl(-)/HCO(-) 3 exchanger in mice affects rate-dependent inotropy and stress-related AKT signaling in heart. *Front Physiol* 2013; **4**: 399 [PMID: 24427143 DOI: 10.3389/fphys.2013.00399]

80   **Ko SB**, Shcheynikov N, Choi JY, Luo X, Ishibashi K, Thomas PJ, Kim JY, Kim KH, Lee MG, Naruse S, Muallem S. A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis. *EMBO J* 2002; **21**: 5662-5672 [PMID: 12411484 DOI: 10.1093/emboj/cdf580]

81   **Shcheynikov N**, Wang Y, Park M, Ko SB, Dorwart M, Naruse S, Thomas PJ, Muallem S. Coupling modes and stoichiometry of Cl-/HCO3- exchange by slc26a3 and slc26a6. *J Gen Physiol* 2006; **127**: 511-524 [PMID: 16606687 DOI: 10.1085/jgp.200509392]

82   **Chernova MN**, Jiang L, Friedman DJ, Darman RB, Lohi H, Kere J, Vandorpe DH, Alper SL. Functional comparison of mouse slc26a6 anion exchanger with human SLC26A6 polypeptide variants: differences in anion selectivity, regulation, and electrogenicity. *J Biol Chem* 2005; **280**: 8564-8580 [PMID: 15548529 DOI: 10.1074/jbc.M411703200]

83   **Ohana E**, Shcheynikov N, Yang D, So I, Muallem S. Determinants of coupled transport and uncoupled current by the electrogenic SLC26 transporters. *J Gen Physiol* 2011; **137**: 239-251 [PMID: 21282402 DOI: 10.1085/jgp.201010531]

84   **Yamamoto T**, Swietach P, Rossini A, Loh SH, Vaughan-Jones RD, Spitzer KW. Functional diversity of electrogenic Na+-HCO3- cotransport in ventricular myocytes from rat, rabbit and guinea pig. *J Physiol* 2005; **562**: 455-475 [PMID: 15550467 DOI: 10.1113/jphysiol.2004.071068]

85   **Vaughan-Jones RD**, Villafuerte FC, Swietach P, Yamamoto T, Rossini A, Spitzer KW. pH-Regulated Na(+) influx into the mammalian ventricular myocyte: the relative role of Na(+)-H(+) exchange and Na(+)-HCO Co-transport. *J Cardiovasc Electrophysiol* 2006; **17** Suppl 1: S134-S140 [PMID: 16686668 DOI: 10.1111/j.1540-8167.2006.00394.x]

86   **Grace AA**, Kirschenlohr HL, Metcalfe JC, Smith GA, Weissberg PL, Cragoe EJ, Vandenberg JI. Regulation of intracellular pH in the perfused heart by external HCO3- and Na(+)-H+ exchange. *Am J Physiol* 1993; **265**: H289-H298 [PMID: 8393626]

87   **Vandenberg JI**, Metcalfe JC, Grace AA. Mechanisms of pHi recovery after global ischemia in the perfused heart. *Circ Res* 1993; **72**: 993-1003 [PMID: 8386598 DOI: 10.1161/01.RES.72.5.993]

88   **Khandoudi N**, Albadine J, Robert P, Krief S, Berrebi-Bertrand I, Martin X, Bevensee MO, Boron WF, Bril A. Inhibition of the cardiac electrogenic sodium bicarbonate cotransporter reduces ischemic injury. *Cardiovasc Res* 2001; **52**: 387-396 [PMID: 11738055 DOI: 10.1016/S0008-6363(01)00430-8]

89   **Schäfer C**, Ladilov YV, Siegmund B, Piper HM. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. *Am J Physiol Heart Circ Physiol* 2000; **278**: H1457-H1463 [PMID: 10775122]

90   **Schäfer C**, Ladilov YV, Schäfer M, Piper HM. Inhibition of NHE protects reoxygenated cardiomyocytes independently of anoxic Ca(2+) overload and acidosis. *Am J Physiol Heart Circ Physiol* 2000; **279**: H2143-H2150 [PMID: 11045947]

91   **Thomas MJ**, Sjaastad I, Andersen K, Helm PJ, Wasserstrom JA, Sejersted OM, Ottersen OP. Localization and function of the Na+/Ca2+-exchanger in normal and detubulated rat cardiomyocytes. *J Mol Cell Cardiol* 2003; **35**: 1325-1337 [PMID: 14596789 DOI: 10.1016/j.yjmcc.2003.08.005]

92   **Petrecca K**, Atanasiu R, Grinstein S, Orlowski J, Shrier A. Subcellular localization of the Na+/H+ exchanger NHE1 in rat myocardium. *Am J Physiol* 1999; **276**: H709-H717 [PMID: 9950874]

93   **Aronsen JM**, Swift F, Sejersted OM. Cardiac sodium transport and excitation-contraction coupling. *J Mol Cell Cardiol* 2013; **61**: 11-19 [PMID: 23774049 DOI: 10.1016/j.yjmcc.2013.06.003]

94   **Villa-Abrille MC**, Petroff MG, Aiello EA. The electrogenic Na+/HCO3- cotransport modulates resting membrane potential and action potential duration in cat ventricular myocytes. *J Physiol* 2007; **578**: 819-829 [PMID: 17138608 DOI: 10.1113/jphysiol.2006.120170]

95   **Saegusa N**, Moorhouse E, Vaughan-Jones RD, Spitzer KW. Influence of pH on Ca^{2+} current and its control of electrical and Ca^{2+} signaling in ventricular myocytes. *J Gen Physiol* 2011; **138**: 537-559 [PMID: 22042988 DOI: 10.1085/jgp.201110658]

96   **Sandmann S**, Yu M, Kaschina E, Blume A, Bouzinova E, Aalkjaer C, Unger T. Differential effects of angiotensin AT1 and AT2 receptors on the expression, translation and function of the Na+-H+ exchanger and Na+-HCO3- symporter in the rat heart after myocardial infarction. *J Am Coll Cardiol* 2001; **37**: 2154-2165 [PMID: 11419902 DOI: 10.1016/S0735-1097(01)01287-6]

97   **Kohout TA**, Rogers TB. Angiotensin II activates the Na+/HCO3- symport through a phosphoinositide-independent mechanism in cardiac cells. *J Biol Chem* 1995; **270**: 20432-20438 [PMID: 7657618 DOI: 10.1074/jbc.270.35.20432]

98   **De Giusti VC**, Garciarena CD, Aiello EA. Role of reactive oxygen species (ROS) in angiotensin II-induced stimulation of the cardiac Na+/HCO3- cotransport. *J Mol Cell Cardiol* 2009; **47**: 716-722 [PMID: 19646989 DOI: 10.1016/j.yjmcc.2009.07.023]

99   **De Giusti VC**, Orlowski A, Aiello EA. Angiotensin II inhibits the electrogenic Na+/HCO3- cotransport of cat cardiac myocytes. *J Mol Cell Cardiol* 2010; **49**: 812-818 [PMID: 20692267 DOI: 10.1016/j.yjmcc.2010.07.018]

100   **Orlowski A**, Ciancio MC, Caldiz CI, De Giusti VC, Aiello EA. Reduced sarcolemmal expression and function of the NBCe1 isoform of the Na$^+$-HCO$_3$$^-$ cotransporter in hypertrophied cardiomyocytes of spontaneously hypertensive rats: role of the renin-angiotensin system. *Cardiovasc Res* 2014; **101**: 211-219 [PMID: 24253522 DOI: 10.1093/cvr/cvt255]

101   **Lebeche D**, Kaprielian R, Hajjar R. Modulation of action potential duration on myocyte hypertrophic pathways. *J Mol Cell Cardiol* 2006; **40**: 725-735 [PMID: 16600293 DOI: 10.1016/j.yjmcc.2006.01.018]

102   **Carmeliet E**. Action potential duration, rate of stimulation, and intracellular sodium. *J Cardiovasc Electrophysiol* 2006; **17** Suppl 1: S2-S7 [PMID: 16686677 DOI: 10.1111/j.1540-8167.2006.00378.x]

103   **De Giusti VC**, Orlowski A, Villa-Abrille MC, de Cingolani GE, Casey JR, Alvarez BV, Aiello EA. Antibodies against the cardiac sodium/bicarbonate co-transporter (NBCe1) as pharmacological tools. *Br J Pharmacol* 2011; **164**: 1976-1989 [PMID: 21595652 DOI: 10.1111/j.1476-5381.2011.01496.x]

104   **Yan S**, Chen Y, Dong M, Song W, Belcher SM, Wang HS. Bisphenol A and 17β-estradiol promote arrhythmia in the female heart via alteration of calcium handling. *PLoS One* 2011; **6**: e25455 [PMID: 21980463 DOI: 10.1371/journal.pone.0025455]

105   **Mason MJ**, Smith JD, Garcia-Soto JJ, Grinstein S. Internal pH-sensitive site couples Cl-(-)HCO3- exchange to Na+-H+ antiport in lymphocytes. *Am J Physiol* 1989; **256**: C428-C433 [PMID: 2537575]

106   **Jiang L**, Chernova MN, Alper SL. Secondary regulatory volume increase conferred on Xenopus oocytes by expression of AE2 anion exchanger. *Am J Physiol* 1997; **272**: C191-C202 [PMID: 9038825]

107   **Belcher SM**, Chen Y, Yan S, Wang HS. Rapid estrogen receptor-mediated mechanisms determine the sexually di-

morphic sensitivity of ventricular myocytes to 17β-estradiol and the environmental endocrine disruptor bisphenol A.

Endocrinology 2012; **153**: 712-720 [PMID: 22166976 DOI: 10.1210/en.2011-1772]

REVIEW

FoxO3a and disease progression

Richard Seonghun Nho, Polla Hergert

Richard Seonghun Nho, Polla Hergert, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Minnesota, Minneapolis, MN 55455, United States
Author contributions: Nho RS and Hergert P both contributed to this paper.
Supported by the National Institutes of Health R01 HL 114662 to Nho R
Correspondence to: Richard Seonghun Nho, PhD, Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Minnesota, Box 276, 420 Delaware Street SE., Minneapolis, Minneapolis, MN 55455, United States. nhoxx002@umn.edu
Telephone: +1-612-6250686   Fax: +1-612-6252174
Received: November 29, 2013 Revised: April 21, 2014
Accepted: May 16, 2014
Published online: August 26, 2014

Abstract

The Forkhead box O (FoxO) family has recently been highlighted as an important transcriptional regulator of crucial proteins associated with the many diverse functions of cells. So far, FoxO1, FoxO3a, FoxO4 and FoxO6 proteins have been identified in humans. Although each FoxO family member has its own role, unlike the other FoxO families, FoxO3a has been extensively studied because of its rather unique and pivotal regulation of cell proliferation, apoptosis, metabolism, stress management and longevity. FoxO3a alteration is closely linked to the progression of several types of cancers, fibrosis and other types of diseases. In this review, we will examine the function of FoxO3a in disease progression and also explore FoxO3a's regulatory mechanisms. We will also discuss FoxO3a as a potential target for the treatment of several types of disease.

Key words: Forkhead box O; Cell proliferation; Apoptosis; Stress; Aging

Core tip: Forkhead box O (FoxO)3a has recently been highlighted as a critical protein that regulates numerous cell functions from proliferation/apoptosis to stress-resistance and aging. FoxO3a has been found to be deregulated in several diseases and FoxO3a targeting approaches are currently underway to treat various types of cancers. This review will describe the current concept of FoxO3a's pathological role in various diseases and elucidate the regulatory mechanisms involved. It will also provide the clinical significance and strategies to target FoxO3a to limit the progression of human diseases.

Nho RS, Hergert P. FoxO3a and disease progression. *World J Biol Chem* 2014; 5(3): 346-354 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/346.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.346

INTRODUCTION

Forkhead box O (FoxO) transcription factors are the human homologues of the *C. elegans* transcription factor DAF-16 and share a highly conserved 110-amino acid DNA binding domain, forkhead box or winged-helix domain[1,2]. Forkhead box proteins comprise more than 100 members in humans, classified from FOXA to FOXR[3-5]. Members of class O share the characteristic of being regulated by the insulin/PI3K/Akt signaling pathway[4]. Four principal members of the mammalian FoxO subfamily, FoxO1, FoxO3a, FoxO4 and FoxO6 have been previously described[3]. Although they seem to bind a common set of DNA sites, FoxO6 is mainly specific to neurons, while the other 3 FoxO family members are expressed in most tissues. These FoxO members are linked to cell survival, cellular proliferation and DNA damage repair response[5,6]. Among them, FoxO3a has recently been studied extensively as a crucial protein that is involved in the regulation of several essential cellular functions (see page 349). Prior studies have shown that FoxO3a functions as a tumor suppressor by regulating expression of genes in-
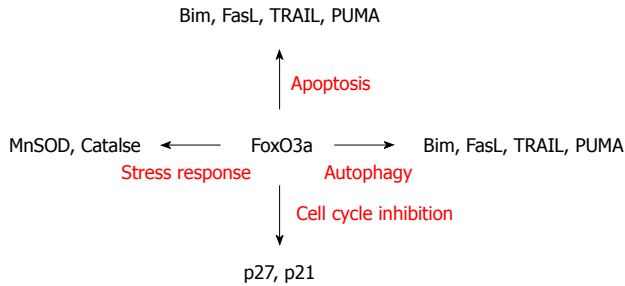
Figure 1 **Forkhead box O3a target genes.** Forkhead box O (FoxO)3a transcriptionally activates several target genes. FoxO3a binds to the promoter of apoptosis inducing genes, such as *Bim*, *FasL* and *TRAIL*, and to the promoter of cell cycle inhibitors, such as p27 and p21. FoxO3a also activates autophagy genes Gabarapl1, ATG12, etc. A recent study showed that FoxO3a also participates in the activation of stress response genes, such as MnSOD and catalase in response to oxidative stress.

volved in apoptosis, cell cycle arrest, oxidative stress resistance and autophagy[3,7-9] (Figure 1). In general, FoxO3a is known to suppress cell cycle progression and promote cell death. Thus, it has been thought that FoxO3a can be an important target to inhibit cancer cell progression. However, recent studies have discovered other functions of FoxO3a, such as stress response and longevity, as described on page 349. FoxO3a alteration is also linked to many different types of disease. Interestingly, FoxO3a increases autophagy to protect cells from environmental stresses[10,11]. Thus, under this situation, unlike the general concept of FoxO3a's role, FoxO3a potentially has a protective role in maintaining a cell's homeostasis. Perhaps the most interesting feature of FoxO3a is its biological role associated with longevity (page 349). Based on this, it becomes clear that FoxO3a has diverse roles in response to many environmental stimuli and these recent findings certainly change our view on the previous roles of FoxO3a. Therefore, from the perspective of disease progression, it is imperative to define the potential role of FoxO3a in cells and elucidate how alteration of FoxO3a is linked to the development of several types of disease.

FOXO3A STRUCTURE

Recent technologies have revealed that the primary structure of FoxO3a contains highly conserved residues of the helix H3 (motif NXXRHXXS/T), which is the main DNA recognition element that binds into a major groove, which comprises the majority of the direct base-specific contacts[1,6]. Recent studies further revealed that FoxO proteins recognize two consensus sequences, 5'-GTAAA(T/C)AA-3' known as the Daf-16 family member-binding element[6,7] and 5'-(C/A)(A/C)AAA(C/T)AA-3' known as the insulin-responsive sequence (IRE)[8,9]. Crystal structure revealed that the recognition helix H3 docked perpendicular to the major groove making extensive contacts with the DNA[7]. FoxO3a contains several crucial domains[12] (Figure 2) such as a nuclear localization signal (NLS), a nuclear export signal (NES) and a transactivation domain (TA).

FOXO3A REGULATORY MECHANISMS

Phosphorylation and dephosphorylation

FoxO3a is regulated by posttranslational modifications such as phosphorylation, acetylation and ubiquitination, each of which affects the transcriptional activity of FoxO proteins[11-16] (Figure 2). The potency of FoxO3a is carefully regulated by phosphorylation. The phosphorylation of FoxO3a by several kinases is well established. Among them, protein kinase B (Akt) is an important kinase that directly phosphorylates FoxOs. In the case of FoxO3a, T32, S253 and S315 residues are phosphorylated by Akt and, in particular, the phosphorylation of S253 is a crucial residue regulating the nuclear/cytoplasmic shuttling of FoxO3a. For example, when cells are cultured in the presence of growth factors or insulin, FoxO3a is phosphorylated by Akt and mainly localized to the cytoplasm, which prevents its transcriptional activity. The phosphorylation event of FoxO3a by Akt facilitates FoxO3a interaction with the 14-3-3 nuclear export protein, further preventing nuclear re-import by concealing nuclear localization signals[13]. Furthermore, the phosphorylation of FoxO3a by activated Akt promotes an association with an ubiquitin E3 ligase, subsequently polyubiquitinating FoxO3a, which facilitates FoxO3a degradation by proteasomes[13-17]. Thus, the activation of Akt is thought to be critical in FoxO3a regulation. However, in some tumors, FoxO3a remains in the cytoplasm even in the absence of active Akt[14]. It has been found that IkB kinase (IKK) phosphorylates FoxO3a at serine 644, thereby inhibiting its transcriptional activity in an Akt-independent manner[15]. The phosphorylation of FoxO3a by IKK also leads to its cytoplasmic localization, although the underlying export mechanism is not understood. The insulin/IGF-1 and integrin-dependent signaling pathways activate Akt *via* PTEN suppression which phosphorylates FoxO3a, thereby rendering it functionally inactive. In contrast, FoxO3a is localized to the nucleus to activate its target genes when growth factors or serum are deprived. Additionally, serum and glucocorticoid regulated kinase (SGK), casein kinase 1 (CK1), dual specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A), janus N-terminal kinase (JNK), mitogen-activated protein kinases (MAPKs), IkappaB kinase (IKKβ), mammalian sterile 20-like kinase 1 (MST1) and AMP activated protein kinase (AMPK) are also known to regulate FoxO3a and other family members[18-23] by phosphorylating multiple residues. Interestingly, SGK1 is transcriptionally up-regulated in response to a variety of external stimuli, including growth factors. SGK1 is also known to phosphorylate the pivotal ser 253 residue, which triggers its location to the cytoplasm, thereby inhibiting its function[19]. In contrast, AMPK activates FoxO3a function. 6 threonine/serine residues (T179, S399, S413, S555, S588 and S626) in mammalian FoxO3a are found to be phosphorylated by AMPK[24,25]. Mutation of these phosphorylation residues to alanine severely impairs its function, yet it does not alter its ability to bind to cognate
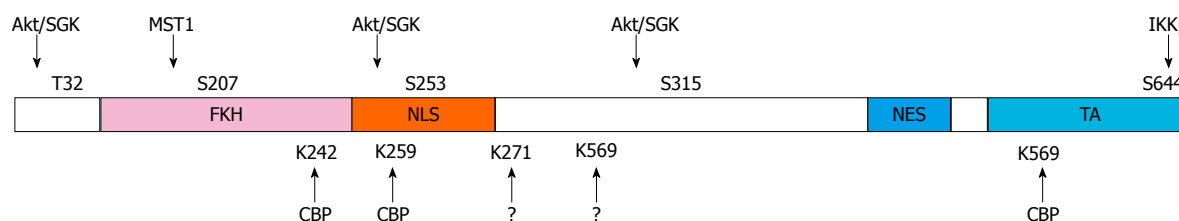
Figure 2 Major phosphorylation and acetylation residues of FoxO3a. Post-translational modification sites of FoxO3a. Shown are sites of serine/threonine phosphorylation by Akt/SGK, MST1, IKKβ or the residues acetylated by CBP or unidentified acetyl transferases (?) on FoxO3a domains[12]. FKH: Forkhead DNA binding domain; NLS: Nuclear localization signal; NES: Nuclear export sequence; TA: Transactivation domain; Akt: Protein kinase B; MST1: Mammalian sterile 20 like kinase-1; CBP: The cyclic–AMP responsive element binding (CREB) binding protein, IKKβ: Ikβ kinase; SGK: Serum-and glucocorticoid-induced protein kinase.
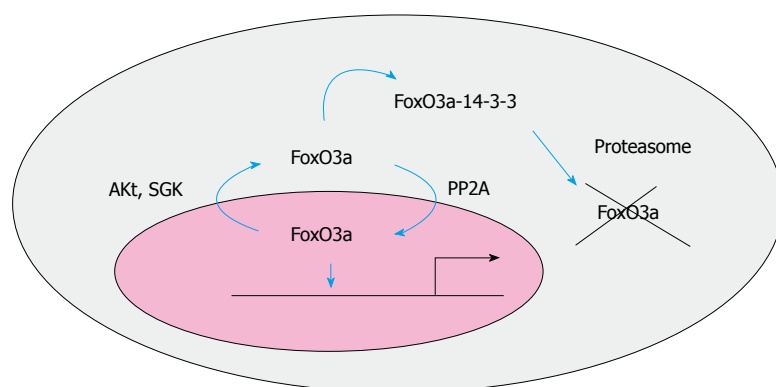
Figure 3 Forkhead box O3a localization by phosphorylation and dephosphorylation. Forkhead box O (FoxO)3a becomes translocated to the cytoplasm when phosphorylated on ser 253 residue by Akt or SGK. FoxO3a is then bound to 14-3-3 and this interaction promotes its degradation by the proteasome. In contrast, FoxO3a is dephosphorylated by protein phosphatase-2A and this opposite event facilitates its relocation into the nucleus, thereby activating its target genes. SGK: Serum-and glucocorticoid-induced protein kinase; Akt: Protein kinase B.

sequences or to participate in nucleocytoplasmic shuttling depending on external cues[25]. Likewise, JNK also phosphorylates FoxO3a, activating FoxO3a function by enhancing its location into the nucleus which subsequently increases its transcriptional activity[18,22].

Unlike kinases, very few phosphatases have been found to regulate FoxO3a. One particular phosphatase, protein phosphatase-2A (PP2A), has been shown to regulate FoxO3a function. Nho *et al*[26] showed that when fibroblasts attach to 2D type collagen coated plates, PP2A activity is suppressed, which facilitates FoxO3a inactivation by enhanced Akt, promoting fibroblast proliferation. But the over-expression of PP2A reverses this inactivation and increases dephosphorylated FoxO3a, thereby suppressing their proliferation. Singh *et al*[24] also demonstrated that FoxO3a interacts with PP2A C/A subunits in HeLA cells, dephosphorylating its T32/S253 residues, which subsequently inhibits the interaction of the 14-3-3 protein to FoxO3a by Akt. This study showed that PP2A is required for the reactivation of FoxO3a by promoting its translocation to the nucleus (Figure 3). Interestingly, recent studies also showed that the adenovirus E1A stabilizes FoxO3a by inducing the expression of PP2A/C, which suppresses βTrCP-mediated degradation of FoxO3a[25]. Thus, these studies clearly suggest that the imbalance between kinases and phosphatase(s) can greatly affect a cell's fate by curbing FoxO3a function and the alteration of these kinases and phosphatases are directly linked to certain disease progression.

Ubiquitin proteasome degradation

As we briefly described above, FoxO3a degradation is

also an important step to regulate its function. The single molecule RING-finger E3 ligase murine double minute 2 (MDM2) promotes ubiquitination of FoxO3a as well as FoxO1 and FoxO4, facilitating their degradation[27]. Intriguingly, knockout or knockdown of MDM2 alone increases FoxO3a protein levels. This effect was shown to be mediated by MDM2-induced polyubiquitination of FoxO proteins[27,28], whereas another study showed that MDM2 catalyzes multiple monoubiquitination of FoxO4 rather than polyubiquitination[28]. When FoxO3a is located to the cytoplasm by Akt, FoxO3a becomes ubiquitinated and this event triggers a proteasome-dependent degradation process. Like MDM2, FoxO3a phosphorylation by IKK also leads to its ubiquitination and degradation[15]. Thus, these studies document that FoxO3a localization in the cytoplasm not only deactivates FoxO3a function but also becomes a crucial step leading to FoxO3a degradation.

Acetylation, transcriptional regulation, microRNA and others

Acetylation also plays an important role in regulating FoxO3a. Oxidative stress triggers FoxO3a acetylation/deacetylation and affects the localization of FoxO3a. For example, protein acetylase CREB binding protein (CBP)[29-31], p300[32,33] and deacetylase Sirt are known to modulate FoxO3a function[34-38], although a precise mechanism describing the effects of acetylation and deacetylation is not known. A recent piece of evidence suggests that the FoxO family is also regulated by microRNA. mir155, mir96 and mir21 are thought to directly regulate FoxO3a, while mir205 regulates FoxO3a *via* its

upstream target PTEN[39-43]. FoxO3a is also known to be regulated by a transcription factor. E2F-1 can bind to the promoter region of FoxO1 and FoxO3a, thereby regulating FoxO3a at the mRNA level[44]. FoxO3a mRNAs are modulated as a function of age in rat muscle, peaking at 6 and 23 mo, suggesting that FoxO3a may also affect longevity in mammals[45].

FOXO3A FUNCTION

Cell proliferation and apoptosis

Perhaps the two most significant cellular processes that are regulated by FoxO transcription factor are the suppression of cell cycle progression and the promotion of apoptosis[46-50]. FoxO3a activation increases cell cycle inhibitor proteins p21 and p27, both of which subsequently suppress G1 to S cell cycle transition[51-54]. Although p27 is transcriptionally regulated by FoxO3a *via* the PI3K/Akt-dependent axis, it has been shown that p27 is also regulated *via* the FoxO3a/NF-κB/c-Myc-dependent pathway. Chandramohan *et al*[55] showed that in WEHI 231 cells, the suppression of PI3K activity promotes a decrease in c-Myc dependent p27 expression *via* NF-κB inhibition. Since NF-κB is frequently altered in many types of cancers and NF-κB transcriptionally activates *c-Myc* gene expression, this finding suggests that p27 is reciprocally regulated by FoxO3a and c-Myc. A recent study further suggests that FoxO3a inhibits NF-κB function and that the alteration of FoxO3a is associated with hyper-proliferative helper T cells, cigarette smoke-induced inflammation, airspace enlargement and chronic obstructive pulmonary disease[56,57]. Likewise, FoxO3a also increases several target genes, such as Bim, TRAIL, PUMA and Fas ligand, which all promote cell apoptosis. For example, FoxO3a directly binds to the promoter region of Bim, causing sympathetic neuron cell death[44]. The activation of the transcription factor FoxO3a led to increased TRAIL transcription and induction of G1 arrest in the absence of v-Abl inhibition; this effect could be inhibited by the expression of a constitutively active Akt mutant in BCR-Abl-transformed human cells. Ghaffari *et al*[49] also demonstrated that cytokine and BCR-Abl suppression of TRAIL transcription is mediated through phosphorylation and inhibition of the FoxO3a transcription factor. This study showed that BCR-Abl-induced inhibition of TRAIL transcription is linked to the tumorigenicity in chronic myeloid leukemia[50]. FoxO3a is also associated with the regulation of PUMA and Noxa proteins in lymphoid and neuroblastoma cells, respectively[58,59]. Thus, these findings clearly demonstrate that FoxO3a-dependent cell cycle arrest and apoptosis induction are important for tumor suppression (Table 1) and further indicate that the pathological alteration of FoxO3a can potentially contribute to the acquisition of uncontrolled cell proliferation and an apoptosis-resistant cell phenotype.

Stress resistant effect

The most recent discovery regarding FoxO3a's function is that it is also associated with stress response and longevity. In contrast to FoxO3a's better known functions of inhibiting cell proliferation and promoting apoptosis as described above, FoxO3a also participates in protecting cells when exposed to unfavorable conditions. This seemingly contradictory effect of FoxO3a has been observed in various cell models and it has been found that the reactive oxygen species (ROS) are linked to the activation of FoxO3a to protect cells from a stress inducing environment[60,61]. In *C. elegans,* DAF-16 is thought to regulate 230 genes on the ablated germ cell line background and most of these genes are related to the resistance of external stress[62,63]. Deregulated ROS induce apoptosis and are associated with various diseases and aging. Sirtuin-1 (Sirt1) decreases ROS levels and promotes cell survival under oxidative stress conditions. Interestingly, FoxO3a and other FoxO family members increase superoxide dismutase (SOD) and protect cells from oxidative stress in a Sirt1-dependent manner[34,38]. A Sirt1/FoxO3a-dependent cell regulatory function that has been linked to stress management was previously studied. Brunet *et al* showed that Sirt1 and FoxO3a form a complex in cells in response to oxidative stress and Sirt1 increases the ability of FoxO3a to induce cell cycle arrest and resistance to oxidative stress but inhibited FoxO3a's function to induce cell death[38]. These results showed that FoxO3a deacetylation by Sirt1 in response to ROS can be an important self defense mechanism to detoxifying harmful reactive molecules, further suggesting that Sirt1 is linked to protect cells from a stress inducing environment by tipping FoxO dependent response away from apoptosis and toward stress resistance[38]. Studies also found that Sirt3, which belongs to class Ⅲ of HDACs, is linked to the resistance of stress inducing environments by detoxifying ROS. The role of Sirt3 and FoxO3a function is particularly well described in myocytes[64]. At the cellular level, when cardiomyocytes are exposed to stressful stimuli, Sirt3 levels are elevated, which subsequently deacetylase FoxO3a and facilitate its location into the nucleus to activate anti-oxidant genes[65]. Among them, catalase (Cat) and manganese superoxide dismutase (MnSOD) are direct targets of detoxifying enzymes by FoxO3a. Thus, the increased level of Cat and MsSoD by FoxO3a activation may efficiently and effectively manage ROS, which can be beneficial for reducing stress induced by ROS. Interestingly, a prior study found a potential FoxO activator as a way to protect cells from oxidative stress. Resveratrol, a polyphenolic flavonoid abundant in red wine with potent antioxidant activity, is known to up-regulate the FoxO family and block caspase 3, 8, and 9 activation, protecting photoreceptor cells from oxidative stress[66]. Thus, it is believed that when cells are exposed to a stress inducing environment, FoxO3a protects cells by utilizing SOD, catalase, *etc.*, and this action is ultimately beneficial to cells. Given the fact that FoxO3a is linked to stress response and cells utilize FoxO3a to respond to ROS, it is a plausible scenario that the activation of FoxO3a under stress inducing conditions triggers the cell's defense sys-

FoxO3a target genes	Cell types
Bim	Neuron cells[48]
TRAIL	Bcr/Abl transformed cells[57]
TRAIL	Chronic myeloid leukemia[46]
PUMA	Lymphoid cells[58]
Noxa	Neuroblastoma[59]
FasL	Glomerular mesangial cells[102]
p27, Caveolin-1	Glomerular mesangial cells[102]
p21	Glomerular mesangial cells[102]

Shown are previously known FoxO3a target genes that regulate cell proliferation and apoptosis in different cell types.

tem, which can protect cells from harmful environments.

Longevity

However, perhaps the most intriguing recent discovery in FoxO3a function is that the *FoxO3a* gene is associated with aging. Because FoxO3a is regulated by insulin-IGF1 signaling (IIS) which influences metabolism and lifespan in model organisms[67], FoxO3a had been proposed to be an ideal candidate to study longevity as the link between FoxO3a and longevity that has previously been described. Willcox *et al*[68] described 3 single nucleotide polymorphisms (SNPs) in the *FoxO3a* gene that were statistically significantly associated with longevity and different aging phenotypes in a sample of long-lived Americans of Japanese ancestry. Furthermore, Flachsbart *et al*[69] found that not only were certain FoxO3a variants very common in 90 year olds, they were even more common in 100 year olds, emphasizing the importance of genetics for aging well. It becomes clear that increases in cellular ROS levels are known to be associated with aging[70-75]. Increased cellular oxidative stress regulates FoxO post-translational modifications and the activation of the FoxO family has been shown to regulate cellular oxidative-stress resistance[76-81]. Interestingly, to support these findings, recent studies suggest a possibility that Sirt3 and FoxO3a have been linked to an extended life span in humans[75-78,82].

FOXO3A IN CLINICAL APPLICATION

FoxO3, FoxO1 and FoxO4 are present at chromosomal translocation break points in cells of rhabdomyosarcomas and acute myeloid leukemia. Among the FoxO family, FoxO3a has been shown to be deregulated in several tumor types, including breast cancer[83-85], prostate cancer[86-88], glioblastoma[89] and leukemia[90,91]. Therefore, FoxO3a has been targeted as a way to treat several types of cancers. Interestingly, Akt, IKK and Erk are three commonly activated oncogenic kinases in human cancers and all three kinases target FoxO3a in an identical manner to inhibit its tumor suppressor function[92]. All three kinase-mediated phosphorylations stimulate FoxO3a ubiquitination, resulting in its proteasomal degradation. Thus, a FoxO3a targeting approach *via* the modulation of above kinases is currently underway. For example,

the chemotherapeutic drugs paclitaxel[93] and KP372-1 (a multiple kinase inhibitor)[30], currently used in the treatment of breast carcinoma, activate FoxO3a by reducing Akt activity. Doxorubicin activates FoxO3a to induce the expression of the multidrug resistance gene ABCB1 (MDR1) in K562 doxorubicin-sensitive leukemic cells[94]. Imatinib activates FoxO3a and induces Bim-dependent apoptosis through inhibition of BCR-ABL in chronic myeloid leukemia[95]. Imatinib also induces erythroid differentiation through repressing ID1 gene transcription by FoxO3a activation[96]. BMS-345541, a selective IKK inhibitor, promotes apoptosis in T-cell acute lymphoblastic leukemia (T-ALL) cell lines[97]. Several pieces of evidence in recent years further suggest that a FoxO3a targeting approach may be helpful for the treatment of other types of human diseases. For example, FoxO3a causes the induction of apoptosis in prostate cancer cells *via* up-regulating PUMA[98]. Low levels of FoxO3a may link to chemotherapy resistance in liver cancer and FoxO3a appears to present antitumor properties in hepatocellular carcinoma[99-101]. FoxO3a also plays a role in the neuroprotective effect of the erythropoietin (EPO) role in Parkinson's disease *via* Akt[102]. Thus, all these studies indicate that as our knowledge for FoxO3a targeting approaches continuously develop, the clinical application of FoxO3 is potentially promising to limit the progression of human diseases in the future.

FUTURE APPLICATION OF FOXO3A

FoxO3a has recently been recognized as a promising therapeutic target to treat cancers and other types of diseases. To improve therapeutic outcomes, FoxO3a-dependent chemosensitization is being currently tested. Studies suggest that precise FoxO3a regulation is essential for homeostasis and if there is deregulation of FoxO3a by environmental factors, such as chronic exposure to ROS or genetic/epigenetic alteration, this pathological condition can directly lead to abnormal proliferation or changes in apoptotic signals, which subsequently are responsible for disease progression. In particular, age-dependent FoxO3a modulation is an interesting concept to help understand the pathogenesis of certain types of disease models. If FoxO3a is a crucial protein mainly deregulated by aging, maintaining optimum FoxO3a activity in a patient's specific clinical condition can be beneficial to minimize age-dependent disease. For example, the preservation of optimum FoxO3a activity using drugs such as paclitaxel may be helpful for patients with age-related diseases. Clearly, more studies are required to elucidate FoxO3a's function as an effective and useful target capable of preventing or limiting the progression of diseases without clinical compromise.

REFERENCES

1   **Clark KL**, Halay ED, Lai E, Burley SK. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* 1993; **364**: 412-420 [PMID: 8332212 DOI:

10.1038/364412a0]

2   **Arden KC**. Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer. *Exp Gerontol* 2006; **41**: 709-717 [PMID: 16806782 DOI: 10.1016/j.exger.2006.05.015]

3   **Huang H**, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007; **120**: 2479-2487 [PMID: 17646672 DOI: 10.1242/jcs.001222]

4   **Dillin A**, Crawford DK, Kenyon C. Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science* 2002; **298**: 830-834 [PMID: 12399591 DOI: 10.1126/science.1074240]

5   **Monsalve M**, Olmos Y. The complex biology of FOXO. *Curr Drug Targets* 2011; **12**: 1322-1350 [PMID: 21443460 DOI: 10.2174/138945011796150307]

6   **Biggs WH**, Cavenee WK, Arden KC. Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. *Mamm Genome* 2001; **12**: 416-425 [PMID: 11353388 DOI: 10.1007/s003350020002]

7   **Furuyama T**, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 2000; **349**: 629-634 [PMID: 10880363 DOI: 10.1042/0264-6021:]

8   **Hall RK**, Yamasaki T, Kucera T, Waltner-Law M, O'Brien R, Granner DK. Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. *J Biol Chem* 2000; **275**: 30169-30175 [PMID: 10913147 DOI: 10.1074/jbc.M004898200]

9   **Hribal ML**, Nakae J, Kitamura T, Shutter JR, Accili D. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 2003; **162**: 535-541 [PMID: 12925703 DOI: 10.1083/jcb.200212107]

10   **Warr MR**, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J, Passegué E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature* 2013; **494**: 323-327 [PMID: 23389440 DOI: 10.1038/nature11895]

11   **Xia N**, Strand S, Schlufter F, Siuda D, Reifenberg G, Kleinert H, Förstermann U, Li H. Role of SIRT1 and FOXO factors in eNOS transcriptional activation by resveratrol. *Nitric Oxide* 2013; **32**: 29-35 [PMID: 23583951 DOI: 10.1016/j.niox.2013.04.001]

12   **Greer EL**, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005; **24**: 7410-7425 [PMID: 16288288 DOI: 10.1038/sj.onc.1209086]

13   **Plas DR**, Thompson CB. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* 2003; **278**: 12361-12366 [PMID: 12517744 DOI: 10.1074/jbc.M213069200]

14   **Brunet A**, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* 2001; **21**: 952-965 [PMID: 11154281 DOI: 10.1128/MCB.21.3.952-965.2001]

15   **Hu MC**, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* 2004; **117**: 225-237 [PMID: 15084260 DOI: 10.1016/S0092-8674(04)00302-2]

16   **Vogt PK**, Jiang H, Aoki M. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle* 2005; **4**: 908-913 [PMID: 15917664 DOI: 10.4161/cc.4.7.1796]

17   **Fu W**, Ma Q, Chen L, Li P, Zhang M, Ramamoorthy S, Nawaz Z, Shimojima T, Wang H, Yang Y, Shen Z, Zhang Y, Zhang X, Nicosia SV, Zhang Y, Pledger JW, Chen J, Bai

18   **Sunters A**, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, Burgering BM, Coombes RC, Lam EW. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. *Cancer Res* 2006; **66**: 212-220 [PMID: 16397234 DOI: 10.1158/0008-5472.CAN-05-1997]

19   **Huang H**, Tindall DJ. Regulation of FOXO protein stability via ubiquitination and proteasome degradation. *Biochim Biophys Acta* 2011; **1813**: 1961-1964 [PMID: 21238503 DOI: 10.1016/j.bbamcr.2011.01.007]

20   **Chatterjee A**, Chatterjee U, Ghosh MK. Activation of protein kinase CK2 attenuates FOXO3a functioning in a PML-dependent manner: implications in human prostate cancer. *Cell Death Dis* 2013; **4**: e543 [PMID: 23492774 DOI: 10.1038/cddis.2013.63]

21   **Gao J**, Yang X, Yin P, Hu W, Liao H, Miao Z, Pan C, Li N. The involvement of FoxO in cell survival and chemosensitivity mediated by Mirk/Dyrk1B in ovarian cancer. *Int J Oncol* 2012; **40**: 1203-1209 [PMID: 22159921 DOI: 10.3892/ijo.2011.1293]

22   **Wang X**, Chen WR, Xing D. A pathway from JNK through decreased ERK and Akt activities for FOXO3a nuclear translocation in response to UV irradiation. *J Cell Physiol* 2012; **227**: 1168-1178 [PMID: 21604264 DOI: 10.1002/jcp.22839]

23   **Clavel S**, Siffroi-Fernandez S, Coldefy AS, Boulukos K, Pisani DF, Dérijard B. Regulation of the intracellular localization of Foxo3a by stress-activated protein kinase signaling pathways in skeletal muscle cells. *Mol Cell Biol* 2010; **30**: 470-480 [PMID: 19917721 DOI: 10.1128/MCB.00666-09]

24   **Nho RS**, Kahm J. beta1-Integrin-collagen interaction suppresses FoxO3a by the coordination of Akt and PP2A. *J Biol Chem* 2010; **285**: 14195-14209 [PMID: 20223831 DOI: 10.1074/jbc.M109.052845]

25   **Singh A**, Ye M, Bucur O, Zhu S, Tanya Santos M, Rabinovitz I, Wei W, Gao D, Hahn WC, Khosravi-Far R. Protein phosphatase 2A reactivates FOXO3a through a dynamic interplay with 14-3-3 and AKT. *Mol Biol Cell* 2010; **21**: 1140-1152 [PMID: 20110348 DOI: 10.1091/mbc.E09-09-0795]

26   **Chiacchiera F**, Simone C. The AMPK-FoxO3A axis as a target for cancer treatment. *Cell Cycle* 2010; **9**: 1091-1096 [PMID: 20190568 DOI: 10.4161/cc.9.6.11035]

27   **Milkiewicz M**, Roudier E, Doyle JL, Trifonova A, Birot O, Haas TL. Identification of a mechanism underlying regulation of the anti-angiogenic forkhead transcription factor FoxO1 in cultured endothelial cells and ischemic muscle. *Am J Pathol* 2011; **178**: 935-944 [PMID: 21281824 DOI: 10.1016/j.ajpath.2010.10.042]

28   **Brenkman AB**, de Keizer PL, van den Broek NJ, Jochemsen AG, Burgering BM. Mdm2 induces mono-ubiquitination of FOXO4. *PLoS One* 2008; **3**: e2819 [PMID: 18665269 DOI: 10.1371/journal.pone.0002819]

29   **Wang F**, Marshall CB, Li GY, Yamamoto K, Mak TW, Ikura M. Synergistic interplay between promoter recognition and CBP/p300 coactivator recruitment by FOXO3a. *ACS Chem Biol* 2009; **4**: 1017-1027 [PMID: 19821614 DOI: 10.1021/cb900190u]

30   **Zeng Z**, Samudio IJ, Zhang W, Estrov Z, Pelicano H, Harris D, Frolova O, Hail N, Chen W, Kornblau SM, Huang P, Lu Y, Mills GB, Andreeff M, Konopleva M. Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia. *Cancer Res* 2006; **66**: 3737-3746 [PMID: 16585200 DOI: 10.1158/0008-5472.CAN-05-1278]

31   **Wang F**, Marshall CB, Yamamoto K, Li GY, Gasmi-Seabrook GM, Okada H, Mak TW, Ikura M. Structures of KIX do-

main of CBP in complex with two FOXO3a transactivation domains reveal promiscuity and plasticity in coactivator recruitment. *Proc Natl Acad Sci USA* 2012; **109**: 6078-6083 [PMID: 22474372 DOI: 10.1073/pnas.1119073109]

32 **Emerling BM**, Weinberg F, Liu JL, Mak TW, Chandel NS. PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3A). *Proc Natl Acad Sci USA* 2008; **105**: 2622-2627 [PMID: 18268343 DOI: 10.1073/pnas.0706790105]

33 **Senf SM**, Sandesara PB, Reed SA, Judge AR. p300 Acetyltransferase activity differentially regulates the localization and activity of the FOXO homologues in skeletal muscle. *Am J Physiol Cell Physiol* 2011; **300**: C1490-C1501 [PMID: 21389279 DOI: 10.1152/ajpcell.00255.2010]

34 **Hori YS**, Kuno A, Hosoda R, Horio Y. Regulation of FOXOs and p53 by SIRT1 modulators under oxidative stress. *PLoS One* 2013; **8**: e73875 [PMID: 24040102 DOI: 10.1371/journal.pone.0073875]

35 **Kim MJ**, Ahn K, Park SH, Kang HJ, Jang BG, Oh SJ, Oh SM, Jeong YJ, Heo JI, Suh JG, Lim SS, Ko YJ, Huh SO, Kim SC, Park JB, Kim J, Kim JI, Jo SA, Lee JY. SIRT1 regulates tyrosine hydroxylase expression and differentiation of neuroblastoma cells via FOXO3a. *FEBS Lett* 2009; **583**: 1183-1188 [PMID: 19285077 DOI: 10.1016/j.febslet.2009.03.007]

36 **Ni HM**, Du K, You M, Ding WX. Critical role of FoxO3a in alcohol-induced autophagy and hepatotoxicity. *Am J Pathol* 2013; **183**: 1815-1825 [PMID: 24095927 DOI: 10.1016/j.ajpath.2013.08.011]

37 **Lai L**, Yan L, Gao S, Hu CL, Ge H, Davidow A, Park M, Bravo C, Iwatsubo K, Ishikawa Y, Auwerx J, Sinclair DA, Vatner SF, Vatner DE. Type 5 adenylyl cyclase increases oxidative stress by transcriptional regulation of manganese superoxide dismutase via the SIRT1/FoxO3a pathway. *Circulation* 2013; **127**: 1692-1701 [PMID: 23536361 DOI: 10.1161/CIRCULATIONAHA.112.001212]

38 **Brunet A**, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; **303**: 2011-2015 [PMID: 14976264 DOI: 10.1126/science.1094637]

39 **Babar IA**, Czochor J, Steinmetz A, Weidhaas JB, Glazer PM, Slack FJ. Inhibition of hypoxia-induced miR-155 radiosensitizes hypoxic lung cancer cells. *Cancer Biol Ther* 2011; **12**: 908-914 [PMID: 22027557 DOI: 10.4161/cbt.12.10.17681]

40 **Lin H**, Dai T, Xiong H, Zhao X, Chen X, Yu C, Li J, Wang X, Song L. Unregulated miR-96 induces cell proliferation in human breast cancer by downregulating transcriptional factor FOXO3a. *PLoS One* 2010; **5**: e15797 [PMID: 21203424 DOI: 10.1371/journal.pone.0015797]

41 **Ling N**, Gu J, Lei Z, Li M, Zhao J, Zhang HT, Li X. microRNA-155 regulates cell proliferation and invasion by targeting FOXO3a in glioma. *Oncol Rep* 2013; **30**: 2111-2118 [PMID: 23970205]

42 **Wang K**, Li PF. Foxo3a regulates apoptosis by negatively targeting miR-21. *J Biol Chem* 2010; **285**: 16958-16966 [PMID: 20371612 DOI: 10.1074/jbc.M109.093005]

43 **Cai J**, Fang L, Huang Y, Li R, Yuan J, Yang Y, Zhu X, Chen B, Wu J, Li M. miR-205 targets PTEN and PHLPP2 to augment AKT signaling and drive malignant phenotypes in non-small cell lung cancer. *Cancer Res* 2013; **73**: 5402-5415 [PMID: 23856247 DOI: 10.1158/0008-5472.CAN-13-0297]

44 **Nowak K**, Killmer K, Gessner C, Lutz W. E2F-1 regulates expression of FOXO1 and FOXO3a. *Biochim Biophys Acta* 2007; **1769**: 244-252 [PMID: 17482685 DOI: 10.1016/j.bbaexp.2007.04.001]

45 **Furuyama T**, Yamashita H, Kitayama K, Higami Y, Shimokawa I, Mori N. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. *Microsc Res Tech* 2002; **59**:

331-334 [PMID: 12424797 DOI: 10.1002/jemt.10213]

46 **Rathbone CR**, Booth FW, Lees SJ. FoxO3a preferentially induces p27Kip1 expression while impairing muscle precursor cell-cycle progression. *Muscle Nerve* 2008; **37**: 84-89 [PMID: 17894357 DOI: 10.1002/mus.20897]

47 **Roy SK**, Chen Q, Fu J, Shankar S, Srivastava RK. Resveratrol inhibits growth of orthotopic pancreatic tumors through activation of FOXO transcription factors. *PLoS One* 2011; **6**: e25166 [PMID: 21980390 DOI: 10.1371/journal.pone.0025166]

48 **Gilley J**, Coffer PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 2003; **162**: 613-622 [PMID: 12913110 DOI: 10.1083/jcb.200303026]

49 **Wilson MK**, McWhirter SM, Amin RH, Huang D, Schlissel MS. Abelson virus transformation prevents TRAIL expression by inhibiting FoxO3a and NF-kappaB. *Mol Cells* 2010; **29**: 333-341 [PMID: 20213318 DOI: 10.1007/s10059-010-0029-8]

50 **Ghaffari S**, Jagani Z, Kitidis C, Lodish HF, Khosravi-Far R. Cytokines and BCR-ABL mediate suppression of TRAIL-induced apoptosis through inhibition of forkhead FOXO3a transcription factor. *Proc Natl Acad Sci USA* 2003; **100**: 6523-6528 [PMID: 12750477 DOI: 10.1073/pnas.0731871100]

51 **van den Heuvel AP**, Schulze A, Burgering BM. Direct control of caveolin-1 expression by FOXO transcription factors. *Biochem J* 2005; **385**: 795-802 [PMID: 15458387 DOI: 10.1042/BJ20041449]

52 **Kops GJ**, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffer PJ, Lam EW, Burgering BM. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 2002; **22**: 2025-2036 [PMID: 11884591 DOI: 10.1128/MCB.22.7.2025-2036.2002]

53 **Hauck L**, Harms C, Grothe D, An J, Gertz K, Kronenberg G, Dietz R, Endres M, von Harsdorf R. Critical role for FoxO3a-dependent regulation of p21CIP1/WAF1 in response to statin signaling in cardiac myocytes. *Circ Res* 2007; **100**: 50-60 [PMID: 17158337 DOI: 10.1161/01.RES.0000254704.92532.b9]

54 **Miyauchi H**, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J* 2004; **23**: 212-220 [PMID: 14713953 DOI: 10.1038/sj.emboj.7600045]

55 **Chandramohan V**, Jeay S, Pianetti S, Sonenshein GE. Reciprocal control of Forkhead box O 3a and c-Myc via the phosphatidylinositol 3-kinase pathway coordinately regulates p27Kip1 levels. *J Immunol* 2004; **172**: 5522-5527 [PMID: 15100294]

56 **Lin L**, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 2004; **21**: 203-213 [PMID: 15308101 DOI: 10.1016/j.immuni.2004.06.016]

57 **Hwang JW**, Rajendrasozhan S, Yao H, Chung S, Sundar IK, Huyck HL, Pryhuber GS, Kinnula VL, Rahman I. FOXO3 deficiency leads to increased susceptibility to cigarette smoke-induced inflammation, airspace enlargement, and chronic obstructive pulmonary disease. *J Immunol* 2011; **187**: 987-998 [PMID: 21690325 DOI: 10.4049/jimmunol.1001861]

58 **You H**, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G, Erlacher M, Villunger A, Mak TW. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* 2006; **203**: 1657-1663 [PMID: 16801400 DOI: 10.1084/jem.20060353]

59 **Obexer P**, Geiger K, Ambros PF, Meister B, Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. *Cell Death Differ* 2007; **14**: 534-547 [PMID: 16888645 DOI: 10.1038/sj.cdd.4402017]

60 **Sengupta A**, Molkentin JD, Paik JH, DePinho RA, Yutzey KE. FoxO transcription factors promote cardiomyocyte

survival upon induction of oxidative stress. *J Biol Chem* 2011; **286**: 7468-7478 [PMID: 21159781 DOI: 10.1074/jbc.M110.179242]

61   **Liu JW**, Chandra D, Rudd MD, Butler AP, Pallotta V, Brown D, Coffer PJ, Tang DG. Induction of prosurvival molecules by apoptotic stimuli: involvement of FOXO3a and ROS. *Oncogene* 2005; **24**: 2020-2031 [PMID: 15674333 DOI: 10.1038/sj.onc.1208385]

62   **Murphy CT**, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature* 2003; **424**: 277-283 [PMID: 12845331 DOI: 10.1038/nature01789]

63   **McCormick M**, Chen K, Ramaswamy P, Kenyon C. New genes that extend Caenorhabditis elegans' lifespan in response to reproductive signals. *Aging Cell* 2012; **11**: 192-202 [PMID: 22081913 DOI: 10.1111/j.1474-9726.2011.00768.x]

64   **Sengupta A**, Molkentin JD, Yutzey KE. FoxO transcription factors promote autophagy in cardiomyocytes. *J Biol Chem* 2009; **284**: 28319-28331 [PMID: 19696026 DOI: 10.1074/jbc.M109.024406]

65   **Sundaresan NR**, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009; **119**: 2758-2771 [PMID: 19652361]

66   **Huang W**, Li G, Qiu J, Gonzalez P, Challa P. Protective effects of resveratrol in experimental retinal detachment. *PLoS One* 2013; **8**: e75735 [PMID: 24040416 DOI: 10.1371/journal.pone.0075735]

67   **Li M**, Chiu JF, Gagne J, Fukagawa NK. Age-related differences in insulin-like growth factor-1 receptor signaling regulates Akt/FOXO3a and ERK/Fos pathways in vascular smooth muscle cells. *J Cell Physiol* 2008; **217**: 377-387 [PMID: 18615585 DOI: 10.1002/jcp.21507]

68   **Willcox BJ**, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD. FOXO3A genotype is strongly associated with human longevity. *Proc Natl Acad Sci USA* 2008; **105**: 13987-13992 [PMID: 18765803 DOI: 10.1073/pnas.0801030105]

69   **Flachsbart F**, Caliebe A, Kleindorp R, Blanché H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A. Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proc Natl Acad Sci USA* 2009; **106**: 2700-2705 [PMID: 19196970 DOI: 10.1073/pnas.0809594106]

70   **Morris BJ**. A forkhead in the road to longevity: the molecular basis of lifespan becomes clearer. *J Hypertens* 2005; **23**: 1285-1309 [PMID: 15942449 DOI: 10.1097/01.hjh.0000173509.45363.dd]

71   **Eelen G**, Verlinden L, Meyer MB, Gijsbers R, Pike JW, Bouillon R, Verstuyf A. 1,25-Dihydroxyvitamin D3 and the aging-related forkhead box O and sestrin proteins in osteoblasts. *J Steroid Biochem Mol Biol* 2013; **136**: 112-119 [PMID: 22989484 DOI: 10.1016/j.jsbmb.2012.09.011]

72   **Kops GJ**, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 2002; **419**: 316-321 [PMID: 12239572 DOI: 10.1038/nature01036]

73   **Giordano S**, Darley-Usmar V, Zhang J. Autophagy as an essential cellular antioxidant pathway in neurodegenerative disease. *Redox Biol* 2014; **2**: 82-90 [PMID: 24494187 DOI: 10.1016/j.redox.2013.12.013]

74   **El Assar M**, Angulo J, Rodríguez-Mañas L. Oxidative stress and vascular inflammation in aging. *Free Radic Biol Med* 2013; **65**: 380-401 [PMID: 23851032 DOI: 10.1016/j.freeradbiomed.2013.07.003]

75   **Liochev SI**. Reactive oxygen species and the free radical theory of aging. *Free Radic Biol Med* 2013; **60**: 1-4 [PMID: 23434764 DOI: 10.1016/j.freeradbiomed.2013.02.011]

76   **van der Horst A**, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 2007; **8**: 440-450 [PMID: 17522590 DOI: 10.1038/nrm2190]

77   **Chen IC**, Chiang WF, Chen PF, Chiang HC. STRESS-responsive deacetylase SIRT3 is up-regulated by areca nut extract-induced oxidative stress in human oral keratinocytes. *J Cell Biochem* 2014; **115**: 328-339 [PMID: 24339251 DOI: 10.1002/jcb.24667]

78   **Zhang S**, Zhao Y, Xu M, Yu L, Zhao Y, Chen J, Yuan Y, Zheng Q, Niu X. FoxO3a modulates hypoxia stress induced oxidative stress and apoptosis in cardiac microvascular endothelial cells. *PLoS One* 2013; **8**: e80342 [PMID: 24278276 DOI: 10.1371/journal.pone.0080342]

79   **Raju I**, Kannan K, Abraham EC. FoxO3a Serves as a Biomarker of Oxidative Stress in Human Lens Epithelial Cells under Conditions of Hyperglycemia. *PLoS One* 2013; **8**: e67126 [PMID: 23805295 DOI: 10.1371/journal.pone.0067126]

80   **Chen SJ**, Zhang W, Tong Q, Conrad K, Hirschler-Laszkiewicz I, Bayerl M, Kim JK, Cheung JY, Miller BA. Role of TRPM2 in cell proliferation and susceptibility to oxidative stress. *Am J Physiol Cell Physiol* 2013; **304**: C548-C560 [PMID: 23302782 DOI: 10.1152/ajpcell.00069.2012]

81   **Li Z**, Zhang H, Chen Y, Fan L, Fang J. Forkhead transcription factor FOXO3a protein activates nuclear factor κB through B-cell lymphoma/leukemia 10 (BCL10) protein and promotes tumor cell survival in serum deprivation. *J Biol Chem* 2012; **287**: 17737-17745 [PMID: 22474286 DOI: 10.1074/jbc.M111.291708]

82   **Tseng AH**, Shieh SS, Wang DL. SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative damage. *Free Radic Biol Med* 2013; **63**: 222-234 [PMID: 23665396 DOI: 10.1016/j.freeradbiomed.2013.05.002]

83   **Yang JY**, Chang CJ, Xia W, Wang Y, Wong KK, Engelman JA, Du Y, Andreeff M, Hortobagyi GN, Hung MC. Activation of FOXO3a is sufficient to reverse mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor chemoresistance in human cancer. *Cancer Res* 2010; **70**: 4709-4718 [PMID: 20484037 DOI: 10.1158/0008-5472.CAN-09-4524]

84   **Chen J**, Gomes AR, Monteiro LJ, Wong SY, Wu LH, Ng TT, Karadedou CT, Millour J, Ip YC, Cheung YN, Sunters A, Chan KY, Lam EW, Khoo US. Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. *PLoS One* 2010; **5**: e12293 [PMID: 20808831 DOI: 10.1371/journal.pone.0012293]

85   **Lam M**, Carmichael AR, Griffiths HR. An aqueous extract of Fagonia cretica induces DNA damage, cell cycle arrest and apoptosis in breast cancer cells via FOXO3a and p53 expression. *PLoS One* 2012; **7**: e40152 [PMID: 22761954 DOI: 10.1371/journal.pone.0040152]

86   **Shukla S**, Bhaskaran N, Maclennan GT, Gupta S. Deregulation of FoxO3a accelerates prostate cancer progression in TRAMP mice. *Prostate* 2013; **73**: 1507-1517 [PMID: 23765843 DOI: 10.1002/pros.22698]

87   **Shukla S**, Bhaskaran N, Babcook MA, Fu P, Maclennan GT, Gupta S. Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoxO pathway. *Carcinogenesis* 2014; **35**: 452-460 [PMID: 24067903 DOI: 10.1093/carcin/bgt316]

88   **Chen Q**, Ganapathy S, Singh KP, Shankar S, Srivastava RK. Resveratrol induces growth arrest and apoptosis through activation of FOXO transcription factors in prostate cancer cells. *PLoS One* 2010; **5**: e15288 [PMID: 21179458 DOI: 10.1371/journal.pone.0015288]

89   **Sunayama J**, Sato A, Matsuda K, Tachibana K, Watanabe E, Seino S, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity. *Stem Cells* 2011; **29**:

1327-1337 [PMID: 21793107]

90   **Ruvolo PP**. The Herculean task of killing cancer cells: suppression of FOXO3A in acute leukemia involves a hydra of multiple survival kinases. *Cell Cycle* 2012; **11**: 2589 [PMID: 22751430 DOI: 10.4161/cc.21233]

91   **Wang W**, Li NN, Du Y, Lv FF, Lin GQ. FoxO3a and nilotinib-induced erythroid differentiation of CML-BC cells. *Leuk Res* 2013; **37**: 1309-1314 [PMID: 23915976 DOI: 10.1016/j.leukres.2013.07.001]

92   **Yang W**, Dolloff NG, El-Deiry WS. ERK and MDM2 prey on FoxO3a. *Nat Cell Biol* 2008; **10**: 125-126 [PMID: 18246039 DOI: 10.1038/ncb0208-125]

93   **Sunters A**, Fernández de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, Coffer PJ, Medema RH, Coombes RC, Lam EW. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 2003; **278**: 49795-49805 [PMID: 14527951 DOI: 10.1074/jbc.M309523200]

94   **Hui RC**, Francis RE, Guest SK, Costa JR, Gomes AR, Myatt SS, Brosens JJ, Lam EW. Doxorubicin activates FoxO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells. *Mol Cancer Ther* 2008; **7**: 670-678 [PMID: 18347152 DOI: 10.1158/1535-7163.MCT-07-0397]

95   **Naka K**, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, Nakao S, Motoyama N, Hirao A. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 2010; **463**: 676-680 [PMID: 20130650 DOI: 10.1038/nature08734]

96   **Birkenkamp KU**, Essafi A, van der Vos KE, da Costa M, Hui RC, Holstege F, Koenderman L, Lam EW, Coffer PJ. FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1. *J Biol Chem* 2007; **282**: 2211-2220 [PMID: 17132628 DOI: 10.1074/jbc.M606669200]

97   **Buontempo F**, Chiarini F, Bressanin D, Tabellini G, Melchionda F, Pession A, Fini M, Neri LM, McCubrey JA, Martelli AM. Activity of the selective IκB kinase inhibitor BMS-345541 against T-cell acute lymphoblastic leukemia: involvement of FOXO3a. *Cell Cycle* 2012; **11**: 2467-2475 [PMID: 22713244 DOI: 10.4161/cc.20859]

98   **Dey P**, Ström A, Gustafsson JÅ. Estrogen receptor β upregulates FOXO3a and causes induction of apoptosis through PUMA in prostate cancer. *Oncogene* 2014; **33**: 4213-4225 [PMID: 24077289 DOI: 10.1038/onc.2013.384]

99   **Jia Y**, Mo SJ, Feng QQ, Zhan ML, OuYang LS, Chen JC, Ma YX, Wu JJ, Lei WL. EPO-dependent activation of PI3K/Akt/FoxO3a signalling mediates neuroprotection in in vitro and in vivo models of Parkinson's disease. *J Mol Neurosci* 2014; **53**: 117-124 [PMID: 24390959 DOI: 10.1007/s12031-013-0208-0]

100 **Carbajo-Pescador S**, Mauriz JL, García-Palomo A, González-Gallego J. FoxO proteins: regulation and molecular targets in liver cancer. *Curr Med Chem* 2014; **21**: 1231-1246 [PMID: 24372208]

101 **Liang C**, Chen W, Zhi X, Ma T, Xia X, Liu H, Zhang Q, Hu Q, Zhang Y, Bai X, Liang T. Serotonin promotes the proliferation of serum-deprived hepatocellular carcinoma cells via upregulation of FOXO3a. *Mol Cancer* 2013; **12**: 14 [PMID: 23418729 DOI: 10.1186/1476-4598-12-14]

102 **Ghosh Choudhury G**, Lenin M, Calhaun C, Zhang JH, Abboud HE. PDGF inactivates forkhead family transcription factor by activation of Akt in glomerular mesangial cells. *Cell Signal* 2003; **15**: 161-170 [PMID: 12464387 DOI: 10.1016/S0898-6568(02)00057-8]

REVIEW

Matrix metalloproteinases and gastrointestinal cancers: Impacts of dietary antioxidants

Sugreev Verma, Kousik Kesh, Nilanjan Ganguly, Sayantan Jana, Snehasikta Swarnakar

Sugreev Verma, Kousik Kesh, Nilanjan Ganguly, Sayantan Jana, Snehasikta Swarnakar, Drug Development Diagnostic and Biotechnology Division, CSIR-Indian Institute of Chemical Biology, Jadavpur, Kolkata 700032, India
Author contributions: All authors contributed to this paper.
Supported by Council of Scientific and Industrial Research, India; (CSIR)-INDEPTH and HUM projects
Correspondence to: Dr. Snehasikta Swarnakar, Drug Development Diagnostic and Biotechnology Division, CSIR-Indian Institute of Chemical Biology, 4 Raja S C Mullick Road, Jadavpur, Kolkata 700032, India. snehasiktas@hotmail.com
Telephone: +91-33-24995824 Fax: +91-33-24735197

Abstract

The process of carcinogenesis is tightly regulated by antioxidant enzymes and matrix degrading enzymes, namely, matrix metalloproteinases (MMPs). Degradation of extracellular matrix (ECM) proteins like collagen, proteoglycan, laminin, elastin and fibronectin is considered to be the prerequisite for tumor invasion and metastasis. MMPs can degrade essentially all of the ECM components and, most MMPs also substantially contribute to angiogenesis, differentiation, proliferation and apoptosis. Hence, MMPs are important regulators of tumor growth both at the primary site and in distant metastases; thus the enzymes are considered as important targets for cancer therapy. The implications of MMPs in cancers are no longer mysterious; however, the mechanism of action is yet to be explained. Herein, our major interest is to clarify how MMPs are tied up with gastrointestinal cancers. Gastrointestinal cancer is a variety of cancer types, including the cancers of gastrointestinal tract and organs, *i.e.*, esophagus, stomach, biliary system, pancreas, small intestine, large intestine, rectum and anus. The activity of MMPs is regulated by its endogenous inhibitor tissue inhibitor of metallopro-

teinase (TIMP) which bind MMPs with a 1:1 stoichiometry. In addition, RECK (reversion including cysteine-rich protein with kazal motifs) is a membrane bound glycoprotein that inhibits MMP-2, -9 and -14. Moreover, α2-macroglobulin mediates the uptake of several MMPs thereby inhibit their activity. Cancerous conditions increase intrinsic reactive oxygen species (ROS) through mitochondrial dysfunction leading to altered protease/anti-protease balance. ROS, an index of oxidative stress is also involved in tumorigenesis by activation of different MAP kinase pathways including MMP induction. Oxidative stress is involved in cancer by changing the activity and expression of regulatory proteins especially MMPs. Epidemiological studies have shown that high intake of fruits that rich in antioxidants is associated with a lower cancer incidence. Evidence indicates that some antioxidants inhibit the growth of malignant cells by inducing apoptosis and inhibiting the activity of MMPs. This review is discussed in six subchapters, as follows.

Key words: Gastrointestinal cancer; Matrix metalloproteinase; Tissue inhibitor of matrix metalloproteinases; Reactive oxygen species; Antioxidants

Core tip: Matrix metalloproteinases (MMPs), a group of zinc dependent endopeptidases, substantially contribute to extra cellular remodelling, angiogenesis, cellular differentiation, proliferation and apoptosis. MMPs are also important regulators of tumor growth both at the primary site and in distant metastasis; thus the enzymes are considered as important targets for cancer therapy. This review describes the roles and regulation of different MMPs and their subsequent actions over different gastrointestinal cancers both in epigenetic and cellular level. Furthermore, this review summarizes the current state of knowledge of dietary antioxidants in preventing gastrointestinal cancer progression as well as mechanism of action.

EPIDEMIOLOGY AND GENETIC BASIS OF GASTRIC CANCERS

As far the incidence rate is concerned, gastric cancer holds the fourth position among the most common cancers in men and fifth in women, from a worldwide perspective. The death rate goes hand to hand with lung cancer, the most frequent cancer globally[1]. Approximately, one million cases of gastric cancer were reported in the year 2008, which accounted for almost 8% of all cancerous incidents throughout the world[1]. Regions of Asia, eastern Europe, South America were highlighted in the reports as the most affected continents[2]. Carcinogenesis in the gastrointestinal tract, accounts for marked geographic variations in incidence and shows morphological heterogeneity. Histologically, gastric cancers are mainly of two types, diffuse and localized intestinal types. Poorly differentiated cancer cells, scattered within the stromal cells diagnosed as diffuse-type gastric cancer (DGC), whereas tubular gland like structures formed by the cancer cells having a few stromal components give rise to intestinal-type gastric cancer (IGC). Recognized as a familial disease many years ago, hereditary diffuse gastric cancer has now been identified as an autosomal dominant cancer susceptibility syndrome. This familial disease was probably most elegantly demonstrated in the family of Napoleon Bonaparte[3,4].

Chromosomal anomalies leading to gastrointestinal cancers

Aneuploidy of chromosomes 4, 8, 17 and 20 in gastric cancer were reported in several studies. Researchers have been trying to identify the precise stages known from Correa's pathway, where these chromosomal anomalies arise. Chromosome 4 and 20 were found to be amplified with the deletion of chromosome 17(p53) in multiple known progressive stages of carcinogenesis including normal gastric mucosa as well as metaplasia, dysplasia and cancer. A significant increase in the levels of aneuploidy was also reported with disease severity. Moreover, in some cases significant positive association was observed between chromosome 4 amplification and infection with *Helicobacter pylori* (*H. pylori*). In the same study, a similar kind of aneuploid condition was induced *in vitro* by exposing a human cell line to hydrogen peroxide suggesting that *H. pylori* induces gastric cancer with the help of reactive oxygen species (ROS) mediated chromosomal aneuploidy[5].

In 1988, Correa proposed the stages of human gastric cancer progression using several stages including gastritis, metaplasia, dysplasia, carcinoma, *etc*. In a study by Sugai *et al*[6] chromosomal allelic losses were tested of multiple cancer related chromosomal loci (1p, 3p, 4p, 5q, 8p, 9p, 13p, 17p, 18q and 22q). In addition, microsatellite instability (MSI) and overexpression of p53 protein were checked in all tumor samples. A prominent 3p allelic loss was observed in the cases of gastric phenotypes, whereas 5q allelic loss was highly associated to the intestinal phenotypes. Both loss of heterozygosity and microsatellite instability were observed in the genetic profiles of the mixed phenotypes. Allelic losses of 5q, 3p and 18q loci were consistent in intra-mucosal carcinomas and allelic losses of 17p, 1p and 9p were associated to submucosal carcinomas, all leading to loss of heterozygosity. MSI was observed only in 6 out of 31 cases of mixed phenotype gastric cancers, while p53 overexpression is observed in most of the cases of differentiated gastric carcinomas[6].

Specific gene mutations and their contributions

In most cases, the molecular expression of several biological markers show no link between the young ($\leqslant$ 45 years) and the aged ($\geqslant$ 45 years) patients, suggesting that early onset of gastric cancers possess different expression patterns of several important biomolecules[7]. Early onset gastric cancer patients may be more susceptible to the genetic factors but these individuals account for 10% or even less of all gastric cancer patients throughout the world[8,9]. Only10% of early onset gastric cancer cases belong to the inherited gastric cancer predisposition syndromes, but the genetic events taking place in the background of these remain largely unclear till date.

Development of tumors, often result from defects in several signaling pathways, including tyrosine phosphorylation, which occurs through the combined actions of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs). About 26% of colorectal cancers and a minute fraction of gastric cancers were reported to have mutations in the *PTP* genes. Mutated PTP originally are tumor suppressor genes, which regulate pathways associated with cell growth and differentiation. Wang *et al*[10] uncovered 83 such somatic mutations by mutational analysis of the six tyrosine phosphatase gene superfamily namely (PTPRF, PTPRG, PTPRT, PTPN3, PTPN13, and PTPN14). Production of truncated proteins lacking phosphatase activity was due to 15 mutations, which were nonsense, frame shift or splice site mutations. Reduced phosphatase activity was resulted in 5 missense mutations in PTPRT. Restoration of wild-type PTPRT expression in human cancer cells tended to cease cell growth[10].

Several studies reported the involvement of different biomolecules for the cause of gastric cancer. E-cadherin, p53, cycloxygenase-2 (COX-2), trefoil factor-1 (TFF-1), β-catenin, p16, c-myc, *etc*., are some of the known molecules. Significant difference in the expression of these markers and some other molecules are found, namely c-jun, HuR, C/EBP-β, *etc*., in early onset of gastric cancer, as well as regular gastric cancer patients. TFF-1 was overexpressed with a comparatively lower level of COX-2 in the early onset gastric cancer, whereas COX-2 overex-

pression and loss of TFF1 was found in regular cancers. Surprisingly, overexpression of COX-2, C/EBP-β in intestinal type gastric cancer was observed[7].

Risk factors for familial and non-familial gastrointestinal cancers

Gastrointestinal carcinomas like, esophageal adenocarcinoma, gastroesophageal junctional adenocarcinoma, *etc.*, often originate from Barrett's esophagus (BE), a chronic gastroesophageal reflux disease[11,12]. When BE and its associated diseases occur in families, they are collectively included within a syndrome named Familial Barrett's Esophagus, categorized as a complex genetic disease[13,14].

The onset of adenocarcinomas is thought to be determined by a combined effect of genetic variation and distinct environmental factors. Chak *et al*[15] determined the relationship between risk factors and the age of onset of these cancers. Family history of BE/cancer occurrence, gastroesophageal reflux symptoms, obesity (defined as body mass index > 30) and other risk factors were assessed in a total of 356 gastroesophageal adenocarcinoma patients. This study reports that both familial and non-familial cancers arise at similar ages, but obesity is associated with a comparatively earlier age of onset.

Appropriate clinical counseling based on the genetics of gastrointestinal cancers always depends on well substantiated data reflecting the risk factors existing through a family. The estimated risk of gastric cancer within a family, however, may differ widely from one another. A group of researchers from Sweden used the updated Swedish Family-Cancer Database to investigate the familial risks of gastric cancer in 5358 patients among the offspring and 36486 patients among the parents. In this investigation, 133 families were identified having one parent and one offspring recognized as patients of gastric cancer, whereas 20 families had two affected offspring. The standardized incidence ratio (SIR) for the families was 1.63, when the parents displayed gastric cancer and the same was 2.93 in the families where the siblings had the disease. Cancer in the corpus (main body of the stomach) was related to high sibling risk (SIR = 7.28). Whenever gastric cancer was diagnosed in the parents, the SIR for cancer in the cardia (the area joining stomach and esophagus) was 1.54. In most cases, upper stomach cancer did show a particular association to esophageal adenocarcinoma. Histological analysis revealed an increase of signet ring cells in cancers. Among the factors, giving rise to high sibling risk in the case of corpus cancer, *H. pylori* infection may be an important one. The association of upper stomach cancer and esophageal adenocarcinoma in families may also lead to important clues on the aetiology of both diseases[16].

Chromatin remodeling and epigenetic modifications as etiological factors

Various carcinogenic pathways and environmental factors may contribute to the aetiologies of gastric cancers[17]. Several genes as well as some of their mutations were identified in a study by exome sequencing of 22 gastric cancer samples. In this way, genes participating in chromatin remodeling were most commonly found to be mutated, leading to alterations in specific pathway. Protein deficiency of AT-rich interactive domain-containing protein 1A (ARID1A) were observed in 83% of gastric cancers with MSI, 73% of those with Epstein-Barr virus (EBV) infection and 11% of those that were not infected with EBV and microsatellite stable. A small division of the disease may arise due to *TP53* (gene encoding p53) mutations, as well as other genetic alterations and modified pathways. Occurrence of these mutations shows a negative correlation with mutations in *TP53*. The significance of chromatin remodeling is highlighted in the context of gastric cancers, which also reveal some new genomic landscapes[18].

Overexpression of claudin-4 (CLDN4), a protein involved in tight junctions is known to be associated to gastric cancers. Increased expression of CLDN4 on the membrane enhances the barrier like function of tight junctions which tends to prevent the migration and invasion of gastric cancer cells, without affecting cell growth. The epigenetic regulation of CLDN4 overexpression and its clinical significance as potential therapeutic targets was reported by Kwon *et al*[19]. DNA hypomethylation parallels to CLDN4 upregulation in both cancerous and non-cancerous gastric tissues. In normal gastric tissues, bivalent histone modifications often lead to repression of CLDN4 expression, whereas loss of repressive histone methylations results in upregulation of CLDN4 in gastric cancer cells[19].

Methylation level of long interspersed element-1 (LINE-1) is associated with esophagus gastric as well as colon cancer progression and prognosis[20]; this helps in assessing tumor heterogeneity and drug efficacy for the personalized treatment of patients with gastrointestinal cancers. Okada *et al*[21] documented that promoter methylation rate of seven genes *TP73*, *BLU*, *FSD1*, *BCL7A*, *MARK1*, *SCRN1*, and *NKX3.1* are higher in EBV-associated gastric carcinomas compare to EBV-negative gastric carcinoma signify the viral-mediated epigenetic alteration in cancer. Report suggested that *H. pyroli* infection induced promoter methylations of THBS1 and GATA-4 gene in the early stages of chronic gastritis and gastric cancer development[22]. Jin *et al*[23,24] reported the enhanced rate of promoter methylation of a transmembrane glycoprotein endoglin and Ras-related associated with diabetes gene in human ESCC. Also, TIMP-3 hypermethylation contributes to the downregulation TIMP-3 protein expression in ESCC and is associated with poor patient survival[25]. Poplineau *et al*[26] reported that a DNA hypomethylation agent enhanced upregulation of MMP-1 gene expression and triggered tumor cell invasion. In contrast, treatment with S-adenosylmethionine, a methyl donor, resulted in activation of TIMP-2 and significant downregulation of MMP-2 and MT1-MMP gene in colorectal cancer[27]. Prognostic values of promoter hypermethylation in patients with gastric cancer

Table 1 Major matrix metalloproteinases studied in cancer biology

Collagenase	Gelatinase	Stromolysin	Matrilysin	Membrane type MMPs	Others
MMP-1	MMP-2	MMP-3	MMP-7	MMP-14	MMP-12
MMP-8	MMP-9	MMP-10	MMP-26	MMP-15	MMP-20
MMP-13		MMP-11		MMP-16	MMP-28
MMP-18		MMP-19		MMP-17	
				MMP-24	
				MMP-25	

MMP: Matrix metalloproteinases.

documented that patients with higher stage of colorectal cancer possess a higher concentration of methylated APC, TIMP-3 and hMLH1 in the serum[28]. Wang *et al*[29] reported a frequent hypermethylation of RASSF1A gene promoter in gastric and colon cancer and predicted its utility as a diagnostic marker.

ELEVATED INDUCTION OF MMPS IN GASTROINTESTINAL CANCERS

The MMPs are comprised of a family of endopeptidases, which can cleave almost every component of the extra cellular matrix (ECM) proteins. It is documented that many non-ECM proteins can also be cleaved by selected MMPs. Structurally, they all have a zinc ion in the catalytic domain and their activity is dependent on divalent ions, mainly, Zn^{2+} and Ca^{2+}[30,31]. There are about 27 different MMPs discovered so far and they are subdivided in groups according to substrate specificity and structural integrity (Table 1). Induction and expression of MMPs are regulated at the level of transcription and translation, respectively. Further complexity of MMPs is the activation from zymogen to active enzyme and, secondly, the mRNA stability of few MMPs play critical role. ProMMPs are converted to active MMPs by intra-molecular cleavage of cysteine bridge between thiol group at the prodomain and Zn^{2+} near the catalytic site. The overall activity depends on the availability of the substrate as well as inhibitor in pericellular space, though a high concentration of MMPs exists near the plasma membrane.

Cancer progression can be explained in six major steps: self-support in growth signals; resistance to growth-inhibitory signals; reduced apoptosis; uncontrolled replication; sustained angiogenesis; and tissue invasion followed by metastasis[32]. Considerable evidence has demonstrated that disease progression in experimental animal models of cancer invasion and metastasis correlate with enhanced secretion of specific MMPs by tumor cells and/or stromal cells. Gastrointestinal cancer can be subdivided into different types, *e.g.*, cancers of upper digestive tract, esophageal cancer, gastric cancer, pancreatic cancer, liver cancer, gallbladder cancer and others like MALT lymphoma, gastrointestinal stromal tumors, cancer of the biliary tree, cancer of the lower digestive tract, colorectal and gastrointestinal carcinoid tumor.

Role of MMPs in esophageal and gastric cancer

Role of MMPs in gastrointestinal cancer has been well studied. IHC analysis of tumor biopsy samples suggest the expression of MMP-1 in 24% of oesophageal cancers, while MMP-2 and MMP-9 in 78% and 70% of samples respectively[33]. Similarly, studies revealed that MMP-13 is localized predominantly in tumor cells; and the presence of MMP-13 together with MT1-MMP is implicated in determining tumor aggressiveness of human oesophageal carcinomas. Etoh *et al*[34] found a significant correlation in survival period for subjects with the expression of MMP-13 and MT1-MMP in tumor tissue. Moreover, the activities of MMP-2, -3, -9, and -10 enzymes were detected in each of the 24 cancer cases. MMP overexpression was reported in tumors in comparison to normal tissue; having elevated levels of the activated form of MMP-3 and -10 in tumors. In addition, MMP-3 and -10 mRNA levels were significantly higher in tumors than paired normal tissues in both the stromal and epithelial component of tissues[35].

One of the important features of the malignant phenotype in both colorectal and gastric cancer is the overexpression of MMP-2 and -9 as well as activation of proMMP-2 to active MMP-2. Expression of MMP-2, -1 and -9 was found in 94%, 73% and 70% respectively in gastric specimens when studied in 74 patients. Conversely, MMP-3 was only present in 27% of tumors while MMP-1 and -9 were present mostly in all intestinal phenotypes of gastric cancer. In addition to MMPs, TIMP-1 and TIMP-2 were detected in approximately 50% of gastric tumors. Progression of gastric cancer is associated with MMP-13 expression along with its coexpression with MT1-MMP and/or MMP-2 that may have a synergistic effect in the progression of the disease[36]. The expression of TIMP-3 was significantly higher than that of MMP-3, and MMP-3/TIMP-3 was lower in gastric cancer tissue at the early stages ($n = 18$) than in that of the advanced stage group ($n = 26$) ($P < 0.05$)[37]. MMP-7 expression has been found to be prognostic marker for metastasis of gastric carcinoma because MMP-7 mRNA as well as protein was pronounced in aggressiveness carcinoma tissues.

Role of specific MMPs in colorectal cancer

The critical event in the process of cancer invasion and metastasis is the degradation of the ECM surrounding

the tumor tissue[38]. This ECM is degraded by the action of a set of proteases, in which several types of MMPs play major role, of which MMP-2 and -9 are most prominent. The basement membrane which prevents an invading epithelial tumor is mainly made up of type IV collagen, which is substrate of MMP-2 and -9. The event of basement membrane degradation promotes epithelial tumor invasion. Higher levels of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP expression have been documented in human colorectal. Murray *et al*[33] demonstrated that MMP-1 in colorectal cancer specimens was linked to a poor prognosis of the disease. This study was later confirmed by performing IHC, FISH, and RT-PCR on 142 samples of colorectal carcinomas[39]. The latent form of MMP-2, *i.e.*, proMMP-2, is expressed in significant levels almost in all normal tissues. *MMP-2* acts as the 'house-keeping' gene due to its importance in normal cellular physiology. While active *MMP-2* is found in neoplastic tissues, it is lacking in most normal tissues. Parsons *et al*[40] in 1992, were the first who described the role of MMP-2 in colorectal cancer and the ratio of MMP-2 to proMMP-2 was 20 fold higher in colorectal cancer specimens in comparison to non-malignant biopsies as judged by gelatin zymography. Parsons *et al*[40] demonstrated increased expression of proMMP-9 in colorectal cancer. The increased activity of proMMP-9 from inflammatory cells may cause an early change in progression from adenoma to carcinoma, when colonic adenoma is compared to normal mucosa. Increased co-expression of MMP-3 and MMP-9 has been found in colorectal tumors. Co-expression of uPA with MMP-9 in colorectal cancers is responsible for the activation of plasminogen to plasmin[41]. Plasmin stimulates proMMP-3 to active MMP-3 which in turn promotes proMMP-9 to active MMP-9, thus, resulting in colorectal cancer progression[41]. Excess MMP-9 expression in colorectal cancer contributes to the inflammation related to neoplasms but not to aggressive tumors[42]. Low levels of microsatellite instability and poor prognosis is observed with increased expression of MMP-3 in colorectal cancer. Moreover 90% of colonic adenocarcinomas demonstrated high levels of MMP-7 expression. Studies on surgically resected colorectal cancer specimens elucidated the clinical importance of MMP-7 expression in this cancer type. It demonstrated that overexpression of MMP-7 in colorectal cancer (measured by IHC and in situ hybridization) directly relates to nodal or distant metastasis[43,44]. On the contrary, MMP-12 overexpression is associated with increased survival in colorectal cancer because of its influence as protective factor presumably by inhibiting tumor angiogenesis[43]. In fact, inhibition of tumor growth with upregulation of MMP-12, also known as macrophage metalloprotease, is well accepted. It was reported by Dong *et al*[45] that macrophages capable of producing MMP-12 in tumors that are responsible for increased production of angiostatin, an inhibitor of tumor neovascularization[45,46]. High expression of MMP-13 results in poor survival of colon cancer patients. Colorectal tumor biopsy specimens were examined for the identification of

MMP-13 by Leeman *et al*[47]. MMP-13 was found in 91% of cases and was localized to cytosol of tumor tissues. Significantly higher activity of MMP-13 was observed in malignant than the non-malignant tissues. Moreover, plasmin, MT1-MMP and MMP-2 are key molecules in the production of active MMP-13. Active MMP-13 was found to be responsible for activation of MMP-9 during cancer progression[48].

MMPs polymorphism in tumor formation

Unlike classical oncogenes, MMPs are not upregulated by gene amplification or mutations. The increased MMP expression in tumours is mainly due to transcriptional changes rather than genetic alterations. The only two reported genetic alterations in cancer cells are translocation of the *MMP-23* gene in neuroblastoma[49] and amplification of the *MT5-MMP* gene[50]. Polymorphisms in MMP promoters also affect gene transcription and influence cancer susceptibility (Figure 1). The estimated number of single nucleotide polymorphisms (SNPs) in the human genome is 10 million, while only a small part of these polymorphisms are functionally relevant. The differences in allele transcription caused by polymorphisms in the MMP promoters are subtle compared with the overexpression that arises from the amplification of oncogenes. Most of the functional SNPs are located in the promoter region of the *MMP-1,-2,-3,-9* and *-7* genes that are associated with gastric cancer risk.

MMP1-1607 1G/2G polymorphism was found to be associated with gastric cancer risk as presence of extra guanine (2G) creates a binding site for Ets-1 transcription factor that enhances transcription of MMP-1. Bradbury *et al*[51] reported an elevated esophageal cancer risk in 1G/2G and 2G/2G carrier. MMP-1 protein expression is higher in tumors from gastric cancer patients who carry the 2G allele not 1G homozygotes[52]. Moreover, 2G homozygotes are more likely to develop invasive tumors. Dey *et al*[52] reported that MMP-1 promoter polymorphism is significantly associated with lower stomach tumor formation. MMP-1 -1607 1G/2G polymorphism is also involved with colon cancer risks.

MMP-2 polymorphism was investigated mainly in the promoter region, *e.g.*, MMP-2 -1306 C/T, -735 C/T, -790T/G, -955A/C, and -1575G/A in the context of gastrointestinal cancer risk[53]. Studies reported association of gastrointestinal cancer risk with -1306 C/T and -735 C/T polymorphic site worldwide. Price *et al*[54] characterized genetic variants in the human MMP-2 -1306 C/T allele-specific transcriptional regulation. The common C>T transition at -1306 disrupts a Sp1-type promoter site (CCACC box), leading to lower promoter activity with the T allele[54]. On the other hand, G to A substitution at the MMP-2 -1575 site reduces gene expression due to a reduction of estrogen receptor-α binding to A allele[55]. Fruh *et al*[56] found the presence of CC allele at MMP-2 -1306 position in *H. pylori* infected individuals which provide protection against esophagus adenocarcinoma. Studies also reported that presence of CC allele at
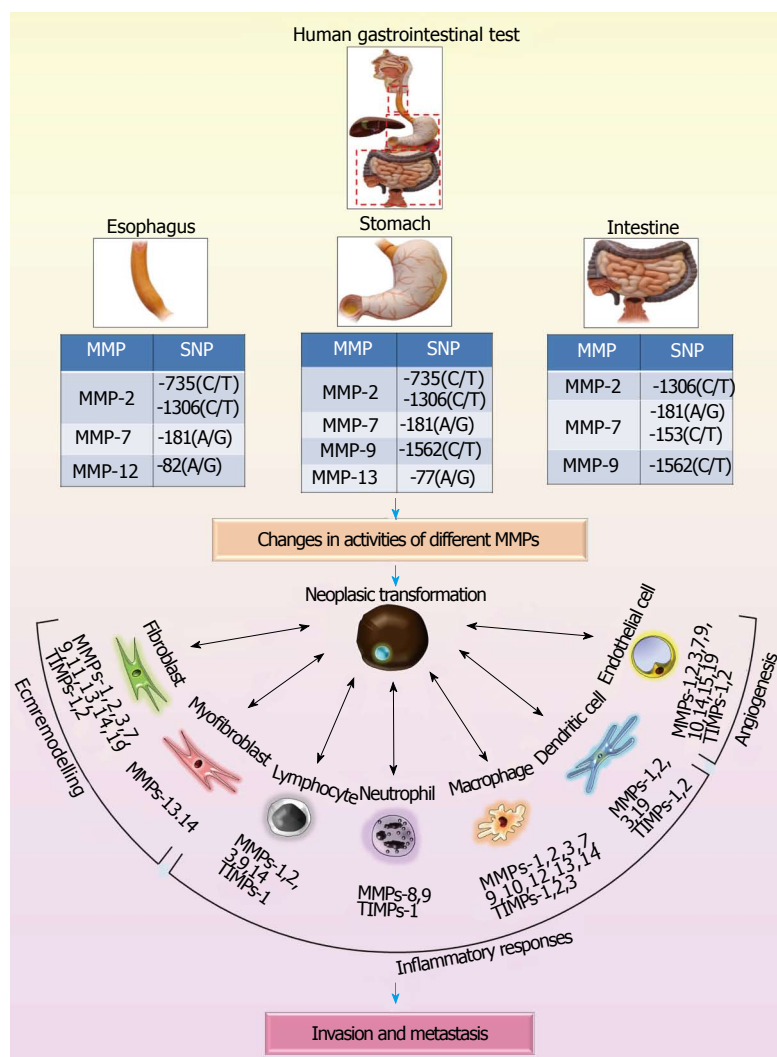
Figure 1 Matrix metalloproteinases polymorphism in gastrointestinal cancers. Single nucleotide polymorphism (SNP) for matrix metalloproteinases (MMP) genes in gastrointestinal organs (e.g., esophagus, stomach and intestine) of human has been reported. These SNPs are involved in changing MMPs activities in neoplastic transformation of gastric tissues in cancer patients. In addition to cancerous cells, the secretion of MMPs by fibroblasts, myofibroblasts, lymphocytes, neutrophils, macrophages, dendritic cells and endothelial cells has been documented. Both MMPs and tissue inhibitor of metalloproteinases (TIMPs) are important in regulation of extracellular matrix (ECM) remodeling, inflammatory responses and angiogenesis for cancer invasion and metastasis.

-1306 site increases the risk of ESCC, although Chen et al[54,57] did not observe any positive association of MMP-2 -1306 C/T polymorphism with ESCC in Mongolian population suggests differences in genetic susceptibility between Han-ethnic Chinese and the Mongolian population. However, both positive and negative influences of MMP-2 -1306 C/T polymorphism with gastric cancer in Asian and Caucasian population were reported[58]. Studies were performed to evaluate the association of MMP-2 -1306 C/T polymorphism with colon cancer risk[59]. Langer et al[53,60] reported that presence of CC or CT genotype enhances the survival rate of colon cancer patients. There is also a significant association of MMP-2 -735 C/T polymorphism with esophageal cancer risks[54].

MMP-9 over expression is associated with almost all the hallmark steps of cancer progression that make MMP-9 an ideal candidate gene for genetic association studies. Functional polymorphisms, e.g., MMP-9 promoter (-90CA(n), -1562C/T) as well as structural region (R279Q, P574R, R668Q) polymorphism were studied, assuming it might influence the MMP-9 gene expression or protein activity. MMP-9 polymorphism and gastrointestinal cancer risk is apparent in both Chinese and Caucasian populations[61,62]. In a hospital based case control study in

Chinese population, MMP-9 polymorphism in individual carrying RR genotype at P574R have increased risk of ESCC while R279Q and R668Q polymorphism has no association with cancer risk. In contrast Fang et al[63] reported that individuals having RR genotype at R279Q site have enhanced risk towards colon cancer. Tang et al[62] showed R279Q and P574R polymorphism were associated with lymph node metastasis of gastric cancer. Positive association of MMP-9 -1562 C/T polymorphism and colon cancer risk has been reported by Woo et al[61] in Korean population. Other studies reported a higher incident of lymph node metastasis in gastric and colon cancer patients having CC genotype at -1562 bp[64,65].

Association of SNPs in MMP-3 promoter and gastrointestinal cancer has been investigated in MMP-3 -1171 6A/5A site, since transcription repressor bind strongly with 6A allele leading to reduced gene expression. Bradbury et al[51] suggested a positive association of EA risk with 6A/5A or 5A/5A carrier. In addition, Zhang et al[64] found a higher ESCC risk among smokers having the 5A allele and reported that an elevated risk of lymph node metastasis in patients having 5A allele instead of the 6A allele. Interestingly, Dey et al[52] reported that the frequency of homozygotes for the 6A allele is lower in

gastric cancer patients than in controls of eastern Indian population[66]. On contrary, only one study performed in Japanese population that showed higher incidents of colon cancer in individuals having 6A/6A genotype.

Two common functional MMP-7 SNPs (-181A/G, -153C/T) are believed to control gene expression in several diseases, including gastrointestinal cancer[67-69]. MMP-7 up-regulation was significantly related to the promoter activity variation of the –181A/G alleles. Jormsjö *et al*[68] reported that the expression and promoter activity of the MMP7 -181G allele was higher in G over A and, attributed to the formation of a putative binding site (NGAAN) for a heat-shock transcription factor to G-allele. On the contrary, Richards *et al*[69] reported that elevated plasma MMP-7 level in AA genotype was governed by the G to A transition in -181 bp resulting in higher binding for the forkhead box A2 transcription factor to AA genotype. Studies reported a positive association of MMP-7 -181A/G polymorphism in esophagus, and gastric adenocarcinoma in Chinese population with an increased gastric cancer risk in G allele carrier[70]. However, in contrast, Kubbent *et al*[58] reported more AA and less AG in gastric cancer group. Moreover, MMP-7, -181 A/G and -153 C/T polymorphism is also significantly associated with colon cancer risk[71].

REGULATION OF MMPS IN GASTROINTESTINAL CANCER

An understanding of the MMP regulation in different cellular processes, *e.g.*, apoptosis, angiogenesis, invasion, metastasis as well as immune function is important for early prognosis and better therapeutics of gastrointestinal cancers. The regulation of MMPs goes awry at any or all cellular processes during cancer development[30]. The regulatory mechanisms shared among different cellular processes might control the invasive property of cells. The presence of MMP-1, -2, -3, -9, and MT1-MMP mRNA and protein in gastric and colorectal cancer tissues are evident from IHC and FISH assay. It is also known that MMPs are produced by inflammatory and fibroblast cells, in the vicinity of cancer cells. Among various signaling pathways, mitogen-activated protein kinases (MAPKs) pathways are important in the regulation of MMP induction as most of MMP promoters contain AP-1 and NFκB-binding sites, the downstream target of MAPK pathways. NFκB and AP-1 activity are significantly enhanced during cancer progression. JNK pathway induces MMP expression through activation as well as nuclear translocation of multiple transcription factors such as Jun D, ATF and most of the MMP promoter contain putative-binding sites for these DNA-binding proteins[72]. It is now conceivable that the function of MMPs is not only confined to invasion and metastasis steps of cancer but they also facilitate initial phases of cancer development. In cancer, special emphasis has been placed on the degradation of type IV collagen, a major protein component of basement membranes that can be cleaved

by MMP-2 and -9. Disease progression in experimental animal models of cancer invasion and metastasis correlate with enhanced secretion of specific MMPs by tumor cells and/or stromal cells. Specific MMPs appear to have different functions depending on the stage of cancer and tissue type.

MMPs regulate apoptosis
MMPs especially, MMP-3, -7, -9 and -11 regulate apoptosis by degrading matrix protein. MMPs have both apoptotic and anti-apoptotic actions on endothelial and epithelial cells by cleaving adhesion molecule, *e.g.*, VE-cadherin[73], PECAM-1[74] and E-cadherin[75]. Detachment of adhesion molecules from the membrane is prerequisite for apoptosis to occur. Degradation of laminin by MMP-3 is another example of enhanced apoptosis in mammary epithelial cells possibly by degrading laminin[76]. MMP-7 releases the Fas ligand from the membrane which then induces apoptosis of neighboring cells, or decreases cancer-cell apoptosis, depending on the system[77]. MMPs might also negatively regulate cancer-cell growth, by means of activation of TGF-β or generation of pro-apoptotic molecules such as Fas ligand or TNF-α. By producing heparin-binding epidermal growth factor (HBEGF) from the latent form, *i.e.*, pro-HBEGF, MMP-7 promotes cell survival which is opposes the apoptosis *via* tyrosine kinase-mediated pathway. Moreover, MMP-11 also inhibits cancer cell apoptosis, as indicated by Wu *et al*[78], who showed that over expression of MMP-11 decreases spontaneous apoptosis in tumor xenografts. In contrast, MMP11-null mice show a higher rate of apoptosis compared to wild-type when challenged with cancer cells. MMP-11 inhibits apoptosis by the mechanism of releasing IGFs, known to can act as survival factors[79]. Although MMP-9 and -11 decrease cancer cell apoptosis, they increase apoptosis during development[78,80].

MMPs regulate angiogenesis
Angiogenesis, the formation of new capillaries from pre-existing vessels, is associated with several physiological processes as well as pathological conditions. Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells and is required for tumor-induced angiogenesis. The activation of MMPs is governed by VEGF and then activated MMPs degrade collagen and ECM proteins of basement membrane thereby aiding in the migration of endothelial cells. Cleavage of collagen type I allow endothelial cells to invade the tumor stroma during vessel formation and MMP promote the process by degrading collagen[81]. MMP-2, -9 and -14 directly regulate angiogenesis, and MMP-19 might also be important as it is expressed in blood vessel[82]. Furthermore, reduced MMP-2 expression resulted in decreased angiogenesis in cancer cells in chicken chorioallantoic membrane model. Tumor angiogenesis is significantly inhibited in mice deficient in MMP-2 in comparison with wild type mice[83]. Cleavage of collagen type IV by MMP-2 exposes a cryptic, αvβ3 integrin binding site within the collagen that
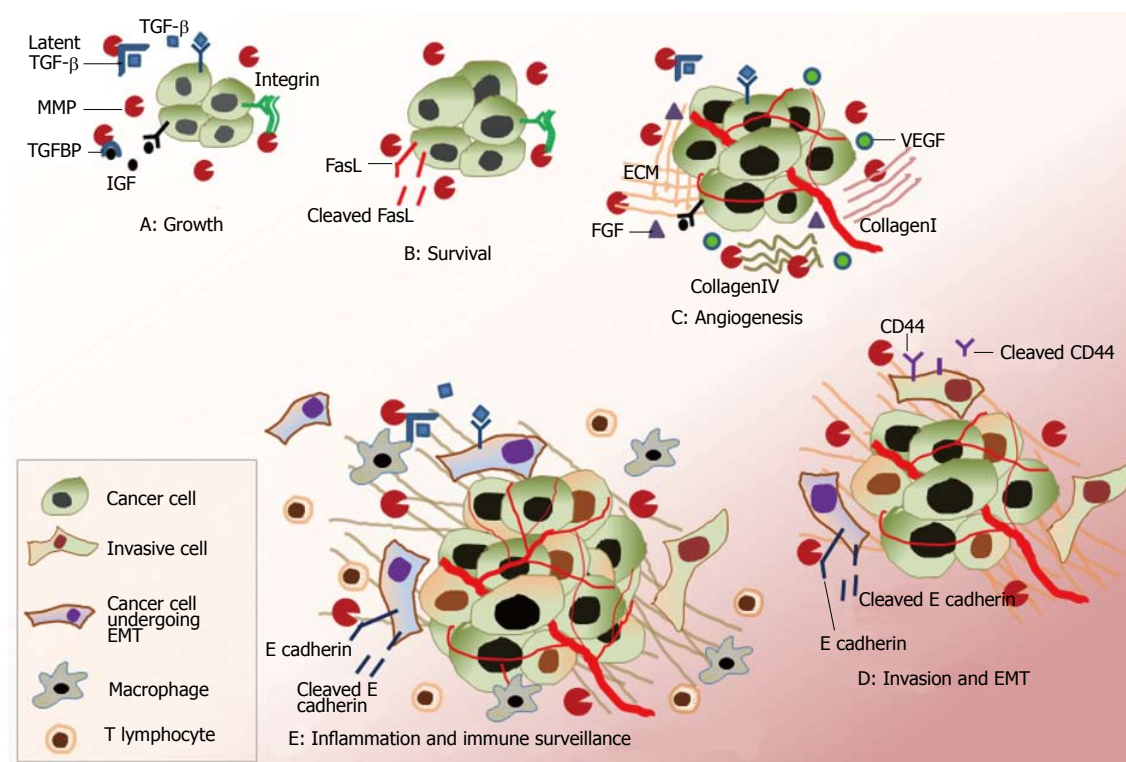
Figure 2 Roles of matrix metalloproteinases in cancer progression. The matrix metalloproteinases (MMPs) play complex but important roles during different stages of cancer progression. A and B: Growth and survival. MMP modulates cellular growth by cleaving different cellular components. It promotes cellular growth by releasing IGF from insulin growth factor-binding protein (IGF-BP). MMP-7 promotes cell survival by resisting apoptosis through cleaving Fas ligand (FasL). MMP modulates integrin signalling by regulating the extracellular matrix (ECM), which regulates growth. MMP activates transforming growth factor-β (TGF-β) from its latent TGF-β complex, which plays important roles in tumour development; C: Angiogenesis. MMP promotes angiogenesis through recruitment of VEGF, FGF. Angiogenesis is further promoted by degradation of extracellular component like collagen I, IV, fibrin, *etc*., which also act as pro-angiogenic factors; D: Invasion and epithelial to mesenchymal transition (EMT). MMP modulates invasion by degrading specific cellular components, including E-cadherin and CD-44. MMP is involved in mesenchymal transition through cleavage of E-cadherin and modulating TGF-β signaling. MMP-3 is directly involved in EMT, whereas MMP-9 has roles in differentiation; E: Inflammation and immune surveillance. MMPs also regulate immune reactions against the cancer cells. MMP-mediated TGF-β activation inhibits T lymphocyte proliferation. MMPs also modulate cancer-cell sensitivity to natural killer cells and leukocyte accumulation by cleaving different chemokines and cytokine families. VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factors.

helps in migration of endothelial cells both *in vitro* and *in vivo* model[84].

MMP-9 is a key player for pathological angiogenesis as revealed by experiments done in transgenic models of tumour progression, in the K14-HPV16 skin cancer model[85]. In contrast, the angiogenesis process is unaffected by MMP-2 in skin cancer. Both MMP-14 and MMP-9 null mice have impaired angiogenesis during development[86]. Cleavage of plasminogen by MMP-2, -3, -7, -9 and -12 generates angiostatin[87], and MMP-3, -9, -12, -13 and -20 are involved in the generation of endostatin, a C-terminal fragment of the basement membrane collagen type XVIII[45,88]. Both angiostatin and endostatin reduce endothelial cell proliferation[46]; endostatin also inhibits endothelial cell invasion by acting as an inhibitor of MMP-14 and -2[89]. By degrading fibrin matrix of blood vessels, MMP-14 promotes cell invasion and thus increase angiogenesis. In contrast, MMP-12 inhibits tumor angiogenesis by inhibiting endothelial cell invasion *via* a different pathway that mediated by urokinase-type plasminogen activator receptor.

MMPs regulate invasion and metastasis

One of the foremost and major steps in invasion is migration of cancer cells from the site of origin to the docking site. The cleavage of ECM is essential for detachment of cancerous cells from neighboring. Cleavage of laminin-5 by MMP-2 and -14 generates a cryptic site that facilitates cell migration[90]. This is supported by the fact of colocalization of laminin-5 and MMP-14 in human cancer specimen and abundance of degraded laminin in tumor tissues[91]. In addition, the main receptor for hyaluronan, CD44 is cleaved by MMP-14, thus, extracellular domain is released, thereby facilitating tissue invasion (Figure 2). Moreover, cell migration is hampered when the cleavage site is mutated[92]. In addition to binding to the ECM, CD44 also binds MMP-9, thereby localizing the enzyme to the cell surface that promotes tumor invasion and angiogenesis, as confirmed by overexpression of the extracellular domain of CD44 and suppression of tumor invasiveness[93]. Thus, cancer cell migration is regulated by cycles of MMP activity or localized MMP activity, not by continuously high activity. During metas-

tasis, cancer cells first cross the epithelial basement membrane and invade the surrounding stroma, followed by invasion to blood or lymphatic vessels; then extravasate to new tissues and establish growth of new proliferating cells in new tissues. The role of MMPs in metastasis was evidenced by *in vitro* invasion assays and *in vivo* xenograft metastasis assays. Overexpression of MMP-2, -3, -13 and -14 promotes invasion as documented through either optic nerve explants or cell culture in matrigel[94-96]. In addition, metastasis is reduced in the MMP-2 and MMP-9 null mice as compared with wild-type mice. There is no linear relationship between MMP-2 expression and cell invasion, rather cells expressing intermediate levels of active MMP-2 are the most invasive. The localization of specific MMPs to specialized surfaces on the cell membrane is necessary for their ability to promote invasion. Endothelial cell-adhesion molecule E-cadherin is associated with cancer progression, as it is cleaved by MMP-3 or -7[97]. The released fragment of E-cadherin promotes tumor cell invasion in a paracrine manner *in vitro*[98,99]. MMP-2, -9 and -14 are known to localize to invadopodia. Moreover, MMP-2 is recruited to invadopodia by either binding to $\alpha5\beta3$ integrin[100] or by binding to MMP-14. MMP-14 is recruited to invadopodia by means of its transmembrane and cytoplasmic domains. Overexpression of MMP-14 increases the number of cancer cells in an experimental metastasis assay. Furthermore, the docking of cancer cells at the secondary site, the late events in the metastatic process also involves MMP activity.

MMPs and the immune responses to cancer

Inflammatory reactions by tumour-specific cytotoxic T lymphocytes, natural killer cells, neutrophils and macrophages are a key mechanism of cellular carcinoma[101]. The immune system is capable of recognizing and attacking cancer cells, while cancer cells somehow escape immune surveillance. MMPs are involved in the escape mechanisms. Although the immune response helps to delay tumour progression, chronic inflammation is also associated with various cancers including cancers those of gastric mucosa, large bowel, and liver[101]. In animal models, mast cells, neutrophils and macrophages are contributors to the progression of cancer. Inflammatory cells synthesize several MMPs, including -9, -12 and -14 and stimulate cancer progression. Indeed, MMP-9 null mice are less prone to skin cancer[85]. Especially, MMP-9 can cleave interleukin-2 receptor (IL-2R)-α and thereby suppress the proliferation of the T lymphocytes[102]. MMP-9 also can act on IL-8, and, thus increases the activity by several folds. MMP-2 cleaves the monocyte chemoattractant protein-3, and the cleaved fragment not only is inactivated but also becomes an antagonist to the receptors[103]. Furthermore, CXCL12 (also known as stromal-cell-derived factor 1) is cleaved and inactivated by MMP-1, -3, -9, -13 and -14[104]. CXCL12 is a ligand for the CXC chemokine receptor 4 (CXCR4) on leukocytes. Inhibition of the binding of CXCL12 to CXCR4 greatly reduces metastasis to lung and lymph nodes

in breast cancer[105]. MMP-11 acts on $\alpha1$-proteinase-inhibitor and the cleaved product altered sensitivity of tumor cells towards natural killer cells[106]. Moreover, few MMPs also activate TGF-β an important inhibitor of the T-lymphocyte response against tumors[107]. MMPs play indirect roles in proliferation of cancer cells by acting on growth factors that entangled into ECM (Figure 3). First, membrane-bound precursors of some growth factors, *e.g.*, TGF-β, are released by MMPs or ADAMs[107]. Second, degradation of growth factors by MMPs makes them available in pericellular space, *e.g.*, MMPs can cleave IGF-BP to release IGF[108]. Finally, cell proliferation by growth factors occurs through integrin signaling.

MAJOR IMPACT OF OXIDATIVE STRESS ON GASTROINTESTINAL DISEASES

For many years, researchers have recognized ROS only as causative factor in pathological processes, although the opinion has now changed. ROS were shown to meet the criteria for signalling molecules and they have significant roles in biological functions. This section deals with involvement of ROS in physiological and pathological processes and, subsequent responses in cancer cells under oxidative stress.

Generation of ROS in biological systems

ROS are often byproducts of mitochondrial function that are constantly generated and eliminated from physiological systems. ROS are oxygen containing reactive species, that may contain an unpaired electron, *e.g.*, superoxide radical ($O_2 \cdot$), hydroxyl radical ($OH \cdot$) and/or non-radical molecules, such as hydrogen peroxide (H_2O_2). $O_2 \cdot$ are formed by chemical reduction of molecular oxygen, by electrons that escape from complex I and III of electron transport chain and $O_2 \cdot$ is than dismutated to H_2O_2. Nearly 2% of molecular oxygen consumed during mitochondrial respiration ends up as $O_2 \cdot$[109,110]. Apart from mitochondria, ROS can be generated from a family of trans-membrane proteins, known as NADPH oxidases (Nox). These enzymes catalyse NADPH dependent reduction of molecular oxygen to $O_2 \cdot$[111].

Increased ROS generation can induce lipid peroxidation and protein oxidation, hampering normal cellular processes. In addition, ROS can target mitochondrial DNA (mDNA) more effectively due to its proximity and lack of protective histones and limited repair mechanism. mDNA does not contain any introns and encodes for 13 respiratory complexes essential for electron transport chain and oxidative phosphorylation. Mutation of mDNA results in aberrant mitochondrial proteins during ATP generation; this induces further ROS production. Moreover, ROS cause nuclear DNA damage through DNA oxidation (by formation of 8-oxo-G, 8-oxo-dG), which contributes to mutation[109].

Cancer cells are known to be metabolically active and require elevated amount of energy to support their functionality. To meet their ATP need, cancer cells exhibit an increased dependency to glycolysis which slows down
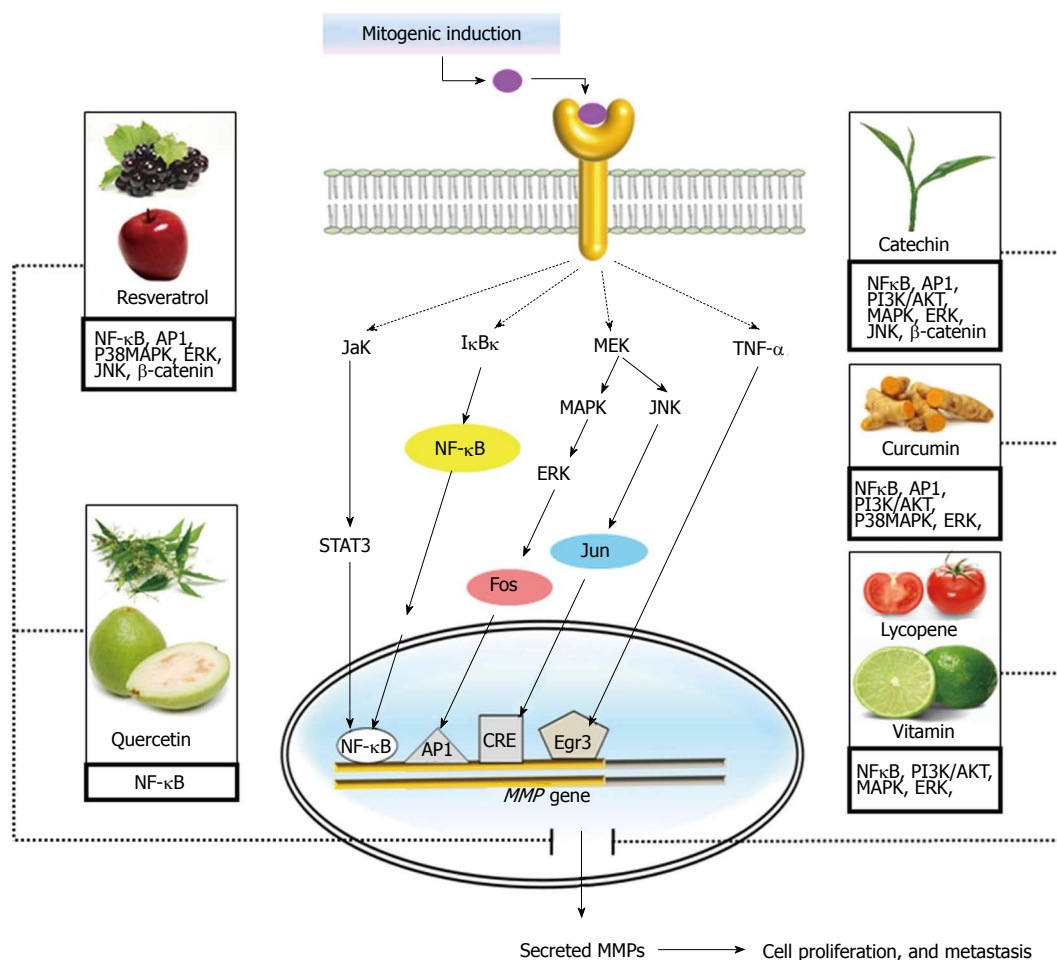
Figure 3 Role of dietary antioxidants in modulating matrix metalloproteinases action. Different ingredients in diet possess antioxidant activity. They act on various signalling pathways and transcription factors which modulate the synthesis and secretion of matrix metalloproteinases (MMPs). Therefore regulates MMP-dependent cellular processes, including proliferation and metastasis. TNF: Tumor necrosis factor; MAPK: Mitogen-activated protein kinases; MEK: Methyl ethyl ketone.

mitochondrial energy production. This phenomenon of stimulation of glycolysis in cancer cells that inhibit mitochondrial respiration, is known as "Crabtree effect"[112]. However, inhibition of glycolysis in tumor cells may result impairment of mitochondrial oxidative phosphorylation and subsequently associated with hypoxia and higher amounts of ROS generation and accumulation of ROS-mediated reaction products[113].

Oxidative stress and gastrointestinal diseases

Oxidative stress plays important roles in pathogenesis of gastro-intestinal diseases, which include mucosal damage, gastro-intestinal ulcers, and cancer. Although gastric ulcer can be generated by different factors, *e.g.*, non-steroidal anti-inflammatory drugs (NSAIDs), thermal stress, ethanol, and *H. pylori* infection, leading to oxidative damage through free radical generation (specially OH) and subsequent apoptotic responses of gastric mucosa[114]. In addition, gastro-intestinal diseases are associated to increased oxidative stress and oxidants levels, such as glutathione, lipid peroxidation, myeloperoxidases, protein carbonyl, *etc*[115]. Furthermore, pathogens are directly involved in aggravating oxidative stress; for

example, complete eradication of *H. pylori* is reported to attenuate oxidative stress in gastric mucosa[116].

Although certain types of gastro-intestinal inflammations, like ulcerative colitis, hepatitis, *H. pylori* infection, are more prone to develop cancer, the reasons are still not well elucidated. Inflammation and subsequent elevated oxidative stress might be the factors for aggravating chronic inflammation and inducing malignant transformation. Transgenic mice expressing hepatitis B protein in liver develop chronic hepatitis with elevated levels of 8-oxo-dG, leading to hepatocellular carcinoma[117]. It is well accepted that inflammation is always accompanied with elevated oxidative stress in cancer. Gastric cancer patients (with normal renal and hepatic functions) are found to have significantly increased lipid peroxidation levels[118]. Gastric carcinoma patients have significantly higher myeloperoxidase activity than controls, both before and after operation, although total antioxidant status was decreased post-operation[119]. Gastric cancers are also associated with augmented protein oxidation, although no differences are found in oxidative stress parameters and antioxidant enzyme activities between anti-*H. pylori* IgG positive and negative gastric cancer patients[120].

Cellular responses in cancer cells under oxidative stress

ROS, such as O_2^- and H_2O_2, have roles in proliferation and cellular growth that contribute to the development of cancer. Cancer cells also exhibit different cellular responses under oxidative stress, which include senescence, autophagy and apoptotic responses; although it is still not well understood, how these cellular pathways assume priority to become activated under particular conditions. H_2O_2 can directly modulate autophagic responses during nutritional starvation through Atg4 (autophagin-1) expression and accumulation of LC3-PE on the autophagosomal membrane[121]. In addition, ROS-induced autophagy is dependent on constitutive expression of Atg, baclin, hypoxia induced factor-1 (HIF-1) and BNIP3[122]. Mutations in *ATG* genes (like *ATG2B, ATG5, ATG9B, and ATG12*) are involved in gastric and colorectal carcinomas and may contribute to cancer development by deregulating the autophagy process. Moreover, increased autophagy (through upregulation of Atg5 and Atg7) is also involved in *in vitro* malignant transformation by K-Ras[123]. ROS and ROS-mediated signalling pathways are also involved in senescence responses. Overexpression of p21, along with increased ROS production, directly induces the senescence phenotype; while inhibition of p21-mediated ROS accumulation can rescue cells from senescence[124]. In addition, sub-lethal doses of H_2O_2 can induce senescence-like growth arrest at the G1 stage *via* up regulation of p53 and p21. Anti-apoptotic Bcl-2 family members antagonise ROS generation during apoptosis. Moreover, ROS generation is accompanied by cellular apoptosis due to lipid peroxidation and mitochondrial depolarization.

Increased ROS production in cancer cells is associated with constant activation of transcription factors including NFκB and AP-1. Moreover, a recent study found that activation of TLR4 promotes gastric cancer by increasing mitochondrial ROS generation through NFκB activation[125]. Oncogenic signals are involved in ROS generation that promote metastasis in gastric cancer[126]. The oncogene c-myc has been reported to develop genetic instability, DNA damage and mitigation of p53 function through ROS production[127]. RAS2 mutation promotes oxidative stress by restricting mitochondrial respiration into non-phosphorylating state. K-Ras is also involved in malignant transformation through ROS mediated JNK activation[123]. ROS and Rac1b are involved in MMP-3 mediated epithelial to mesenchymal transition (EMT). ROS also stimulate the expression of Snail and cause damage to DNA and subsequent genomic instability[128]. Reports have suggested a possible association of Nox and Nox-mediated ROS generation for carcinogenesis[111]. Nox1 is involved in *H. pylori*-induced gastric cancer and in angiogenic responses for tumour formation[129,130]. Nox2 is involved in phagocytosis; Nox4 controls cell survival in different cancers, including gastric, colon and pancreatic cancers[111,131]. Increased ROS can cause detachment of JNK associated glutathione-S-transferase (GST)-π

and thus facilitates JNK activation. In addition, H_2O_2 mediated JNK activation also causes downregulation of JNK phosphatases[132]. Mice that have an inactive c-Jun that lacks JNK phosphorylation site or deficient in JNK displayed reduced tumor development[133,134]. Moreover, increased ROS productions in oncogenically transformed cells potentiate JNK and p38 MAP kinases activation[135].

Role of endogenous antioxidants during oxidative injury

Endogenous antioxidants are essential for maintenance and neutralization of perturbed oxidative free radical status. Superoxide dismutases (SOD) are important antioxidants, as Mn-dependent SOD (Mn-SOD) null mice cannot survive after birth[136]. Heterozygous Mn-SOD null mice can survive, however, show higher incidences of lymphomas and adenocarcinomas[137]. In addition, Zn-dependent SOD (Zn-SOD) knockdown mice develop hepatic cancer in late stages of life[138]. Mice lacking catalase also exhibit elevated cancer incidences[139]. Glutathione peroxidases (GPx) also have significant roles as antioxidants. Simultaneous knock out of GPx-1 and -2 in mice leads to gastrointestinal cancer[140].

Clinical publications have reported different levels of endogenous antioxidants in gastrointestinal cancers. GST, GPx and SOD activities are reported to be significantly elevated in colorectal cancers, than adjacent normal tissues[141]. Stomach adenocarcinoma and esophageal squamous cell carcinoma show significantly increased Mn-SOD expression as compared to noncancerous cells[142]. In clinical studies with gastric and colorectal cancer, GPx, SOD, glucose-6-phosphate dehydrogenase (G6PD), malonaldehyde and glutathione reductase were found elevated in the malignant phenotype[143]. However, because of the sustained oxidative stress conditions, these antioxidants are insufficient in cancer, eventually resulting in decreased antioxidant levels in several cancers[144]. Moreover, modulated expressions of Mn-SOD are reported due to mutations in the promoter region, abnormal methylation, loss of heterozygosity or mutation in the coding sequences[145]. These differential results of cellular antioxidant SOD cause metabolic chaos, due to different types and grades of malignancy[146].

DIETARY ANTIOXIDANTS AS A MODULATOR OF MMPs IN GASTROINTESTINAL CANCER

Human diet contains a mixture of oxidants and antioxidants substances. Dietary and endogenous antioxidants are important for cellular protection by reacting and/or eliminating oxidizing free radicals. The question of whether antioxidant supplements might protect against cancer has drawn much attention since the mid '80s and different antioxidants were extensively studied thereafter, although the results of the investigations are mixed and contradictory. Antioxidant and endogeneous redox enzymes act as the first-line defense against ROS in all

cellular compartments and also extracellularly. The most important of these enzymes include SOD, GPx, catalase and peroxiredoxins. The specific role of above enzymes in carcinogenesis is still unambiguous since their roles in ROS detoxification are well known. It is noteworthy that we do not yet fully understand the chemopreventive role of phytochemicals as antioxidants, or as modulators of other processes related to carcinogenesis. In this section, we highlight the effects of dietary antioxidants in prevention of cancers with particular emphasis on the regulation of MMPs activity.

Tea polyphenol and catechin

Tea, derived from the plant *Camellia sinensis*, is the most consumed beverage worldwide and it is grown in over 30 countries around the world, exclusively in the subtropical and tropical zones[147]. It is processed in different ways in different parts of the world to produce green, black, or Oolong tea. Both green and black teas have been studied for their health benefits, particularly for prevention and treatment of inflammatory diseases as well as cancer. Green tea is rich in polyphenolic substances, which include flavonoid, flavanols, and flavinidols all of which have antioxidant properties. The most common flavonal in tea is catechin. The most active and abundant catechin in green tea is epigallactocatechin-3-gallate (EGCG) that has been shown to inhibit cancer cell growth *in vitro* and *in vivo*. In black tea the major polyphenols are theaflavin and thearubigin.

During the last decade several epidemiological studies have linked tea consumption, especially green tea to a reduced risk of cancer in humans. Morse *et al*[148] documented the beneficial effects of the polyphenol fractions of green tea, the polyphenol fractions of black tea, *i.e.*, EGCG and theaflavins against N-nitrosomethylbenzylamine (NMBA)-induce esophageal cancer in rat. Wang *et al*[149] investigated both protective and therapeutic effects of green tea and black tea extract on esophageal tumorigenesis in rats. A population-based case-control study in Shanghai indicated that tea consumption was strongly associated with reduced colorectal cancer incident. Green tea polyphenol supplementation during the initiation or postinitiation period significantly lowered azoxymethane-induced tumor incidence in rats[150]. In addition, catechin and EGCG reduced colon tumor incidence in a 1, 2-dimethylhydrazine (DMH)-induced intestinal cancer.

Green tea polyphones were shown to prevent cancer cell proliferation and invasion. EGCG has been shown to inhibit NFκB activity in human colon cancer cells[151]. Several studies indicate that chemopreventive properties of EGCG can also mediated by inhibition of MMP induction. EGCG inhibited the PMA-induced cell invasiveness and MMP-9 expression in human gastric cancer adenocarcinoma (AGS) cells[152]. Fassina *et al*[152] documented that EGCG (25-100 µmol/L) inhibits the MMP-2 and -9 in endothelial cells. EGCG inhibited the activity and expression of MT1MMP, a protein responsible for the activation of MMP-2 as examined by Annabi *et al*[153]. Onoda

et al[154] found that gastric cancer cell lines, *e.g.*, MKN-1, MKN-28, MKN-45, NUGC-3 and TMK-1 are sensitive to EGCG treatment with NUGC-3 being the most sensitive. Furthermore, EGCG suppresses Met signaling in HCT116 human colon cancer cells[155]. In another study, EGCG may exert at least part of its anticancer effect by inhibiting angiogenesis through blocking the induction of VEGF and binding to its receptors. EGCG has been shown to affect MMP-2 and -9 activities both directly and indirectly in endothelial cells thereby inhibiting or delaying cancer invasion and metastasis. Concanavalin A-induced activation of MMP-2 and activity of MT1-MMP has been reduced by EGCG[156]. Catechin, another major component of tea, prevents vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression[157]. Black tea polyphenols inhibit DMH-induced colorectal cancer by inhibiting MMP-7 induction *via* Wnt/β-catenin pathway[158]. Hwang *et al*[159] showed the apoptotic effect of EGCG in HT-29 colon cancer cells that was mediated by AMPK signaling. Hence, catechin and EGCG exert strong anticancer activity by targeting transcription factors like NFκB, and AP-1 which involve in the regulation of mainly MMP-2, -9 and -7 activities. Specifically, EGCG regulates angiogenesis and apoptosis *via* changing expression of VEGF, uPA, IGF-1, EGFR, cell cycle regulatory proteins and in turn affects NFκB, PI3-K/Akt, ERK, JNK, Ras/Raf/MAPK and AP-1 signaling pathways, thereby acting as chemopreventive agent[160].

Curcumin

Curcuma longa (Zingiberaceae family) rhizomes have been traditionally used in the south Asian countries for the treatment of a variety of inflammatory conditions and different diseases including carcinomas[161]. The pharmacological properties of curcumin are attributed mainly to the curcumin (diferuloylmethane)-(1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione) a hydrophobic polyphenol present in the rhizome. Curcumin is a potent antioxidant that acts as a free radical scavenger[162]. Curcumin possesses a wide range of pharmacological activities including anti-inflammatory, chemo-preventive, and antimicrobial and wound healing effects[162-164]. Curcumin which is also known as turmeric, has been shown to exhibit dose dependent chemo-preventive effects in several gastrointestinal cancers including colon, duodenal, stomach, esophageal and oral carcinogenesis[165,166]. *In vivo* and *in vitro* studies have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth[167]. These protective effects of curcumin are attributed mainly to its antioxidant properties and investigated for the purpose of developing novel drugs. It reduces carcinogen-induced tumorigenesis in the fore-stomach and N-ethyl-N'-nitro-nitrosoguanidine-induced duodenal tumors. Lower incidences of bowel cancer in Indians, possibly due to the use of turmeric during food preparation. The molecular basis of anti-carcinogenic and chemopreventive effects

of curcumin is targeted to transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signaling molecules[166].

Koo *et al*[168] showed that curcumin and 5-fluorouracil (5-FU) additively inhibited the growth of gastric carcinoma cells. In another study, curcumin reversed the multidrug resistance of a human gastric carcinoma cell line[169]. Curcumin exhibited both preventive and therapeutic effects on the incidence and multiplicity of fore-stomach tumors induced by benzopyrene in mice[170]. A dietary supplementation of 0.15% curcumin reduces intestinal tumor formation in Min$^{-/-}$ mice by 63%. Curcumin induces apoptosis and prevents adenoma development in the intestinal tract in mice[171]. Tetrahydrocurcumin (THC) significantly decreases DMH-induced colon carcinogenesis[172]. Shpitz *et al*[173] showed that curcumin and celecoxib synergistically inhibit colorectal cancer progression in DMH-induced rat model. Several studies documented the inhibitory effects of curcumin on AOM-induced colon cancer[174]. Curcumin inhibits the expression of MMP-9 both *in vitro* and *in vivo* and thereby inhibits tumor invasion and metastasis. Bimonte *et al*[175] reported that curcumin inhibits the expression of MMP-9 in orthotopically implanted pancreatic tumors. Curcumin causes a significant reduction of tumor volume, and MMP-9 activity in a xenografted model[176]. Curcumin also reduces the expression of major MMPs *via* reduced NFκB activity and AP-1 transcription[176]. Lin *et al*[177] reported that curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion *via* suppression of MMP-9 secretion. Curcumin prevents human colon cancer, colo-205 cells migration through the inhibition of NFκB/p65 and downregulation of cyclooxygenase-2 and MMP-2 expression[178]. Lin *et al*[179] reported that curcumin inhibits SDF-1α-induced invasion of human esophageal carcinoma cells by down regulating MMP-2 promoter activity as well as suppressing the formation of lipid raft-associated Rac1/PI3K/Akt signaling complexes. In conclusion, curcumin appears to have a significant potential in the treatment of multiple diseases that are due to oxidative stress. Thus, various inflammatory pathways ultimately act on MMP transcription or expression during prevention of various types of cancer by curcumin.

Resveratrol

Resveratrol (trans-3,4',5-trihydroxystilbene), a non-flavonoid polyphenolic antioxidant, has attracted considerable attention due to its anti-oxidant, anti-cancer, and anti-inflammatory properties. It is in abundance in grapes and grape products such as wine, moderately abundant in blueberries, and sparsely abundant in other plants. Resveratrol is a scavenger of hydroxyl, superoxide, and other radicals and thus acts as a potent antioxidant. It protects against ROS-mediated lipid peroxidation in cell membranes and DNA damage. Resveratrol enhances the expression and/or the activity of drug metabolizing phase I/II enzymes such as Mn-SOD, GST, cytochrome P450 reductase, quinine oxidoreductase, NAD(P)H: quinone

oxidoreductase (NQO1), quinone reductase (QR), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL); thereby protecting against oxidative DNA damages[180]. Several studies demonstrated that resveratrol exhibits strong chemopreventive effects in various experimentally induced tumor models as well as inhibits proliferation and induces apoptosis of various cancerous or transformed cells. Oral or intra-peritoneal administration of resveratrol decreases expressions of COX-1, COX-2, and PGE2 reducing the number and size of esophageal tumors in N-nitrosomethylbenzylamine-induce rat tumor model[181]. Similarly, oral administration of resveratrol limits the formation of aberrant crypt foci and tumors in the colon of rats that are treated with chemical carcinogen, *i.e.*, 1,2-dimethylhydrazine (DMH) or azoxymethane. Resveratrol prevented the formation of colon tumors and reduced the formation of small intestinal tumors by 70% in APC$^{-/+}$ mice[182]. Resveratrol inhibits MMP-9 expression and invasion of human hepatocellular carcinoma cells. Resveratrol modulates all three MAP kinases namely ERK1/2, JNK, and p38 MAPK. Resveratrol impairs the expression of EMT-related genes (E-cadherin, N-cadherin, vimentin, MMP-2, and -9) in pancreatic cancer cells and inhibits proliferation, migration, and invasion[183]. Ji *et al*[184] demonstrated that resveratrol possesses chemopreventive effects in HCT116 CRC through inhibition of MMP-7 *via* Wnt/β-catenin signalling pathway. Weng *et al*[185] suggested that resveratrol and its related methoxy analogue MR-3 might exert anti-invasive activity against hepatoma cells through regulation of MMP-2 and -9 as well as TIMPs. Harikumar *et al*[186] reported that resveratrol can enhance chemopreventive activity of gemcitabine *in vitro* and *in vivo* mouse model of pancreatic cancer. In summary, anticancer activity of resveratrol is augmented by upregulation of TIMP and downregulation of MMP-9 expression.

Quercetin

Quercetin or 3,5,7,3',4'-tetrahydroxyflavone is a flavonoid found abundantly in plant-derived foods and has been shown to possess several health beneficial activities including anti-tumor, anti-inflammation and anti-proliferation; it has recently gained attention due to its potential anticancer activity. As an antioxidant it possesses the most potent ROS scavenging activity and provides protection against the development of variety of cancers by ameliorating ROS-mediated cellular damages[187]. Moreover, it reduces the level of oxidative enzymes, such as xanthine oxidase (XOD), lipoxygenase and NADPH oxidase, thereby preventing free radical-induced cellular damage[187]. *In vivo* studies have been performed to depict the chemopreventive properties of quercetin on different cancers. Volate *et al*[188] found that food supplementation of approximately 3% quercetin exerts significantly beneficial effects by decreasing precancerous lesions through induction of cellular. Dihal *et al*[189] reported that quercetin inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. Quercetin was found to be protective

against hepatocarcinoma that was generated by N-nitros-odiethylamine and, was accompanied by the maintenance of a correct intracellular oxidant/antioxidant status. Quercetin prevents 4-nitroquinoline 1-oxide-induced oral carcinogenesis during the initiation/post initiation phases of carcinogen treatment. A limited number of experiments were conducted to investigate the effects of quercetin in the regulation of MMP activity in gastrointestinal cancer. It has been found that quercetin supplementation did not alter MMP-2 or TIMP-2 gene transcription or plasma protein levels but TIMP-1 gene expression and plasma protein levels decreased significantly. Recently, Lai *et al*[190] reported that migration and invasion of SAS human oral cancer was inhibited by quercetin *via* downregulation of MMP-2 and -9 in a NFκB dependent pathway. They also showed significant reduction of the MMP-7, -10 protein levels by quercetin treatment.

Other promising dietary antioxidants

The roles of few more dietary antioxidants *e.g.*, melatonin, lycopene, retinoic acid, vitamin C and vitamin E in prevention of cancer through regulation of MMPs are discussed below. Melatonin is a naturally occurring antioxidant synthesized mainly by the pineal gland of vertebrates and also found in many edible plant products[191]. Recent research documents that consumption of tropical fruit enhances the serum melatonin level as well as raises the antioxidant capacity of blood serum[191,192]. Melatonin retards the development of cancer in different animal models and possesses strong anti-proliferative and pro-apoptotic effects in various cancer cells. Sharman *et al*[193] reported that long term exposure to dietary melatonin reduces tumor number and size in aged male mice. In addition, melatonin inhibits the growth of murine gastric carcinoma cells by upregulation of p21, Bax and down regulation of Bcl-2. Decreased expressions of melatonin receptor in various cancers also suggest the importance of melatonin signaling in cancer development[194]. Hong *et al*[195] reported that melatonin treatment induces apoptosis, autophagy, and senescence in human colorectal cancer cells. A few report suggested that melatonin prevents gastrointestinal cancer development by modulation of MMP functions. Melatonin induces apoptosis and reduces invasiveness of HepG2 cells *in vitro* through TIMP-1 upregulation and attenuation of MMP-9 activity *via* NFκB signal pathway[196]. Rudra *et al*[192] reported that melatonin reduces MMP-9 activity in AGS cell line and binds directly to its active site.

Two separate studies reported the chemo-preventive effects of lycopene in human colon cancer. Tang *et al*[197] evaluated the chemo-preventive effects of lycopene and fish oil in a mouse xenograft model of colon cancer. They found that inhibition of tumor growth and progression by the augmenting p21 (CIP1/WAF1) and p27 (Kip1) expression, and suppression of MMP-7, MMP-9, COX-2 and PGE2, PCNA, β-catenin, cyclin D1 and c-Myc proteins[197]. *In vitro* study by Lin *et al*[198] suggested the inhibitory effects of lycopene on tumor progression,

where cell invasion was inhibited by down regulation of MMP-7 expression *via* blocking MAPK/ERK and PI3K/Akt signaling pathways. Adachi *et al*[199] reported that naturally occurring retinoid, all-trans retinoic acid (ATRA), 9-cis retinoic acid and 13-cis retinoic acid are helpful in the prevention and therapy of colon cancer. ATRA prevents tumor invasion in mice and inhibits *in vitro* invasion of colon cancer cells by down regulation of MMP-7 expression. Moreover, Park *et al*[200] reported that retinol reduces the invasive potential of retinoic acid resistance colon cancer cells by decreasing MMP-1,-2,-7,-9 expressions and activity. Retinol reduces the metastatic potential of colon cancer cells *via* down regulation of MMP induction in a retinoic acid receptor-independent mechanism. β-ionone, the derivative product of carotenoids, is the precursor of vitamin A also possesses anti-proliferative activity in cancer cells[201]. Liu *et al*[202] found that that γ-tocotrienol inhibit gastric cancer cell (SGC-7901) proliferation by reducing MMP-1 and -2 activity *via* modulating the expression of their inhibitor TIMP-1 and TIMP-2. Dietary supplementation of naturally occurring antioxidants, ascorbic acid reduces the size of colon xenograft cancer by downregulation of MMP-9 and VEGF in nude mice[203]. Vitamin E (γ-tocotrienol) effectively inhibits the growth of human gastric cancer in a xenograft mouse model. γ-tocotrienol inhibited the proliferation of gastric cancer cell lines, *via* inhibiting the NFκB mediated upregulation of MMP-9 and VEGF[204].

CONCLUSION

The immense complexity of cancer disease is not yet fully characterized despite numerous advances in modern molecular biology. Complexity in cancer cells arise from heterogeneity of tumor microenvironment, inflammatory stimuli, immune responses, diet effects as well as intestinal micobiota. All these factors determine whether the fate of cancer cells undergo apoptosis or proliferation or even develop resistance to drugs. Cancer is a multifactorial disease, which varies from patient to patient. Even the complexity lies in a certain tumor cells that may refer to tumors with diverse genetics. In fact, every cancer types is unique. Hence, intramolecular heterogeneity poses another dimension during cancer progression. Significant intra-tumor heterogeneity is present in many patients, thus drug resistance may develop. Sequencing technology is used to monitor clonal dynamics of cancer cells. In this context, careful attention should be given to detect minor clones of clinical significance. Tumor heterogenity was documented by the Cancer Genome Atlas and the Cancer Genome Analysis projects.

In the future, personalised cancer medicine may be possible by accounting for both interpatient and intrapatient heterogeneity. Additionally, new therapeutic strategies are important for targeting cellular conditions like cellular senescence rather than targeting a particular biomolecule. Given the complexity of cancer, it is unlikely universal therapeutic strategy will be employed

for different cancer types and stages. The MMP family of enzymes occupies a major importance in the field of gastric cancer research. Literature in last two decades of MMP biochemistry and cancer biology supports the possibility of particular MMP in particular types of cancer, including gastrointestinal cancer. Both basic and applied research is needed to decipher the mechanisms of cancer progression and regulatory roles of particular MMP associated with different cancer types. Drug discovery efforts have uncovered pharmacological inhibitors of different MMPs. Specific MMP inhibitors at a specific dose would be an important achievement to treat particularly gastric cancer and to halt to the progression of these diseases.

ACKNOWLEDGMENTS

REFERENCES

1   **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]
2   **Wadhwa R**, Song S, Lee JS, Yao Y, Wei Q, Ajani JA. Gastric cancer-molecular and clinical dimensions. *Nat Rev Clin Oncol* 2013; **10**: 643-655 [PMID: 24061039 DOI: 10.1038/nrclinonc]
3   **Murakami T**. Pathomorphological diagnosis. Definition and gross classification of early gastric cancer. *Gann Monogr Cancer Res* 1971: 11
4   **Sokoloff B**. Predisposition to cancer in the bonaparte family. *Am J Surgery* 1938; **40**: 673-678
5   **Williams L**, Jenkins GJ, Doak SH, Fowler P, Parry EM, Brown TH, Griffiths AP, Williams JG, Parry JM. Fluorescence in situ hybridisation analysis of chromosomal aberrations in gastric tissue: the potential involvement of Helicobacter pylori. *Br J Cancer* 2005; **92**: 1759-1766 [PMID: 15827559 DOI: 10.1038/sj.bjc.6602533]
6   **Sugai T**, Habano W, Uesugi N, Jao YF, Nakamura S, Abe K, Takagane A, Terashima M. Three independent genetic profiles based on mucin expression in early differentiated-type gastric cancers--a new concept of genetic carcinogenesis of early differentiated-type adenocarcinomas. *Mod Pathol* 2004; **17**: 1223-1234 [PMID: 15154009 DOI: 10.1038/sj.bjc.6602533]
7   **Milne AN**, Carvalho R, Morsink FM, Musler AR, de Leng WW, Ristimäki A, Offerhaus GJ. Early-onset gastric cancers have a different molecular expression profile than conventional gastric cancers. *Mod Pathol* 2006; **19**: 564-572 [PMID: 16474375 DOI: 10.1038/modpathol]
8   **Kokkola A**, Sipponen P. Gastric carcinoma in young adults. *Hepatogastroenterology* 2001; **48**: 1552-1555 [PMID: 11813570]
9   **Kikuchi S**, Nakajima T, Nishi T, Kobayashi O, Konishi T, Inaba Y, Wada O, Satou H, Ishibashi T, Ichikawa S, Okamoto N, Hirata T, Kubo T, Sato N, Miki K, Myoga A. Association between family history and gastric carcinoma among young adults. *Jpn J Cancer Res* 1996; **87**: 332-336 [PMID: 8641962]
10 **Wang Z**, Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Silliman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang TL, Riggins G, Powell SM, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 2004; **304**: 1164-1166 [PMID: 15155950 DOI: 10.1126/science]
11 **Shaheen N**, Ransohoff DF. Gastroesophageal reflux, barrett esophagus, and esophageal cancer: scientific review. *JAMA* 2002; **287**: 1972-1981 [PMID: 11960540 DOI: 10.1001/jama.287.15.1972]
12 **Lagergren J**, Bergström R, Lindgren A, Nyrén O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med* 1999; **340**: 825-831 [PMID: 10080844 DOI: 10.1056/NEJM199903183401101]
13 **Chak A**, Ochs-Balcom H, Falk G, Grady WM, Kinnard M, Willis JE, Elston R, Eng C. Familiality in Barrett's esophagus, adenocarcinoma of the esophagus, and adenocarcinoma of the gastroesophageal junction. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1668-1673 [PMID: 16985029 DOI: 10.1158/1055-9965]
14 **Sappati Biyyani RS**, Chessler L, McCain E, Nelson K, Fahmy N, King J. Familial trends of inheritance in gastro esophageal reflux disease, Barrett's esophagus and Barrett's adenocarcinoma: 20 families. *Dis Esophagus* 2007; **20**: 53-57 [PMID: 17227311 DOI: 10.1111/j.1442-2050]
15 **Chak A**, Falk G, Grady WM, Kinnard M, Elston R, Mittal S, King JF, Willis JE, Kondru A, Brock W, Barnholtz-Sloan J. Assessment of familiality, obesity, and other risk factors for early age of cancer diagnosis in adenocarcinomas of the esophagus and gastroesophageal junction. *Am J Gastroenterol* 2009; **104**: 1913-1921 [PMID: 19491834 DOI: 10.1038/ajg.2009.241]
16 **Hemminki K**, Sundquist J, Ji J. Familial risk for gastric carcinoma: an updated study from Sweden. *Br J Cancer* 2007; **96**: 1272-1277 [PMID: 17406355 DOI: 10.1038/sj.bjc.6603722]
17 **Bosman F**, Carneiro F, Hruban R, Theise N. Who classification of tumours of the digestive system iarc. The International Agency for Research on Cancer, 2010: 3
18 **Wang K**, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J, Leung SY. Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. *Nat Genet* 2011; **43**: 1219-1223 [PMID: 22037554 DOI: 10.1038/ng.982]
19 **Kwon MJ**, Kim SH, Jeong HM, Jung HS, Kim SS, Lee JE, Gye MC, Erkin OC, Koh SS, Choi Y-L, Park CK, Shin YK. Claudin-4 overexpression is associated with epigenetic derepression in gastric carcinoma. *Lab Invest* 2011; **91**: 1652-1667 [PMID: 21844869 DOI: 10.1038/ labinvest. 2011.117]
20 **Baba Y**, Watanabe M, Murata A, Shigaki H, Miyake K, Ishimoto T, Iwatsuki M, Iwagami S, Yoshida N, Oki E, Sakamaki K, Nakao M, Baba H. LINE-1 hypomethylation, DNA copy number alterations, and CDK6 amplification in esophageal squamous cell carcinoma. *Clin Cancer Res* 2014; **20**: 1114-1124 [PMID: 24423610 DOI: 10.1158/1078-0432.CCR-13-1645]
21 **Okada T**, Nakamura M, Nishikawa J, Sakai K, Zhang Y, Saito M, Morishige A, Oga A, Sasaki K, Suehiro Y, Hinoda Y, Sakaida I. Identification of genes specifically methylated in Epstein-Barr virus-associated gastric carcinomas. *Cancer Sci* 2013; **104**: 1309-1314 [PMID: 23829175 DOI: 10.1111/cas.12228]
22 **Alvarez MC**, Ladeira MS, Scaletsky IC, Pedrazzoli J, Ribeiro ML. Methylation pattern of THBS1, GATA-4, and HIC1 in pediatric and adult patients infected with Helicobacter pylori. *Dig Dis Sci* 2013; **58**: 2850-2857 [PMID: 23765259 DOI: 10.1007/s10620-013-2742-6]
23 **Jin Z**, Zhao Z, Cheng Y, Dong M, Zhang X, Wang L, Fan X, Feng X, Mori Y, Meltzer SJ. Endoglin promoter hypermethylation identifies a field defect in human primary esophageal cancer. *Cancer* 2013; **119**: 3604-3609 [PMID: 23893879 DOI: 10.1002/cncr.28276]
24 **Jin Z**, Feng X, Jian Q, Cheng Y, Gao Y, Zhang X, Wang L, Zhang Y, Huang W, Fan X, Chen S, Yu H, Zhao Z, Dong M, Liu J, Mori Y, Meltzer SJ. Aberrant methylation of the Ras-

related associated with diabetes gene in human primary esophageal cancer. *Anticancer Res* 2013; **33**: 5199-5203 [PMID: 24222170]

25   **Ninomiya I**, Kawakami K, Fushida S, Fujimura T, Funaki H, Takamura H, Kitagawa H, Nakagawara H, Tajima H, Kayahara M, Ohta T. Quantitative detection of TIMP-3 promoter hypermethylation and its prognostic significance in esophageal squamous cell carcinoma. *Oncol Rep* 2008; **20**: 1489-1495 [PMID: 19020732 DOI: 10.3892/or_00000170]

26   **Poplineau M**, Schnekenburger M, Dufer J, Kosciarz A, Brassart-Pasco S, Antonicelli F, Diederich M, Trussardi-Régnier A. The DNA hypomethylating agent, 5-aza-2′-deoxycytidine, enhances tumor cell invasion through a transcription-dependent modulation of MMP-1 expression in human fibrosarcoma cells. *Mol Carcinog* 2013 [PMID: 24038389 DOI: 10.1002/mc.22071]

27   **Hussain Z**, Khan MI, Shahid M, Almajhdi FN. S-adenosylmethionine, a methyl donor, up regulates tissue inhibitor of metalloproteinase-2 in colorectal cancer. *Genet Mol Res* 2013; **12**: 1106-1118 [PMID: 23661436 DOI: 10.4238/2013.April.10.6]

28   **Leung WK**, To KF, Chu ES, Chan MW, Bai AH, Ng EK, Chan FK, Sung JJ. Potential diagnostic and prognostic values of detecting promoter hypermethylation in the serum of patients with gastric cancer. *Br J Cancer* 2005; **92**: 2190-2194 [PMID: 15942635 DOI: 10.1038/sj.bjc.6602636]

29   **Wang YC**, Yu ZH, Liu C, Xu LZ, Yu W, Lu J, Zhu RM, Li GL, Xia XY, Wei XW, Ji HZ, Lu H, Gao Y, Gao WM, Chen LB. Detection of RASSF1A promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients. *World J Gastroenterol* 2008; **14**: 3074-3080 [PMID: 18494062 DOI: 10.3748/wjg.14.3074]

30   **Egeblad M**, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002; **2**: 161-174 [PMID: 11990853 DOI: 10.1038/nrc745]

31   **Tezvergil-Mutluay A**, Agee KA, Hoshika T, Carrilho M, Breschi L, Tjäderhane L, Nishitani Y, Carvalho RM, Looney S, Tay FR, Pashley DH. The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. *Dent Mater* 2010; **26**: 1059-1067 [PMID: 20688380 DOI: 10.1016/j.dental.2010.07.006]

32   **Susnow N**, Zeng L, Margineantu D, Hockenbery DM. Bcl-2 family proteins as regulators of oxidative stress. *Semin Cancer Biol* 2009; **19**: 42-49 [PMID: 19138742 DOI: 10.1016/j.semcancer.2008.12.002]

33   **Murray GI**, Duncan ME, O'Neil P, McKay JA, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol* 1998; **185**: 256-261 [PMID: 9771478 DOI: 10.1002/(SICI)1096-9896]

34   **Etoh T**, Inoue H, Yoshikawa Y, Barnard GF, Kitano S, Mori M. Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness. *Gut* 2000; **47**: 50-56 [PMID: 10861264 DOI: 10.1136/gut.47.1.50]

35   **Mukherjee S**, Roth MJ, Dawsey SM, Yan W, Rodriguez-Canales J, Erickson HS, Hu N, Goldstein AM, Taylor PR, Richardson AM, Tangrea MA, Chuaqui RF, Emmert-Buck MR. Increased matrix metalloproteinase activation in esophageal squamous cell carcinoma. *J Transl Med* 2010; **8**: 91 [PMID: 20920372 DOI: 10.1186/1479-5876-8-91]

36   **Elnemr A**, Yonemura Y, Bandou E, Kinoshita K, Kawamura T, Takahashi S, Tochiori S, Endou Y, Sasaki T. Expression of collagenase-3 (matrix metalloproteinase-13) in human gastric cancer. *Gastric Cancer* 2003; **6**: 30-38 [PMID: 12673424 DOI: 10.1007/s101200300004]

37   **Liu HQ**, Song S, Wang JH, Zhang SL. Expression of MMP-3 and TIMP-3 in gastric cancer tissue and its clinical significance. *Oncol Lett* 2011; **2**: 1319-1322 [PMID: 22848309 DOI: 10.3892/ol.2011.399]

38   **Liabakk N-B**, Talbot I, Smith RA, Wilkinson K, Balkwill F.

39   **Shiozawa J**, Ito M, Nakayama T, Nakashima M, Kohno S, Sekine I. Expression of matrix metalloproteinase-1 in human colorectal carcinoma. *Mod Pathol* 2000; **13**: 925-933 [PMID: 11007031]

40   **Parsons SL**, Watson SA, Collins HM, Griffin NR, Clarke PA, Steele RJ. Gelatinase (MMP-2 and -9) expression in gastrointestinal malignancy. *Br J Cancer* 1998; **78**: 1495-1502 [PMID: 9836483]

41   **Inuzuka K**, Ogata Y, Nagase H, Shirouzu K. Significance of coexpression of urokinase-type plasminogen activator, and matrix metalloproteinase 3 (stromelysin) and 9 (gelatinase B) in colorectal carcinoma. *J Surg Res* 2000; **93**: 211-218 [PMID: 11027463 DOI: 10.1006/jsre.2000.5952]

42   **Nielsen BS**, Timshel S, Kjeldsen L, Sehested M, Pyke C, Borregaard N, Danø K. 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. *Int J Cancer* 1996; **65**: 57-62 [PMID: 8543396 DOI: 10.1002/(SICI)1097-0215]

43   **Zucker S**, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev* 2004; **23**: 101-117 [PMID: 15000152 DOI: 10.1023/A: 1025867130437]

44   **Adachi Y**, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. *Gut* 1999; **45**: 252-258 [PMID: 10403738 DOI: 10.1136/gut.45.2.252]

45   **Dong Z**, Kumar R, Yang X, Fidler IJ. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 1997; **88**: 801-810 [PMID: 9118223 DOI: 10.1016/S0092-8674(00)81926-1]

46   **O'Reilly MS**, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; **79**: 315-328 [PMID: 7525077 DOI: 10.1016/0092-8674(94)90200-3]

47   **Leeman MF**, McKay JA, Murray GI. Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer. *J Clin Pathol* 2002; **55**: 758-762 [PMID: 12354802 DOI: 10.1136/jcp.55.10.758]

48   **Knäuper V**, Will H, López-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem* 1996; **271**: 17124-17131 [PMID: 8663255]

49   **Gururajan R**, Lahti JM, Grenet J, Easton J, Gruber I, Ambros PF, Kidd VJ. Duplication of a genomic region containing the Cdc2L1-2 and MMP21-22 genes on human chromosome 1p36.3 and their linkage to D1Z2. *Genome Res* 1998; **8**: 929-939 [PMID: 9750192 DOI: 10.1101/gr.8.9.929]

50   **Llano E**, Pendás AM, Freije JP, Nakano A, Knäuper V, Murphy G, López-Otin C. Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase a overexpressed in brain tumors. *Cancer Res* 1999; **59**: 2570-2576 [PMID: 10363975]

51   **Bradbury PA**, Zhai R, Hopkins J, Kulke MH, Heist RS, Singh S, Zhou W, Ma C, Xu W, Asomaning K, Ter-Minassian M, Wang Z, Su L, Christiani DC, Liu G. Matrix metalloproteinase 1, 3 and 12 polymorphisms and esophageal adenocarcinoma risk and prognosis. *Carcinogenesis* 2009; **30**: 793-798 [PMID: 19321798 DOI: 10.1093/carcin/bgp065]

52   **Dey S**, Ghosh N, Saha D, Kesh K, Gupta A, Swarnakar S. Matrix metalloproteinase-1 (MMP-1) Promoter polymorphisms are well linked with lower stomach tumor formation in eastern Indian population. *PLoS One* 2014; **9**: e88040 [PMID: 24505369 DOI: 10.1371/journal.pone.0088040]

53   **Langers AM**, Verspaget HW, Hommes DW, Sier CF. Single-

nucleotide polymorphisms of matrix metalloproteinases and their inhibitors in gastrointestinal cancer. *World J Gastrointest Oncol* 2011; **3**: 79-98 [PMID: 21731908 DOI: 10.4251/wjgo.v3.i6.79]

54 **Chen XB**, Chen GL, Liu JN, Yang JZ, Yu DK, Lin DX, Tan W. [Genetic polymorphisms in STK15 and MMP-2 associated susceptibility to esophageal cancer in Mongolian population]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2009; **43**: 559-564 [PMID: 19954064]

55 **Harendza S**, Lovett DH, Panzer U, Lukacs Z, Kuhnl P, Stahl RA. Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. *J Biol Chem* 2003; **278**: 20490-20499 [PMID: 12657623 DOI: 10. 10.1074/jbc. M211536200]

56 **Früh M**, Zhou W, Zhai R, Su L, Heist RS, Wain JC, Nishioka NS, Lynch TJ, Shepherd FA, Christiani DC, Liu G. Polymorphisms of inflammatory and metalloproteinase genes, Helicobacter pylori infection and the risk of oesophageal adenocarcinoma. *Br J Cancer* 2008; **98**: 689-692 [PMID: 18253117 DOI: 10.1038/sj.bjc.6604234]

57 **Li Y**, Sun DL, Duan YN, Zhang XJ, Wang N, Zhou RM, Chen ZF, Wang SJ. Association of functional polymorphisms in MMPs genes with gastric cardia adenocarcinoma and esophageal squamous cell carcinoma in high incidence region of North China. *Mol Biol Rep* 2010; **37**: 197-205 [PMID: 19562509 DOI: 10.1007/s11033-009-9593-4]

58 **Kubben FJ**, Sier CF, Meijer MJ, van den Berg M, van der Reijden JJ, Griffioen G, van de Velde CJ, Lamers CB, Verspaget HW. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. *Br J Cancer* 2006; **95**: 744-751 [PMID: 16940985 DOI: 10.1038/sj.bjc.6603307]

59 **Saeed HM**, Alanazi MS, Parine NR, Shaik J, Semlali A, Alharbi O, Azzam N, Aljebreen A, Almadi M, Shalaby MA. Matrix metalloproteinase-2 (-1306 c& gt; t) promoter polymorphism and risk of colorectal cancer in the Saudi population. *Asian Pac J Cancer Prev* 2013; **14**: 6025-6030 [PMID: 24289619]

60 **Langers AM**, Sier CF, Hawinkels LJ, Kubben FJ, van Duijn W, van der Reijden JJ, Lamers CB, Hommes DW, Verspaget HW. MMP-2 geno-phenotype is prognostic for colorectal cancer survival, whereas MMP-9 is not. *Br J Cancer* 2008; **98**: 1820-1823 [PMID: 18506186 DOI: 10.1038/sj.bjc.6604380]

61 **Woo M**, Park K, Nam J, Kim JC. Clinical implications of matrix metalloproteinase-1, -3, -7, -9, -12, and plasminogen activator inhibitor-1 gene polymorphisms in colorectal cancer. *J Gastroenterol Hepatol* 2007; **22**: 1064-1070 [PMID: 17608852 DOI: 10.1111/j.1440-1746.2006]

62 **Tang Y**, Zhu J, Chen L, Chen L, Zhang S, Lin J. Associations of matrix metalloproteinase-9 protein polymorphisms with lymph node metastasis but not invasion of gastric cancer. *Clin Cancer Res* 2008; **14**: 2870-2877 [PMID: 18451255 DOI: 10.1158/1078-0432]

63 **Fang WL**, Liang WB, He H, Zhu Y, Li SL, Gao LB, Zhang L. Association of matrix metalloproteinases 1, 7, and 9 gene polymorphisms with genetic susceptibility to colorectal carcinoma in a Han Chinese population. *DNA Cell Biol* 2010; **29**: 657-661 [PMID: 20662554 DOI: 10.1089/dna]

64 **Matsumura S**, Oue N, Nakayama H, Kitadai Y, Yoshida K, Yamaguchi Y, Imai K, Nakachi K, Matsusaki K, Chayama K, Yasui W. A single nucleotide polymorphism in the MMP-9 promoter affects tumor progression and invasive phenotype of gastric cancer. *J Cancer Res Clin Oncol* 2005; **131**: 19-25 [PMID: 15565457 DOI: 10.1007/s00432-004-0621-4]

65 **Xing LL**, Wang ZN, Jiang L, Zhang Y, Xu YY, Li J, Luo Y, Zhang X. Matrix metalloproteinase-9-1562C& gt; T polymorphism may increase the risk of lymphatic metastasis of colorectal cancer. *World J Gastroenterol* 2007; **13**: 4626-4629 [PMID: 17729419]

66 **Dey S**, Stalin S, Gupta A, Saha D, Kesh K, Swarnakar S.

Matrix metalloproteinase3 gene promoter polymorphisms and their haplotypes are associated with gastric cancer risk in eastern Indian population. *Mol Carcinog* 2012; **51** Suppl 1: E42-E53 [PMID: 22121090 DOI: 10.1002/mc.21837]

67 **Ke P**, Wu ZD, Wen HS, Ying MX, Long HC, Qing LG. Current evidence on associations between the MMP-7 (-181A& gt; G) polymorphism and digestive system cancer risk. *Asian Pac J Cancer Prev* 2013; **14**: 2269-2272 [PMID: 23725125]

68 **Jormsjö S**, Whatling C, Walter DH, Zeiher AM, Hamsten A, Eriksson P. Allele-specific regulation of matrix metalloproteinase-7 promoter activity is associated with coronary artery luminal dimensions among hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1834-1839 [PMID: 11701474 DOI: 10.1161/hq1101.098229]

69 **Richards TJ**, Park C, Chen Y, Gibson KF, Peter Di Y, Pardo A, Watkins SC, Choi AM, Selman M, Pilewski J, Kaminski N, Zhang Y. Allele-specific transactivation of matrix metalloproteinase 7 by FOXA2 and correlation with plasma levels in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L746-L754 [PMID: 22268124 DOI: 10.1152/ajplung.00319.2011]

70 **Zhang J**, Jin X, Fang S, Wang R, Li Y, Wang N, Guo W, Wang Y, Wen D, Wei L, Dong Z, Kuang G. The functional polymorphism in the matrix metalloproteinase-7 promoter increases susceptibility to esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma and non-small cell lung carcinoma. *Carcinogenesis* 2005; **26**: 1748-1753 [PMID: 15930031 DOI: 10.1093/carcin/bgi144]

71 **Ghilardi G**, Biondi ML, Erario M, Guagnellini E, Scorza R. Colorectal carcinoma susceptibility and metastases are associated with matrix metalloproteinase-7 promoter polymorphisms. *Clin Chem* 2003; **49**: 1940-1942 [PMID: 14578330 DOI: 10.1373/clinchem.2003.018911]

72 **Cirillo G**, Casalino L, Vallone D, Caracciolo A, De Cesare D, Verde P. Role of distinct mitogen-activated protein kinase pathways and cooperation between Ets-2, ATF-2, and Jun family members in human urokinase-type plasminogen activator gene induction by interleukin-1 and tetradecanoyl phorbol acetate. *Mol Cell Biol* 1999; **19**: 6240-6252 [PMID: 10454570]

73 **Herren B**, Levkau B, Raines EW, Ross R. Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence for a role for caspases and metalloproteinases. *Mol Biol Cell* 1998; **9**: 1589-1601 [PMID: 9614196 DOI: 10.1091/mbc.9.6.1589]

74 **Ilan N**, Mohsenin A, Cheung L, Madri JA. PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics. *FASEB J* 2001; **15**: 362-372 [PMID: 11156952 DOI: 10.1096/fj.00-0372com]

75 **Steinhusen U**, Weiske J, Badock V, Tauber R, Bommert K, Huber O. Cleavage and shedding of E-cadherin after induction of apoptosis. *J Biol Chem* 2001; **276**: 4972-4980 [PMID: 11076937 DOI: 10.1074/jbc.M006102200]

76 **Sympson CJ**, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 1994; **125**: 681-693 [PMID: 8175886]

77 **Powell WC**, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr Biol* 1999; **9**: 1441-1447 [PMID: 10607586 DOI: 10.1016/S0960-9822(00)80113-X]

78 **Wu E**, Mari BP, Wang F, Anderson IC, Sunday ME, Shipp MA. Stromelysin-3 suppresses tumor cell apoptosis in a murine model. *J Cell Biochem* 2001; **82**: 549-555 [PMID: 11500932 DOI: 10.1002/jcb.1181]

79 **Baserga R**. The contradictions of the insulin-like growth

factor 1 receptor. *Oncogene* 2000; **19**: 5574-5581 [PMID: 11114737]

80 **Swarnakar S**, Paul S, Singh LP, Reiter RJ. Matrix metalloproteinases in health and disease: regulation by melatonin. *J Pineal Res* 2011; **50**: 8-20 [PMID: 20964709]

81 **Seandel M**, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. *Blood* 2001; **97**: 2323-2332 [PMID: 11290594 DOI: 10.1182/blood.V97.8.2323]

82 **Kolb C**, Mauch S, Peter HH, Krawinkel U, Sedlacek R. The matrix metalloproteinase RASI-1 is expressed in synovial blood vessels of a rheumatoid arthritis patient. *Immunol Lett* 1997; **57**: 83-88 [PMID: 9232430 DOI: 10.1016/S0165-2478(97)00057-6]

83 **Itoh T**, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* 1998; **58**: 1048-1051 [PMID: 9500469]

84 **Xu J**, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC. Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 2001; **154**: 1069-1079 [PMID: 11535623 DOI: 10.1083/jcb.200103111]

85 **Coussens LM**, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000; **103**: 481-490 [PMID: 11081634]

86 **Zhou Z**, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci USA* 2000; **97**: 4052-4057 [PMID: 10737763 DOI: 10.1073/pnas.060037197]

87 **Cornelius LA**, Nehring LC, Harding E, Bolanowski M, Welgus HG, Kobayashi DK, Pierce RA, Shapiro SD. Matrix metalloproteinases generate angiostatin: effects on neovascularization. *J Immunol* 1998; **161**: 6845-6852 [PMID: 9862716]

88 **Gorrin-Rivas MJ**, Arii S, Furutani M, Mizumoto M, Mori A, Hanaki K, Maeda M, Furuyama H, Kondo Y, Imamura M. Mouse macrophage metalloelastase gene transfer into a murine melanoma suppresses primary tumor growth by halting angiogenesis. *Clin Cancer Res* 2000; **6**: 1647-1654 [PMID: 10815882]

89 **Kim YM**, Jang JW, Lee OH, Yeon J, Choi EY, Kim KW, Lee ST, Kwon YG. Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase. *Cancer Res* 2000; **60**: 5410-5413 [PMID: 11034081]

90 **Giannelli G**, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 1997; **277**: 225-228 [PMID: 9211848 DOI: 10.1126/science.277.5323.225]

91 **Koshikawa N**, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J Cell Biol* 2000; **148**: 615-624 [PMID: 10662785 DOI: 10.1083/jcb.148.3.615]

92 **Kajita M**, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol* 2001; **153**: 893-904 [PMID: 11381077 DOI: 10.1083/jcb.153.5.893]

93 **Yu Q**, Stamenkovic I. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev* 1999; **13**: 35-48 [PMID: 9887098 DOI: 10.1101/gad.13.1.35]

94 **Beliën AT**, Paganetti PA, Schwab ME. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. *J Cell Biol* 1999; **144**: 373-384 [PMID: 9922462 DOI: 10.1083/jcb.144.2.373]

95 **Deryugina EI**, Luo GX, Reisfeld RA, Bourdon MA, Strongin A. Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res* 1997; **17**: 3201-3210 [PMID: 9413149]

96 **Ala-Aho R**, Johansson N, Baker AH, Kähäri VM. Expression of collagenase-3 (MMP-13) enhances invasion of human fibrosarcoma HT-1080 cells. *Int J Cancer* 2002; **97**: 283-289 [PMID: 11774278 DOI: 10.1002/ijc.1619]

97 **Noë V**, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001; **114**: 111-118 [PMID: 11112695]

98 **Sternlicht MD**, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999; **98**: 137-146 [PMID: 10428026 DOI: 10.1016/S0092-8674(00)81009-0]

99 **Lochter A**, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 1997; **139**: 1861-1872 [PMID: 9412478 DOI: 10.1083/jcb.139.7.1861]

100 **Brooks PC**, Strömblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 1996; **85**: 683-693 [PMID: 8646777 DOI: 10.1016/S0092-8674(00)81235-0]

101 **Coussens LM**, Werb Z. Inflammatory cells and cancer: think different! *J Exp Med* 2001; **193**: F23-F26 [PMID: 11257144 DOI: 10.1084/jem.193.6.F23]

102 **Sheu BC**, Hsu SM, Ho HN, Lien HC, Huang SC, Lin RH. A novel role of metalloproteinase in cancer-mediated immunosuppression. *Cancer Res* 2001; **61**: 237-242 [PMID: 11196168]

103 **McQuibban GA**, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 2002; **100**: 1160-1167 [PMID: 12149192]

104 **McQuibban GA**, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000; **289**: 1202-1206 [PMID: 10947989 DOI: 10.1126/science.289.5482.1202]

105 **Müller A**, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; **410**: 50-56 [PMID: 11242036 DOI: 10.1038/35065016]

106 **Kataoka H**, Uchino H, Iwamura T, Seiki M, Nabeshima K, Koono M. Enhanced tumor growth and invasiveness in vivo by a carboxyl-terminal fragment of alpha1-proteinase inhibitor generated by matrix metalloproteinases: a possible modulatory role in natural killer cytotoxicity. *Am J Pathol* 1999; **154**: 457-468 [PMID: 10027404 DOI: 10.1016/S0002-9440(10)65292-3]

107 **Yu Q**, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; **14**: 163-176 [PMID: 10652271 DOI: 10.1101/gad.14.2.163]

108 **Mañes S**, Mira E, Barbacid MM, Ciprés A, Fernández-Resa P, Buesa JM, Mérida I, Aracil M, Márquez G, Martínez-A C. Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3. *J Biol Chem* 1997; **272**: 25706-25712 [PMID: 9325295]

109 **Murphy MP**. How mitochondria produce reactive oxygen species. *Biochem J* 2009; **417**: 1-13 [PMID: 19061483 DOI: 10.1042/BJ20081386]

110 **Gogvadze V**, Orrenius S, Zhivotovsky B. Mitochondria

in cancer cells: what is so special about them? *Trends Cell Biol* 2008; **18**: 165-173 [PMID: 18296052 DOI: 10.1016/j.tcb.2008.01.006]

111 **Meitzler JL**, Antony S, Wu Y, Juhasz A, Liu H, Jiang G, Lu J, Roy K, Doroshow JH. NADPH oxidases: a perspective on reactive oxygen species production in tumor biology. *Antioxid Redox Signal* 2014; **20**: 2873-2889 [PMID: 24156355 DOI: 10.1089/ars.2013.5603]

112 **Ibsen KH**. The Crabtree effect: a review. *Cancer Res* 1961; **21**: 829-841 [PMID: 13717358]

113 **Wu M**, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol* 2007; **292**: C125-C136 [PMID: 16971499 DOI: 10.1152/ajpcell.00247.2006]

114 **Ding SZ**, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, Dirden-Kramer B, Boldogh I, Ernst PB, Crowe SE. Helicobacter pylori infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infect Immun* 2007; **75**: 4030-4039 [PMID: 17562777 DOI: 10.1128/IAI.00172-07]

115 **Ganguly K**, Swarnakar S. Chronic gastric ulceration causes matrix metalloproteinases-9 and -3 augmentation: alleviation by melatonin. *Biochimie* 2012; **94**: 2687-2698 [PMID: 22959068 DOI: 10.1016/j.biochi.2012.08.004]

116 **Banerjee A**, Mukhopadhyay AK, Paul S, Bhattacharyya A, Swarnakar S. Unveiling the intricacies of helicobacter pylori-induced gastric inflammation: T helper cells and matrix metalloproteinases at a crossroad. Current topics in Gastritis-2012 Mozsik G, editor. *InTech* 2013 [DOI: 10.5772/46056]

117 **Hagen TM**, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1994; **91**: 12808-12812 [PMID: 7809125]

118 **Reddy EP**, Reddy VS, Chandra Mouli K, Rao P. Physiological antioxidant system and oxidative stress in stomach cancer patients with normal renal and hepatic function. *Online J Health Allied Sci* 2010: 8

119 **Czygier M**, Kamocki Z, Ławicki S, Szmitkowski M. [The plasma level of myeloperoxidase (MPO) and total antioxidant status (TAS) in gastric cancer patients after surgery]. *Przegl Lek* 2010; **67**: 443-445 [PMID: 21387751]

120 **Noyan T**, Guducuoglu H, Ilhan M. A study of oxidative stress parameters in anti-helicobacter pylorus immunoglobulin g positive and negative gastric cancer patients. *Yonsei Med J* 2009; **50**: 677-682 [PMID: 19881972 DOI: 10.3349/ymj.2009.50.5.677]

121 **Huang J**, Lam GY, Brumell JH. Autophagy signaling through reactive oxygen species. *Antioxid Redox Signal* 2011; **14**: 2215-2231 [PMID: 20874258 DOI: 10.1089/ars.2010.3554]

122 **Gibson SB**. A matter of balance between life and death: targeting reactive oxygen species (ROS)-induced autophagy for cancer therapy. *Autophagy* 2010; **6**: 835-837 [PMID: 20818163 DOI: 10.4161/auto.6.7.13335]

123 **Kim MJ**, Woo SJ, Yoon CH, Lee JS, An S, Choi YH, Hwang SG, Yoon G, Lee SJ. Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. *J Biol Chem* 2011; **286**: 12924-12932 [PMID: 21300795 DOI: 10.1074/jbc.M110.138958]

124 **Macip S**, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW, Aaronson SA. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *EMBO J* 2002; **21**: 2180-2188 [PMID: 11980715 DOI: 10.1093/emboj/21.9.2180]

125 **Yuan X**, Zhou Y, Wang W, Li J, Xie G, Zhao Y, Xu D, Shen L. Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production. *Cell*

Death Dis 2013; **4**: e794 [PMID: 24030146 DOI: 10.1038/cddis.2013.334]

126 **Liu L**, Sun L, Zhao P, Yao L, Jin H, Liang S, Wang Y, Zhang D, Pang Y, Shi Y, Chai N, Zhang H, Zhang H. Hypoxia promotes metastasis in human gastric cancer by up-regulating the 67-kDa laminin receptor. *Cancer Sci* 2010; **101**: 1653-1660 [PMID: 20491781 DOI: 10.1111/j.1349-7006]

127 **Vafa O**, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, Wahl GM. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell* 2002; **9**: 1031-1044 [PMID: 12049739 DOI: 10.1016/S1097-2765(02)00520-8]

128 **Radisky DC**, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 2005; **436**: 123-127 [PMID: 16001073 DOI: 10.1038/nature03688]

129 **Tominaga K**, Kawahara T, Sano T, Toida K, Kuwano Y, Sasaki H, Kawai T, Teshima-Kondo S, Rokutan K. Evidence for cancer-associated expression of NADPH oxidase 1 (Nox1)-based oxidase system in the human stomach. *Free Radic Biol Med* 2007; **43**: 1627-1638 [PMID: 18037128 DOI: 10.1016/j.freeradbiomed.2007.08.029]

130 **Arbiser JL**, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS, Lambeth JD. Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proc Natl Acad Sci USA* 2002; **99**: 715-720 [PMID: 11805326]

131 **Block K**, Gorin Y. Aiding and abetting roles of NOX oxidases in cellular transformation. *Nat Rev Cancer* 2012; **12**: 627-637 [PMID: 22918415 DOI: 10.1038/nrc3339]

132 **Chen YR**, Shrivastava A, Tan TH. Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. *Oncogene* 2001; **20**: 367-374 [PMID: 11313966]

133 **Chen N**, Nomura M, She QB, Ma WY, Bode AM, Wang L, Flavell RA, Dong Z. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. *Cancer Res* 2001; **61**: 3908-3912 [PMID: 11358804]

134 **Behrens A**, Jochum W, Sibilia M, Wagner EF. Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. *Oncogene* 2000; **19**: 2657-2663 [PMID: 10851065]

135 **Benhar M**, Dalyot I, Engelberg D, Levitzki A. Enhanced ROS production in oncogenically transformed cells potentiates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and sensitization to genotoxic stress. *Mol Cell Biol* 2001; **21**: 6913-6926 [PMID: 11564875 DOI: 10.1128/MCB.21.20.6913-6926.2001]

136 **Li Y**, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995; **11**: 376-381 [PMID: 7493016 DOI: 10.1038/ng1295-376]

137 **Van Remmen H**, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang TT, Nelson J, Strong R, Richardson A. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* 2003; **16**: 29-37 [PMID: 14679299]

138 **Elchuri S**, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ, Huang T-T. Cuznsod deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 2004; **24**: 367-380 [PMID: 15531919 DOI: 10.1038/sj.onc.1208207]

139 **Ishii K**, Zhen LX, Wang DH, Funamori Y, Ogawa K, Taketa K. Prevention of mammary tumorigenesis in acatalasemic

mice by vitamin E supplementation. *Jpn J Cancer Res* 1996; **87**: 680-684 [PMID: 8698615]

140 **Chu FF**, Esworthy RS, Chu PG, Longmate JA, Huycke MM, Wilczynski S, Doroshow JH. Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. *Cancer Res* 2004; **64**: 962-968 [PMID: 14871826 DOI: 10.1158/0008-5472.CAN-03-2272]

141 **Kanbagli O**, Ozdemirler G, Bulut T, Yamaner S, Aykaç-Toker G, Uysal M. Mitochondrial lipid peroxides and antioxidant enzymes in colorectal adenocarcinoma tissues. *Jpn J Cancer Res* 2000; **91**: 1258-1263 [PMID: 11123424]

142 **Janssen AM**, Bosman CB, van Duijn W, Oostendorp-van de Ruit MM, Kubben FJ, Griffioen G, Lamers CB, van Krieken JH, van de Velde CJ, Verspaget HW. Superoxide dismutases in gastric and esophageal cancer and the prognostic impact in gastric cancer. *Clin Cancer Res* 2000; **6**: 3183-3192 [PMID: 10955802]

143 **Scibior D**, Skrzycki M, Podsiad M, Czeczot H. Glutathione level and glutathione-dependent enzyme activities in blood serum of patients with gastrointestinal tract tumors. *Clin Biochem* 2008; **41**: 852-858 [PMID: 18394427 DOI: 10.1016/j.clinbiochem.2008.03.005]

144 **Oberley LW**, Bize IB, Sahu SK, Leuthauser SW, Gruber HE. Superoxide dismutase activity of normal murine liver, regenerating liver, and H6 hepatoma. *J Natl Cancer Inst* 1978; **61**: 375-379 [PMID: 210289]

145 **Hernandez-Saavedra D**, McCord JM. Paradoxical effects of thiol reagents on Jurkat cells and a new thiol-sensitive mutant form of human mitochondrial superoxide dismutase. *Cancer Res* 2003; **63**: 159-163 [PMID: 12517793]

146 **Hwang TS**, Choi HK, Han HS. Differential expression of manganese superoxide dismutase, copper/zinc superoxide dismutase, and catalase in gastric adenocarcinoma and normal gastric mucosa. *Eur J Surg Oncol* 2007; **33**: 474-479 [PMID: 17129702]

147 **Yang CS**, Landau JM. Effects of tea consumption on nutrition and health. *J Nutr* 2000; **130**: 2409-2412 [PMID: 11015465]

148 **Morse MA**, Kresty LA, Steele VE, Kelloff GJ, Boone CW, Balentine DA, Harbowy ME, Stoner GD. Effects of theaflavins on N-nitrosomethylbenzylamine-induced esophageal tumorigenesis. *Nutr Cancer* 1997; **29**: 7-12 [PMID: 9383778 DOI: 10.1080/01635589709514595]

149 **Yang G**, Zheng W, Xiang YB, Gao J, Li HL, Zhang X, Gao YT, Shu XO. Green tea consumption and colorectal cancer risk: a report from the Shanghai Men's Health Study. *Carcinogenesis* 2011; **32**: 1684-1688 [PMID: 21856996 DOI: 10.1093/carcin/bgr186]

150 **Caderni G**, De Filippo C, Luceri C, Salvadori M, Giannini A, Biggeri A, Remy S, Cheynier V, Dolara P. Effects of black tea, green tea and wine extracts on intestinal carcinogenesis induced by azoxymethane in F344 rats. *Carcinogenesis* 2000; **21**: 1965-1969 [PMID: 11062155 DOI: 10.1093/carcin/21.11.1965]

151 **Peng G**, Dixon DA, Muga SJ, Smith TJ, Wargovich MJ. Green tea polyphenol (-)-epigallocatechin-3-gallate inhibits cyclooxygenase-2 expression in colon carcinogenesis. *Mol Carcinog* 2006; **45**: 309-319 [PMID: 16508969 DOI: 10.1002/mc.20166]

152 **Kim HS**, Kim MH, Jeong M, Hwang YS, Lim SH, Shin BA, Ahn BW, Jung YD. EGCG blocks tumor promoter-induced MMP-9 expression via suppression of MAPK and AP-1 activation in human gastric AGS cells. *Anticancer Res* 2004; **24**: 747-753 [PMID: 15161022]

153 **Annabi B**, Lachambre MP, Bousquet-Gagnon N, Page M, Gingras D, Beliveau R. Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. *Biochim Biophys Acta* 2002; **1542**: 209-220 [PMID: 11853893 DOI: 10.1016/S0167-4889(01)00187-2]

154 **Onoda C**, Kuribayashi K, Nirasawa S, Tsuji N, Tanaka M, Kobayashi D, Watanabe N. (-)-Epigallocatechin-3-gallate induces apoptosis in gastric cancer cell lines by down-regulating survivin expression. *Int J Oncol* 2011; **38**: 1403-1408 [PMID: 21344159 DOI: 10.3892/ ijo.2011.951]

155 **Larsen CA**, Dashwood RH. (-)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. *Arch Biochem Biophys* 2010; **501**: 52-57 [PMID: 20361925 DOI: 10.1016/j.abb.2010.03.017]

156 **Cheng XW**, Kuzuya M, Nakamura K, Liu Z, Di Q, Hasegawa J, Iwata M, Murohara T, Yokota M, Iguchi A. Mechanisms of the inhibitory effect of epigallocatechin-3-gallate on cultured human vascular smooth muscle cell invasion. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1864-1870 [PMID: 16051878 DOI: 10.1161/01.ATV.0000179675.49619.9b]

157 **El Bedoui J**, Oak MH, Anglard P, Schini-Kerth VB. Catechins prevent vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression. *Cardiovasc Res* 2005; **67**: 317-325 [PMID: 15885676 DOI: 10.1016/j.cardiores.2005.03.017]

158 **Patel R**, Ingle A, Maru GB. Polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway. *Toxicol Appl Pharmacol* 2008; **227**: 136-146 [PMID: 18037152 DOI: 10.1016/j.taap.2007.10.009]

159 **Hwang JT**, Ha J, Park IJ, Lee SK, Baik HW, Kim YM, Park OJ. Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett* 2007; **247**: 115-121 [PMID: 16797120 DOI: 10.1016/j.canlet.2006.03.030]

160 **Shankar S**, Ganapathy S, Srivastava RK. Green tea polyphenols: biology and therapeutic implications in cancer. *Front Biosci* 2007; **12**: 4881-4899 [PMID: 17569617]

161 **Ammon HP**, Wahl MA. Pharmacology of Curcuma longa. *Planta Med* 1991; **57**: 1-7 [PMID: 2062949 DOI: 10.1055/s-2006-960004]

162 **Barzegar A**, Moosavi-Movahedi AA. Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin. *PLoS One* 2011; **6**: e26012 [PMID: 22016801]

163 **Swarnakar S**, Ganguly K, Kundu P, Banerjee A, Maity P, Sharma AV. Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer. *J Biol Chem* 2005; **280**: 9409-9415 [PMID: 15615723 DOI: 10.1074/jbc.M413398200]

164 **Jana S**, Paul S, Swarnakar S. Curcumin as anti-endometriotic agent: implication of MMP-3 and intrinsic apoptotic pathway. *Biochem Pharmacol* 2012; **83**: 797-804 [PMID: 22227273 DOI: 10.1016/j.bcp.2011.12.030]

165 **Rajasekaran SA**. Therapeutic potential of curcumin in gastrointestinal diseases. *World J Gastrointest Pathophysiol* 2011; **2**: 1-14 [PMID: 21607160 DOI: 10.4291/wjgp.v2.i1.1]

166 **Aggarwal BB**, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 2003; **23**: 363-398 [PMID: 12680238]

167 **Nagpal M**, Sood S. Role of curcumin in systemic and oral health: An overview. *J Nat Sci Biol Med* 2013; **4**: 3-7

168 **Koo JY**, Kim HJ, Jung KO, Park KY. Curcumin inhibits the growth of AGS human gastric carcinoma cells in vitro and shows synergism with 5-fluorouracil. *J Med Food* 2004; **7**: 117-121 [PMID: 15298755 DOI: 10.1089/1096620041224229]

169 **Tang XQ**, Bi H, Feng JQ, Cao JG. Effect of curcumin on multidrug resistance in resistant human gastric carcinoma cell line SGC7901/VCR. *Acta Pharmacol Sin* 2005; **26**: 1009-1016 [PMID: 16038636 DOI: 10.1111/j.1745-7254]

170 **Anand P**, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 2008; **267**: 133-164 [PMID: 18462866 DOI: 10.1016/j.canlet.2008.03.025]

171 **Perkins S**, Verschoyle RD, Hill K, Parveen I, Threadgill MD, Sharma RA, Williams ML, Steward WP, Gescher AJ. Che-

mopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 535-540 [PMID: 12050094]

172 **Kim JM**, Araki S, Kim DJ, Park CB, Takasuka N, Baba-Toriyama H, Ota T, Nir Z, Khachik F, Shimidzu N, Tanaka Y, Osawa T, Uraji T, Murakoshi M, Nishino H, Tsuda H. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis* 1998; **19**: 81-85 [PMID: 9472697 DOI: 10.1093/carcin/19.1.81]

173 **Shpitz B**, Giladi N, Sagiv E, Lev-Ari S, Liberman E, Kazanov D, Arber N. Celecoxib and curcumin additively inhibit the growth of colorectal cancer in a rat model. *Digestion* 2006; **74**: 140-144 [PMID: 17228149 DOI: 10.1159/000098655]

174 **Huang MT**, Lou YR, Ma W, Newmark HL, Reuhl KR, Conney AH. Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Res* 1994; **54**: 5841-5847 [PMID: 7954412]

175 **Bimonte S**, Barbieri A, Palma G, Luciano A, Rea D, Arra C. Curcumin inhibits tumor growth and angiogenesis in an orthotopic mouse model of human pancreatic cancer. *Biomed Res Int* 2013; **2013**: 810423 [PMID: 24324975 DOI: 10.1155/2013/810423]

176 **Kunnumakkara AB**, Diagaradjane P, Guha S, Deorukhkar A, Shentu S, Aggarwal BB, Krishnan S. Curcumin sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting nuclear factor-kappaB-regulated gene products. *Clin Cancer Res* 2008; **14**: 2128-2136 [PMID: 18381954]

177 **Lin LI**, Ke YF, Ko YC, Lin JK. Curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion in vitro and suppresses matrix metalloproteinase-9 secretion. *Oncology* 1998; **55**: 349-353 [PMID: 9663426]

178 **Su CC**, Chen GW, Lin JG, Wu LT, Chung JG. Curcumin inhibits cell migration of human colon cancer colo 205 cells through the inhibition of nuclear factor kappa B /p65 and down-regulates cyclooxygenase-2 and matrix metalloproteinase-2 expressions. *Anticancer Res* 2006; **26**: 1281-1288 [PMID: 16619535]

179 **Lin ML**, Lu YC, Chen HY, Lee CC, Chung JG, Chen SS. Suppressing the formation of lipid raft-associated Rac1/PI3K/Akt signaling complexes by curcumin inhibits SDF-1α-induced invasion of human esophageal carcinoma cells. *Mol Carcinog* 2014; **53**: 360-379 [PMID: 23192861 DOI: 10.1002/mc.21984]

180 **Goswami SK**, Das DK. Resveratrol and chemoprevention. *Cancer Lett* 2009; **284**: 1-6 [PMID: 19261378 DOI: 10.1016/j.canlet.2009]

181 **Li ZG**, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganoi J, Hashimoto Y, Imamura M. Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis* 2002; **23**: 1531-1536 [PMID: 12189197 DOI: 10.1093/carcin/23.9.1531]

182 **Schneider Y**, Duranton B, Gossé F, Schleiffer R, Seiler N, Raul F. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. *Nutr Cancer* 2001; **39**: 102-107 [PMID: 11588890 DOI: 10.1207/S15327914nc391_14]

183 **Li W**, Ma J, Ma Q, Li B, Han L, Liu J, Xu Q, Duan W, Yu S, Wang F, Wu E. Resveratrol inhibits the epithelial-mesenchymal transition of pancreatic cancer cells via suppression of the PI-3K/Akt/NF-κB pathway. *Curr Med Chem* 2013; **20**: 4185-4194 [PMID: 23992306]

184 **Ji Q**, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J, Li Q. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/β-catenin signal pathway. *PLoS One* 2013; **8**: e78700 [PMID:

185 **Weng CJ**, Wu CF, Huang HW, Wu CH, Ho CT, Yen GC. Evaluation of anti-invasion effect of resveratrol and related methoxy analogues on human hepatocarcinoma cells. *J Agric Food Chem* 2010; **58**: 2886-2894 [PMID: 20131808 DOI: 10.1021/jf904182y]

186 **Harikumar KB**, Kunnumakkara AB, Sethi G, Diagaradjane P, Anand P, Pandey MK, Gelovani J, Krishnan S, Guha S, Aggarwal BB. Resveratrol, a multitargeted agent, can enhance antitumor activity of gemcitabine in vitro and in orthotopic mouse model of human pancreatic cancer. *Int J Cancer* 2010; **127**: 257-268 [PMID: 19908231 DOI: 10.1002/ijc.25041]

187 **Gibellini L**, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, Bertoncelli L, Cooper EL, Cossarizza A. Quercetin and cancer chemoprevention. *Evid Based Complement Alternat Med* 2011; **2011**: 591356 [PMID: 21792362 DOI: 10.1093/ecam/neq053]

188 **Volate SR**, Davenport DM, Muga SJ, Wargovich MJ. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin). *Carcinogenesis* 2005; **26**: 1450-1456 [PMID: 15831530 DOI: 10.1093/carcin/bgi089]

189 **Dihal AA**, de Boer VC, van der Woude H, Tilburgs C, Bruijntjes JP, Alink GM, Rietjens IM, Woutersen RA, Stierum RH. Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. *J Nutr* 2006; **136**: 2862-2867 [PMID: 17056814]

190 **Lai WW**, Hsu SC, Chueh FS, Chen YY, Yang JS, Lin JP, Lien JC, Tsai CH, Chung JG. Quercetin inhibits migration and invasion of SAS human oral cancer cells through inhibition of NF-κB and matrix metalloproteinase-2/-9 signaling pathways. *Anticancer Res* 2013; **33**: 1941-1950 [PMID: 23645742]

191 **Reiter RJ**, Tan DX, Manchester LC, Simopoulos AP, Maldonado MD, Flores LJ, Terron MP. Melatonin in edible plants (phytomelatonin): Identification, concentrations, bioavailability and proposed functions. *World Rev Nutr Diet* 2007; **97**: 211-230 [PMID: 17167296]

192 **Rudra DS**, Pal U, Maiti NC, Reiter RJ, Swarnakar S. Melatonin inhibits matrix metalloproteinase-9 activity by binding to its active site. *J Pineal Res* 2013; **54**: 398-405 [PMID: 23330737 DOI: 10.1111/jpi.12034]

193 **Xu L**, Liu H, Zhang H, Wang RX, Song J, Zhou RX. Growth-inhibitory activity of melatonin on murine foregastric carcinoma cells in vitro and the underlying molecular mechanism. *Anat Rec* (Hoboken) 2013; **296**: 914-920 [PMID: 23564716 DOI: 10.1002/ar.22689]

194 **Nemeth C**, Humpeler S, Kallay E, Mesteri I, Svoboda M, Rögelsperger O, Klammer N, Thalhammer T, Ekmekcioglu C. Decreased expression of the melatonin receptor 1 in human colorectal adenocarcinomas. *J Biol Regul Homeost Agents* 2011; **25**: 531-542 [PMID: 22217986]

195 **Hong Y**, Won J, Lee Y, Lee S, Park K, Chang KT, Hong Y. Melatonin treatment induces interplay of apoptosis, autophagy, and senescence in human colorectal cancer cells. *J Pineal Res* 2014; **56**: 264-274 [PMID: 24484372 DOI: 10.1111/jpi.12119]

196 **Ordoñez R**, Carbajo-Pescador S, Prieto-Dominguez N, García-Palomo A, González-Gallego J, Mauriz JL. Inhibition of matrix metalloproteinase-9 and nuclear factor kappa B contribute to melatonin prevention of motility and invasiveness in HepG2 liver cancer cells. *J Pineal Res* 2014; **56**: 20-30 [PMID: 24117795 DOI: 10.1111/jpi.12092]

197 **Tang FY**, Pai MH, Kuo YH, Wang XD. Concomitant consumption of lycopene and fish oil inhibits tumor growth and progression in a mouse xenograft model of colon cancer. *Mol Nutr Food Res* 2012; **56**: 1520-1531 [PMID: 22961879 DOI: 10.1002/mnfr.201200098]

198 **Lin MC**, Wang FY, Kuo YH, Tang FY. Cancer chemopreventive effects of lycopene: suppression of MMP-7 expression

24244343 DOI: 10.1371/journal.pone.0078700]

and cell invasion in human colon cancer cells. *J Agric Food Chem* 2011; **59**: 11304-11318 [PMID: 21923160 DOI: 10.1021/jf202433f]

199   **Adachi Y**, Itoh F, Yamamoto H, Iku S, Matsuno K, Arimura Y, Imai K. Retinoic acids reduce matrilysin (matrix metalloproteinase 7) and inhibit tumor cell invasion in human colon cancer. *Tumour Biol* 2001; **22**: 247-253 [PMID: 11399950]

200   **Park EY**, Wilder ET, Lane MA. Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells in vitro and decreases matrix metalloproteinase mRNA, protein, and activity levels. *Nutr Cancer* 2007; **57**: 66-77 [PMID: 17516864 DOI: 10.1080/01635580701268238]

201   **Tanaka T**, Shnimizu M, Moriwaki H. Cancer chemoprevention by carotenoids. *Molecules* 2012; **17**: 3202-3242 [PMID: 22418926 DOI: 10.3390/molecules17033202]

202   **Liu HK**, Wang Q, Li Y, Sun WG, Liu JR, Yang YM, Xu WL, Sun XR, Chen BQ. Inhibitory effects of gamma-tocotrienol on invasion and metastasis of human gastric adenocarcinoma SGC-7901 cells. *J Nutr Biochem* 2010; **21**: 206-213 [PMID: 19195866 DOI: 10.1016/j.jnutbio]

203   **Roomi MW**, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vivo antitumor effect of ascorbic acid, lysine, proline and green tea extract on human colon cancer cell HCT 116 xenografts in nude mice: evaluation of tumor growth and immunohistochemistry. *Oncol Rep* 2005; **13**: 421-425 [PMID: 15706410]

204   **Manu KA**, Shanmugam MK, Ramachandran L, Li F, Fong CW, Kumar AP, Tan P, Sethi G. First evidence that γ-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway. *Clin Cancer Res* 2012; **18**: 2220-2229 [PMID: 22351692 DOI: 10.1158/1078-0432.CCR-11-2470]

ORIGINAL ARTICLE

Effects of acute doxorubicin treatment on hepatic proteome lysine acetylation status and the apoptotic environment

Amie J Dirks-Naylor, Samir A Kouzi, Joseph D Bero, Ngan TK Tran, Sendra Yang, Raean Mabolo

Amie J Dirks-Naylor, Samir A Kouzi, Joseph D Bero, Ngan TK Tran, Sendra Yang, Raean Mabolo, School of Pharmacy, Wingate University, Wingate, NC 28174, United States
Author contributions: Dirks-Naylor AJ designed the research, performed the research, analyzed the data, and wrote the manuscipt; Kouzi SA designed the research and performed the research and edited the manuscirpt; Bero JD, Tran NTK, Yang S and Mabolo R performed the research.
Correspondence to: Amie J Dirks-Naylor, PhD, Associate Professor, School of Pharmacy, Wingate University, 515 N. Main Street, Wingate, NC 28174, United States. anaylor@wingate.edu
Telephone: +1-704-2338341 Fax: +1-704-2338332
Received: February 18, 2014 Revised: April 2, 2014
Accepted: June 27, 2014
Published online: August 26, 2014

Abstract

AIM: To determine if doxorubicin (Dox) alters hepatic proteome acetylation status and if acetylation status was associated with an apoptotic environment.

METHODS: Doxorubicin (20 mg/kg; Sigma, Saint Louis, MO; $n = 8$) or NaCl (0.9%; $n = 7$) was administered as an intraperitoneal injection to male F344 rats, 6-wk of age. Once animals were treated with Dox or saline, all animals were fasted until sacrifice 24 h later.

RESULTS: Dox treatment decreased proteome lysine acetylation likely due to a decrease in histone acetyltransferase activity. Proteome deacetylation may likely not be associated with a proapoptotic environment. Dox did not increase caspase-9, -8, or -3 activation nor poly (adenosine diphosphate-ribose) polymerase-1 cleavage. Dox did stimulate caspase-12 activation, however, it likely did not play a role in apoptosis induction.

CONCLUSION: Early effects of Dox involve hepatic proteome lysine deacetylation and caspase-12 activation under these experimental conditions.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Core tip: Doxorubicin (Dox) is an effective chemotherapeutic agent, but known to cause cardiotoxicity and hepatotoxicity. Cellular stress can alter proteome acetylation status in various experimental models, which has been associated with a proapoptotic environment. The effects of Dox on hepatic lysine acetylation status has not been studied. The study revealed five interesting findings that open the door for new areas of investigation: (1) Dox induces proteome lysine deacetylation; (2) lysine deacetylation is, at least in part, due to a decrease in histone acetyltransferase activity; (3) lysine deacetylation is likely not associated with an apoptotic environment; (4) Dox-induced hepatic injury is associated with caspase-12 activation; and (5) caspase-12 activation is not involved in apoptosis induction. These results may in the future translate to lysine acetylation homeostasis and/or caspase-12 as therapeutic targets.

Dirks-Naylor AJ, Kouzi SA, Bero JD, Tran NTK, Yang S, Mabolo R. Effects of acute doxorubicin treatment on hepatic proteome lysine acetylation status and the apoptotic environment. *World J Biol Chem* 2014; 5(3): 377-386 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/377.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.377

INTRODUCTION

Doxorubicin (Dox) is a highly effective therapeutic agent

in treating various cancers, but proven to cause cardiotoxicity. Mechanisms of cardiotoxicity have not been distinctly defined, but published literature has suggested that the induction of oxidative stress, apoptosis and mitochondrial dysfunction are involved[1-3]. In addition to proven toxicity in cardiac tissue, hepatic tissue reveals molecular and morphological signs of toxicity[4-9]. Since oxidative stress has been shown to play a role in both cardiac and hepatic toxicity, the use of antioxidants as a preventative measure has been widely studied. However, supplementation with antioxidants in clinical trials have only shown limited protection[10]. Thus, additional research is essential to determine additional mechanisms involved in Dox-induced toxicities. Therefore, the aim of this study was to determine the role of proteome lysine acetylation dyshomeostasis and apoptosis in Dox-induced hepatic toxicity.

Lysine acetylation is a common posttranslational modification[11]. Lysine acetylation regulates function of histones, proteins affecting the ubiquitin-proteosome system, transcription factors, cytoskeletal components, energy generating enzymes, and oxidative stress defense proteins[11]. In various experimental models, cellular stress has been shown to alter proteome lysine acetylation status, causing deacetylation, and thereby affecting the apoptotic environment[12]. Moreover, normalization of the lysine acetylation status has been shown to prevent stimulation of apoptotic pathways[12]. The effects of Dox on proteome acetylation status is unknown, thus, we aimed to make this determination. We hypothesized that Dox-induced cellular injury and stress would lead to lysine deacetylation and may contribute to Dox-induced toxicity. Acetylation status is determined by activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs)[11,13]. Thus, we also investigated potential mechanisms of deacetylation by assessing general HDAC activity and expression of sirtuins, one of several known classes of HDACs, as well as HAT activity.

Secondly, we aimed to determine if the proteome lysine acetylation status was associated with a proapoptotic environment, as shown in other experimental models. Apoptosis can be activated by a variety of specific signaling pathways (*e.g.*, receptor-mediated, mitochondrial-mediated, endoplasmic reticulum (ER)-mediated, *etc.*), depending on the initiating stimulus, and is associated with a distinct apical caspase[14]. These apical caspases converge on caspase-3, which is considered the central executioner of apoptosis. Activation of caspase-3 is responsible for the demise of the cell and most of the characteristic morphology associated with apoptosis, such as DNA fragmentation, cell shrinkage, and membrane blebbing. Thus, we assessed the content and activation of apical caspases and the activity of caspase-3 to determine if the proteome acetylation status was associated with the apoptotic environment in this experimental model. Furthermore, the effects of Dox on apical caspases and their role in hepatic toxicity have not been previously studied.

MATERIALS AND METHODS

Animals and experimental design

The institutional Research Review Board approved all protocols and procedures. Male F344 rats, 6-weeks of age, were purchased from Charles River (Wilmington, MA). The rats were arbitrarily separated into groups and were housed in a light controlled and temperature (22 ℃ ± 2 ℃) controlled facility. Doxorubicin (20 mg/kg; Sigma, Saint Louis, MO; $n = 8$) or NaCl (0.9%; $n = 7$) was injected intraperitoneal (IP). The dose of doxorubicin used in this study is equivalent to clinical doses used in humans that are pharmacologically scaled for use in rats[15,16]. Food and water was withheld from all animals during the experimental period (24 h). Doxorubicin administration can cause up to an approximate 70% decrease in both food and water intake within several hours of administration and typically persists for numerous days[17]. All other investigations examining the effects of Dox on the liver in laboratory animals did not control for differences in food and water intake, thus the Dox group was nutrient deficient while the control group received plenty of food and water. As a result, it was not possible to distinguish between the effects of Dox and the effects of anorexia. Anorexia has been shown to alter hepatic oxidative stress, autophagy, mitochondrial morphology, survival signaling, and many more processes[18-21]. Thus, to control for Dox-induced anorexia we fasted all animals. After exposure to ether, rats were sacrificed by dislocation of the cervical spine. Tissues were excised immediately following sacrifice, saline rinsed and frozen in liquid nitrogen. Tissues were stored at -80 ℃ until analysis.

Western analysis

Liver samples were homogenized (Power Gen 125, Fisher Scientific, Pittsburgh, PA) in ice-cold phosphate-buffered saline (2.68 mmol/L KCl , 1.75 mmol/L KH$_2$PO$_4$, 137 mmol/L NaCl, 10 mmol/L Na$_2$HPO$_4$, 5 mmol/L EDTA). Ten uL/mL of Halt Phosphatase Inhibitor Cocktail and 10 uL/mL of Halt Protease Inhibitor Cocktail (Pierce Biochemicals, Rockford, IL) was added to the buffer immediately before homogenization. The homogenate was centrifuged at 660 × g at 4 ℃ for 10 min. The supernatant was used for biochemical analysis. The Bicinchoninic Acid Protein Assay Kit (Sigma, Saint Louis, MO) was used to assess protein concentration. Samples were run in quadruplicate.

Protein (proteome) lysine acetylation and protein content of sirtuin 1 (Sirt1), sirtuin 3 (Sirt3), poly (ADP-ribose) polymerase-1 (PARP-1), and procaspase-1, -8, -9, -12 were determined by standard wet Western blot analysis. Proteins (50 ug) were separated on tris/glycine 4%-20% separating polyacrylamide PAGEr Gold Precast Gels (Lonza, Rockland, ME) under denaturing conditions and transferred to nitrocellulose membranes. Membranes were blocked in PBS blocking solution containing 5.0% powdered milk for one hour at room temperature. Membranes were incubated in primary antibody overnight
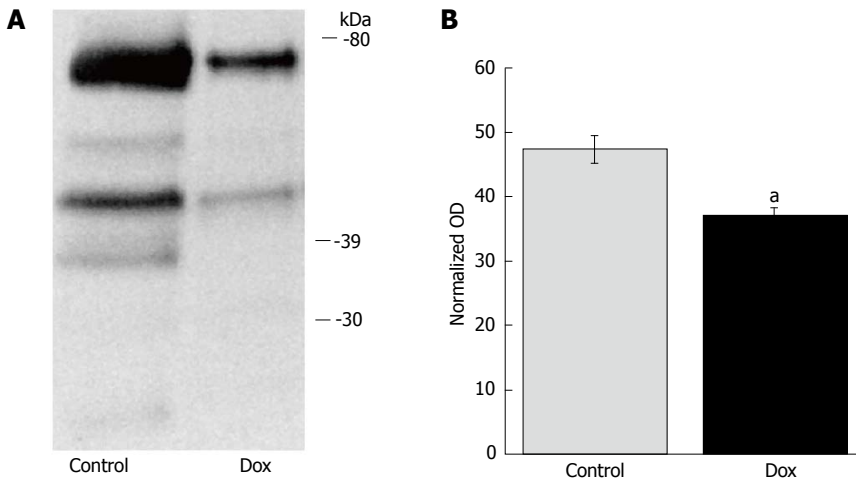
Figure 1 Proteome lysine acetylation status. A: Representative Western blot showing doxorubicin (Dox)-induced proteome lysine deacetylation; B: Graphical representation of OD units determined by whole lane analysis and normalized to Ponceau staining. [a]$P < 0.050$, control *vs* Dox.

at 4 ℃ (dilution of 1:1000; antibodies were purchased from Santa Cruz Biotechnology, INC, Santa Cruz, CA, sc-137254, sc-7150, sc-56036, sc-166320, sc-81663, sc-21747, sc-271014, sc-15404, sc-99143, sc-32268). Membranes were incubated with secondary HRP-linked antibody, with a dilution of 1:10000, for two hours shaking at room temperature. Bands of interest were imaged using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals, Rockford, IL) and the Kodak IS4000R Imaging System (Carestream Health, Inc., New Haven, CT). To assess equal loading of protein, ponceau staining (Pierce Biochemicals, Rockford, IL) of the nitrocellulose membranes was used. Whole lane analysis for each sample was used to determine densitometry of Ponceau staining. Data are presented as arbitrary units of densitometry calculated by subtracting the background intensity from the mean intensity of each band. Arbitrary OD for each band was normalized to the densitometry of Ponceau staining of each lane to account for variances in loading. The utilization of Ponceau staining as a reproducible alternative to actin in assessing equal loading has been validated [22].

HDAC activity
An HDAC Colorimetric Assay (BioVision, Milpitas, CA) was purchased to determine HDAC activity. The manufacturer's instructions were followed. Samples were run in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

HAT activity
Histone acetyltransferse activity was measured using a HAT Colorimetric Assay Kit (BioVision, Milpitas, CA). The manufacturer's instructions were followed. Samples were run in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

Caspase-3 activity
A Caspase-3 Colorimetric Assay Kit (BioVision, Milpitas, CA) was used to determine caspase-3 activity. The manufacturer's instructions were followed. Samples were run

in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

Statistical analysis
For statistical analysis, a Student's *t*-test was used. $P < 0.05$ was deemed statistically significant. Data is presented as mean ± SEM. For all analysis the $n = 8$ for the Dox group and $n = 7$ for the control group.

RESULTS

Proteome lysine acetylation status
Since proteome lysine deacetylation has been associated with pro-apoptotic environment and cellular injury in alternative experimental models, we determined the effects of Dox on acetylation status in the liver. Dox promoted proteome lysine deacetylation of treated animals (42.88 ± 2.06 *vs* 32.03 ± 1.00, $P = 0.002$; control *vs* Dox, respectively; Figure 1). Proteins of molecular weight between 30-80 kDa appear to be most affected, as we did not observe protein bands detected by the lysine acetylation antibody above or below these molecular weights. These results suggest that early effects of acute Dox toxicity involve proteome lysine deacetylation.

Protein content of Sirt1 and Sirt3
Sirtuins are a class of HDACs that have recently been under intense investigation, in part, due to their potential role in longevity[23]. To investigate the potential mechanism of Dox-induced deacetylation, we investigated the effects of Dox on the expression of Sirt1 and Sirt3, two of the most studied lysine deacetylases in the sirtuin family. Despite significant deacetylation, Sirt1 content was not affected by Dox treatment (128.57 ± 6.10 *vs* 122.89 ± 4.09, control *vs* Dox, respectively, $P = 0.44$) nor Sirt3 (27.14 ± 2.69 *vs* 28.00 ± 2.28, control *vs* Dox, respectively, $P = 0.81$; Figure 2).

Interestingly, a prominent band of approximately 45 kDa was detected in the liver with the Sirt1 antibody. Dox treatment increased the content of this species compared to control (139. 0 ± 4.25 *vs* 107.86 ± 4.06, respectively, $P = 0.0001$). It is possible that this species is
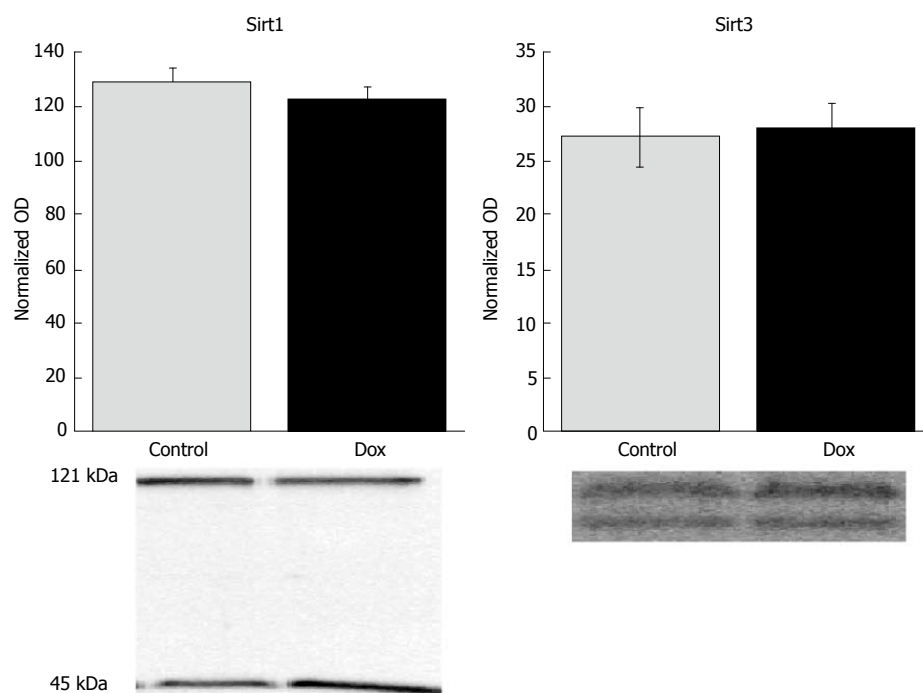
Figure 2 Protein content of Sirt1 and Sirt3. Doxorubicin (Dox) did not affect the expression of Sirt1 or Sirt 3 in liver of treated animals. Representative Western blot shows 120 kDa Sirt1. A 45 kDa species detected by the Sirt1 antibody is also shown. Sirt: Sirtuin.

a cleaved fragment of Sirt1. A recent study has shown, in chondrocytes, that Sirt1 can be cleaved into a 75 kDa fragment that plays a role in regulation of apoptosis[24]. The antibody used in this study does not target the 75 kDa fragment, however, the species detected may be the approximately 45 kDa remnant of the cleaved Sirt1.

HDAC activity

We also assessed general HDAC activity to determine if deacetylases might be responsible for the Dox-induced proteome lysine deacetylation. We did not observe a difference between Dox treated and control animals (0.120 $\pm$ 0.020 *vs* 0.151 $\pm$ 0.017, respectively, $P = 0.29$; Figure 3A). Thus, the data suggests that HDACs may not be responsible for the observed proteome lysine deacetylation in Dox treated animals.

HAT activity

Since the proteome deacetylation did not appear to be due to increased HDAC activity, we assessed HAT activity. Dox decreased hepatic HAT activity (0.209 $\pm$ 0.006 *vs* 0.189 $\pm$ 0.004, $P = 0.017$, Figure 3B). Thus, the data suggest that the Dox-induced proteome deacetylation is, at least in part, due to decreased HAT activity in livers of treated animals.

Expression and activation of apical caspases

Caspase-9: To evaluate the influence of Dox treatment on the stimulation of apoptosis mediated by mitochondria, we determined the content of procaspase-9 and the presence of any cleavage products representing the active caspase. Dox treatment increased procaspase-9 content in the liver (46.86 $\pm$ 1.66 *vs* 56.22 $\pm$ 1.96, control *vs* Dox,

respectively, $P = 0.003$; Figure 4). No cleavage products were detected. The results suggest that activation of the mitochondrial-mediated apoptotic pathway may not contribute to early mechanisms of hepatic toxicity.

Caspase-8: To evaluate the influence of Dox treatment on the stimulation of receptor-mediated apoptosis, we determined the content of procaspase-8 and any potential cleavage products. Dox treatment did not affect content of procaspase-8 or its activation (33.81 $\pm$ 2.38 *vs* 33.99 $\pm$ 2.40, control *vs* Dox, respectively, $P = 0.96$; Figure 5). There was no statistical difference in the presence of lower molecular weight bands between treatment groups (data not shown). The results suggest that activation of caspase-8 by receptor-mediated mechanisms may not contribute to early mechanisms causing acute hepatic toxicity.

Caspase-12: Caspase-12 has been implicated in stimulating apoptosis in response to ER dysfunction and calcium dyshomeostasis[25-27], therefore, we assessed the content of procaspase-12 and any potential cleavage products. Hepatic procaspase-12 (50 kDa) content in Dox treated animals tended to be lower than those of control animals (38.66 $\pm$ 5.82 *vs* 56.0 $\pm$ 6.98, respectively) , however statistical significance was not reached ($P = 0.075$). The content of the cleavage product of caspase-12 (40 kDa) was significantly increased by Dox treatment (78.11 $\pm$ 7.73 *vs* 43.0 $\pm$ 5.91, Dox *vs* control, respectively, $P = 0.004$; Figure 6).

Caspase-1: Caspase-1 is an inflammatory caspase involved in the processing of proinflammatory cytokines.
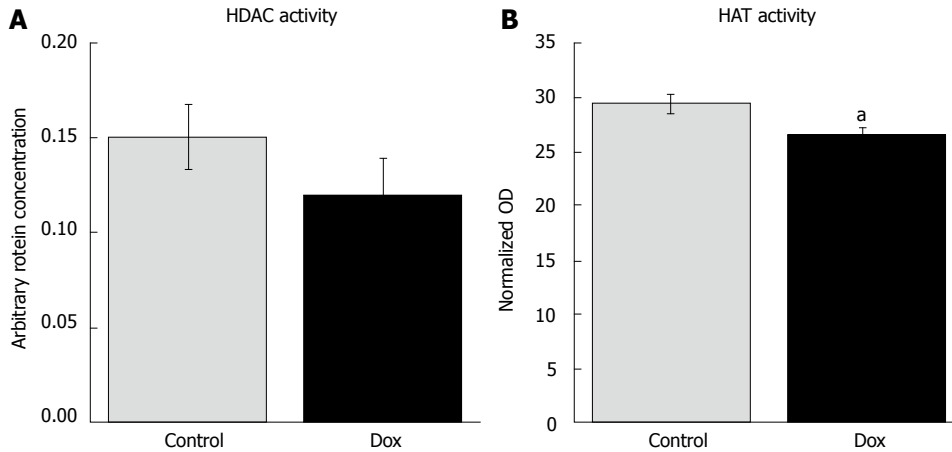
Figure 3 Histone deacetylase and histone acetyltransferase activity. A: Conventional histone deacetylase (HDAC) activity was not affected by doxorubicin (Dox) treatment; B: Dox decreased hepatic histone acetyltransferase (HAT) activity. [a]*P* < 0.050, 0.209 ± 0.006 *vs* 0.189 ± 0.004.
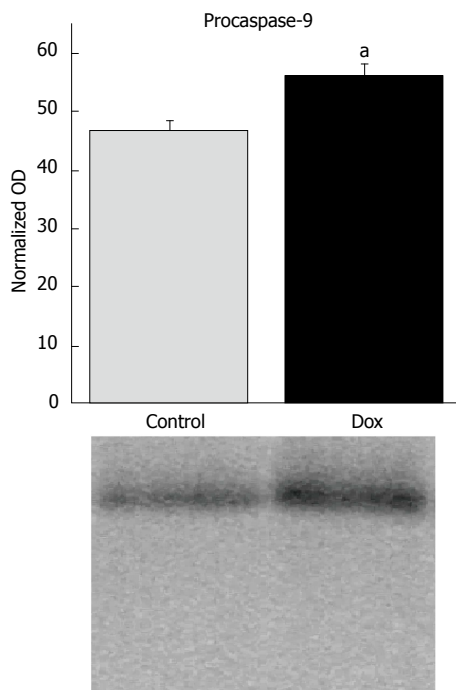
Figure 4 Protein content of procaspase-9. Doxorubicin (Dox) increased the hepatic expression of procaspase-9. Representative Western blots show the band corresponding to procaspase-9; no bands of lower molecular weight representing cleavage products were present. [a]*P* < 0.050, control *vs* Dox.

Figure 5 Protein content of procaspase-8. Doxorubicin (Dox) did not affect hepatic content of procaspase-8. Furthermore, there was no difference in the presence of lower molecular weight bands between treatment groups.
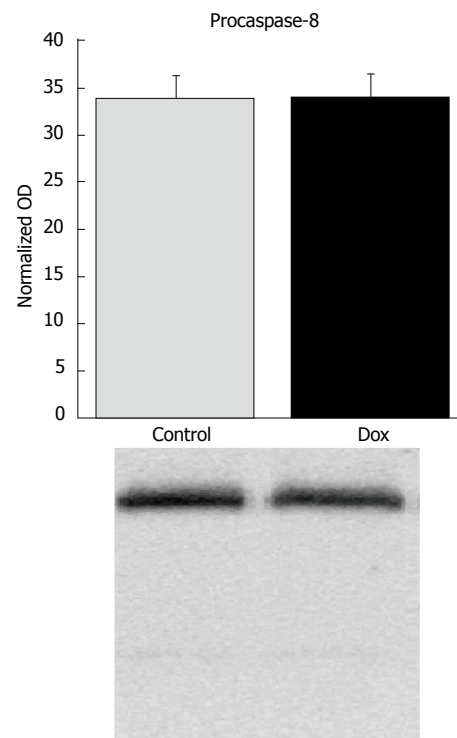
Aside from its implicated role in apoptosis, caspase-12 has been shown to play a role in the inflammatory process[28]. Caspase-12 is a dominant negative effector of caspase-1. Since we observed processing of caspase-12, we assessed expression and activation of caspase-1 to determine if processing of caspase-12 affected expression or activation of caspase-1. Dox treatment did not affect expression of procaspase-1 (52.9 ± 10.33 *vs* 38.2 ± 6.23, control *vs* Dox, respectively, *P* = 0.22). There was no statistical difference in the presence of lower molecular weight bands between treatment groups (data not shown), suggesting that Dox did not lead to its activation (Figure 7).

Markers of apoptosis: Caspase-3 is considered the central executioner of apoptosis and cleaves a plethora of substrates which lead to the demise of the cell and to the classical morphological characteristics of apoptosis, such as DNA fragmentation. Thus, we measured caspase-3 activity to assess activation of apoptosis. Dox did not affect the activity of caspase-3 activity in treated animals (0.193 ± 0.061) compared to control animals (0.134 ± 0.016, *P* = 0.45, Figure 8A). PARP-1 is an enzyme involved in DNA repair. Upon stimulation of apoptosis, PARP-1 is cleaved by various proteases, including caspase-3, in order to disengage the repair process. Thus, PARP-1 cleavage
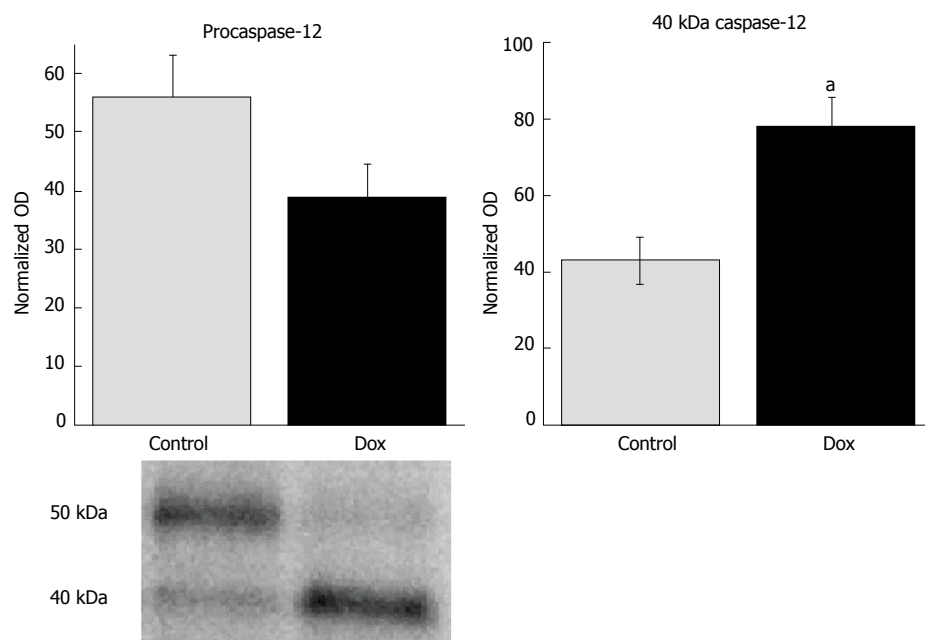
Figure 6 Protein content of procaspase-12 and 40 kDa cleavage product. Doxorubicin (Dox) treatment did not significantly affect the hepatic content of procaspase-12. However, Dox did induce activation of caspase-12 leading to increased content of the 40 kDa cleavage product. Representative Western blot shows the 50 kDa procaspase-12 and the 40 kDa cleaved product. [a]$P < 0.050$, Dox *vs* control.
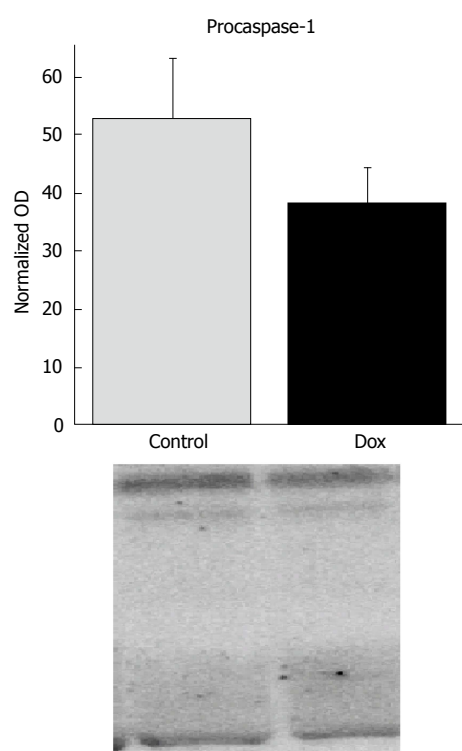

Figure 7 Protein content of procaspase-1. Doxorubicin (Dox) treatment did not affect the hepatic content of procaspase-1. Furthermore, there was no difference in the presence of lower molecular weight bands between treatment groups.

is a commonly used marker of apoptosis. In the current study, Dox treatment did not lead to the cleavage and inactivation of PARP-1. We analyzed PARP-1 and all cleavage products, but only included the data for the 89 kDa cleavage product since this is the product produced *via* cleavage by caspase-3 (88.86 ± 5.83 *vs* 91.1 ± 8.59, control *vs* Dox, respectively, $P = 0.84$, Figure 8B and C). The results suggest that apoptosis did not occur in response to acute Dox treatment, at least 24 h post injection under these experimental conditions. Figure 9 shows Ponceau staining of the samples used in all of the representative Western blots.

DISCUSSION

Recently, it has been shown that cellular stress can cause cellular deacetylation of nuclear and cytoplasmic proteins and that normalization of the acetylation status can protect cells from injury and death[12,29]. Furthermore, caloric restriction, an intervention shown to provoke protection against cellular insults and to increase longevity, increases acetylation status of the mitochondrial proteome in the liver, heart, and kidney[30]. However, the effects were tissue specific in that caloric restriction did not alter acetylation status of the mitochondrial proteome in the brain and caused deacetylation in brown adipose tissue[30]. Since proteome lysine deacetylation may be a marker of cellular stress in various tissues, we investigated the effects of Dox on proteome lysine acetylation status in the liver which was previously unknown. It was found that acute Dox treatment promoted lysine deacetylation which is consistent with the notion that an imbalance in acetylation status favoring deacetylation may signify cellular stress in the liver. Proteins most affected by Dox appear to be those with a molecular weight between 30-80 kDa.

Acetylation status is determined by the activities of HATs and HDACs. Sirt1 and Sirt3 are members of the
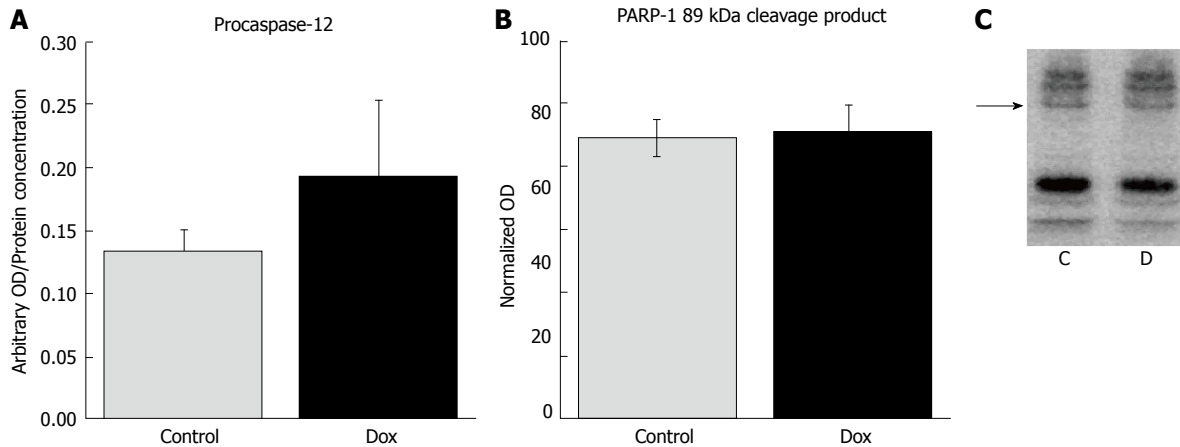
Figure 8 Markers of apoptosis. A: Caspase-3 activity was not affected by Dox treatment; B: Graphical representation of results for the 89 kDa cleavage product of poly (ADP-ribose) polymerase-1 (PARP-1); C: Representative Western blot of PARP-1 cleavage. The arrow indicates the 89 kDa cleavage product. C: Control; D: Dox.
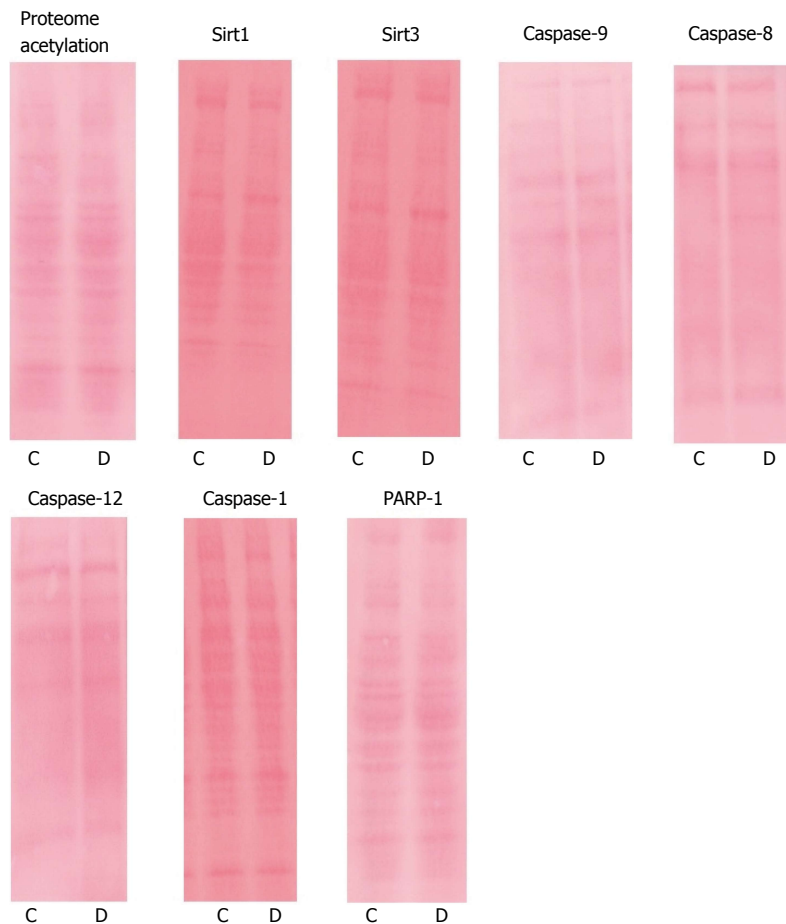
Figure 9 Ponceau staining. Ponceau staining of samples shown in Western blots photos. C: Control; D: Dox.

class Ⅲ HDACs, nicotinamide adenine dinucleotide (NAD⁺)-dependent enzymes, and are the most studied of the seven sirtuins[13]. Sirt1 primarily targets nuclear and some cytoplasmic proteins while Sirt3 targets mitochondrial proteins. Although acute Dox treatment induced proteome lysine deacetylation, Dox did not affect the protein content of Sirt1 or Sirt3 nor the overall HDAC activity. However, it did decrease HAT activity. These results suggest that proteome lysine deacetylation may not be due to a generalized increase in HDAC activity and may rather be due to a decrease in HAT activity.

No studies have previously reported the influence of Dox administration on protein levels of Sirt1 or Sirt3. However, a previous study reported that Dox administration increased the mRNA content of Sirt1 in the heart of treated mice[2]. The discrepancy could be due to tissue specific differences or differences in experimental design. A reduction in food intake and low nutrient availability

increases the expression of Sirt1, as well as Sirt3[31-33]. To control for changes in food intake, we fasted both the control and treatment groups. Zhang *et al* did not control for Dox-induced changes in food intake and thus it cannot be determined if changes in Sirt1 expression are a direct effect of Dox or an indirect effect of anorexia.

Western analysis of Sirt1 revealed two prominent bands of 120 kDa and 45 kDa in the liver. The 120 kDa band corresponds to the expected molecular weight of Sirt1. It is unknown what the 45 kDa species is at this time. However, since this species was responsive to Dox treatment we searched the literature for possibilities. Three studies by the same group reported that Sirt1 can be cleaved under inflammatory conditions into a 75 kDa fragment in chondrocytes[24,34,35]. This fragment is void of deacetylation activity but shown to associate with cytochrome c on the mitochondrial membrane and to prevent apoptosis[24]. The cleavage of human Sirt1 occurs at amino acid residue 533 with the N-terminus being the 75 kDa fragment[34]. Our C-terminus Sirt1 antibody does not detect the N-terminus 75 kDa fragment, but may be detecting the C-terminus 45 kDa fragment. The content of this fragment increased with Dox treatment in the liver, possibly representing an increase in Sirt1 cleavage as a mechanism to prevent apoptosis in stressed hepatocytes. Future investigation is required to identify this Dox-responsive species.

To determine if proteome deacetylation was associated with a proapoptotic environment, as shown in other experimental models, we assessed the content and activation of several apical caspases associated with specific apoptotic stimuli. Activity of caspase-3 and cleavage of PARP-1 were also assessed. To our knowledge, this is the first investigation on the effects of acute Dox administration on the hepatic expression and activation of apical caspases. Our data suggests that acute Dox treatment is not likely associated with apoptosis induction. Dox administration did not stimulate the activation of caspase-9, caspase-8, caspase-3 or induce cleavage of PARP-1 in the liver under these experimental conditions. However, Dox treatment did increase the activation of caspase-12. Caspase-12 has been implicated in both apoptosis and inflammation. Early work suggested that in response to ER stress and calcium dyshomeostasis, caspase-12 can be activated by m-calpain and then induces apoptosis *via* cleavage of caspase-9 with consequent activation of caspase-3[25-27]. Our data suggests that caspase-12 may not be involved in apoptosis induction since we did not detect an increase in caspase-9 cleavage, caspase-3 activity or PARP-1 cleavage. In fact, more recent reports suggest that caspase-12 is incapable of cleaving caspase-9 or caspase-3. It was reported that the catalytic activity of caspase-12 was confined to autoprocessing and was unable to cleave other substrates[28,36]. Thus, caspase-12 may be playing a role in the inflammatory process rather than apoptosis. Caspase-12 has been shown to be an inhibitor of caspase-1. Caspase-12 binds procaspase-1 and prevents its activation and consequent cytokine process-

ing[28]. Both procaspase-12 and the processed form of caspase-12 have been found to be a part of the caspase-1 inhibitory complex[28]. However, the role of caspase-12 cleavage in regulating the inflammatory response is unknown at this time, but has been suggested as a means for temporal limitation of the inhibitory effect of caspase-12[28]. The effects of Dox on the expression and activation of caspase-1 were assessed, but we did not observe an effect. Procaspase-12 has also been shown to have an inhibitory effect on NF-κB[37]. Thus, autoprocessing of caspase-12 may lead to decreased suppression of NF-κB and increased expression of anti-apoptotic proteins helping to protect the liver from Dox-induced apoptosis. This hypothesis is speculative and further studies are required to discern the role of caspase-12 in Dox-induced injury. In summary, lysine deacetylation likely is not associated with an apoptotic environment. However, a limitation is the lack of deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to verify the lack of apoptosis induction. With this limitation, it cannot be conclusively ruled out that apoptosis independent of caspase activation or PARP-1 cleavage did not occur.

Although, our results suggest that Dox does not likely induce apoptosis, at least at this early time point under these experimental conditions, a previous study reported that Dox may induce apoptosis in the liver *via* the accumulation of p53, decreased expression of Bcl-xL, and cytochrome c release from mitochondria[5]. Cytochrome c release stimulates apoptosis *via* caspase-9 activation, however, they did not assess caspase-9 activity. Our results differ from Patel *et al*[5] in that we found that Dox does not increase caspase-9 activation. In the current study, we report that Dox increases the content of procaspase-9 with no evidence of cleavage products. We have also found that Dox decreases the activity of caspase-9 and mitophagy in hepatic tissue of these animals (data will be published elsewhere). We hypothesize that the decreased caspase-9 activity may be due to increased mitophagy, by the elimination of damaged mitochondria susceptible to cytochrome c release[38] and thus may be a protective response to an initial insult induced by Dox. Our results may contrast from Patel et al. due to differences in experimental design as there were several relevant disparities[5]. First, Patel *et al*[5] used an extremely high dose of Dox (60 mg/kg) which was three times the dose used in the current study. Secondly, our animals were fasted to control for the anorexic effect of Dox, while Patel *et al*[5] did not account for this variable. Therefore, the Dox animals were severely deficient in nutrients while the control animals were not. Thirdly, Patel *et al*[5] assessed Dox induced hepatic damage 48 h after treatment, while we examined toxicity at 24 h post treatment. It is possible that hepatic apoptosis may occur at a later time point due to the indirect effects of progressing peripheral organ damage, such as progression of heart failure. Animals treated with Dox usually show signs of heart failure within days of treatment which varies depending on the dose administered. Heart failure is known to cause liver damage[39].

Alternatively, complete fasting (compared to a 50%-70% reduction) of the animals had an unintended protective effect against Dox-induced hepatic apoptosis. Recently, it was shown that fasting prior to Dox treatment, but then fed ad libitum upon treatment, protected against cardiac toxicity by means of autophagy induction[40]. The absence of an ad-libitum fed Dox group and control group does not allow the determination of the effects of fasting on the variables assessed in our study. Yet, the objective was simply to eliminate this external variable. More studies are necessary to determine if fasting extends similar protection against hepatotoxicity.

Early effects of acute Dox treatment include altered proteome acetylation homeostasis favoring lysine deacetylation. The mechanism of proteome lysine deacetylation likely involves decreased activity of HATs, rather than increased activity of HDACs. In our experimental model, lysine deacetylation does not appear to be associated with an apoptotic environment. Acute Dox treatment did not increase cleavage of caspase-9, cleavage of caspase-8, cleavage of PARP-1, or caspase-3 activity. Dox treatment did stimulate caspase-12 activation, however, its activation does not appear to play a role in apoptosis induction. Thus, early mechanisms of hepatic toxicity may not involve induction of apoptosis, at least under these experimental conditions. The liver may be able to engage an early protective response against an initial Dox-induced insult. Further damage to the liver, which may include apoptosis, may occur at later time points as damage to peripheral organs progresses and may be an indirect effect of Dox. Alternatively, fasting of the animals in our experimental design may have provoked a protective response which prevented apoptosis. Further studies are required to discern the role of proteome lysine deacetylation, as well as the role of caspase-12, in Dox-induced hepatic toxicity. It is not clear whether lysine deacetylation and caspase-12 activation contribute to toxicity or play a role in a protective response. Clarification will elucidate the potential of acetylation homeostasis and caspase-12 as therapeutic targets against Dox-induced toxicity.

COMMENTS

Background
Doxorubicin-induced toxicity is a major cause of morbidity and mortality in cancer survivors. Thus, mechanisms of toxicity is essential to elucidate in order to prevent doxorubicin-induced tissue damage in vital organs.

Research frontiers
Proteome lysine deacetylation has been shown to be associated with cellular injury in various experimental models. Normalization of the acetylation status has been shown to prevent cell death. However, the role of altered proteome lysine acetylation status in doxorubicin-induced toxicity has not been previously studied.

Innovations and breakthroughs
Doxorubicin treatment has been found to alter the hepatic proteome acetylation status. However, the modulation of the acetylation status was not associated with apoptosis, as shown in other experimental models. The results lay the groundwork for future studies to determine the role of proteome lysine deacetylation in doxorubicin-induced toxicity. Furthermore, caspase-12 was identified as having a role in doxorubicin-induced hepatic toxicity, which may be either pathological or protective.

Applications
Proteome lysine acetylation and caspase-12 may be potential therapeutic targets to prevent doxorubicin-induced toxicity.

Terminology
Acetylation is a common posttranslational modification in which an acetyl group is covalently attached to a protein. Apoptosis is cellular suicide or programmed cell death.

Peer Review
The authors describe the evaluation of the effect of doxorubicin on proteome acetylation status and found that decreased proteome lysine acetylation was associated with decreased activity of histone deacetylases. Overall, the paper is well written.

REFERENCES

1   **Pointon AV**, Walker TM, Phillips KM, Luo J, Riley J, Zhang SD, Parry JD, Lyon JJ, Marczylo EL, Gant TW. Doxorubicin in vivo rapidly alters expression and translation of myocardial electron transport chain genes, leads to ATP loss and caspase 3 activation. *PLoS One* 2010; **5**: e12733 [PMID: 20856801 DOI: 10.1371/journal.pone.0012733]

2   **Zhang S**, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012; **18**: 1639-1642 [PMID: 23104132 DOI: 10.1038/nm.2919]

3   **Childs AC**, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2: Bax ratio. *Cancer Res* 2002; **62**: 4592-4598 [PMID: 12183413]

4   **Koçkar MC**, Nazıroğlu M, Celik O, Tola HT, Bayram D, Koyu A. N-acetylcysteine modulates doxorubicin-induced oxidative stress and antioxidant vitamin concentrations in liver of rats. *Cell Biochem Funct* 2010; **28**: 673-677 [PMID: 21104935 DOI: 10.1002/cbf.1707]

5   **Patel N**, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. *Toxicol Appl Pharmacol* 2010; **245**: 143-152 [PMID: 20144634 DOI: 10.1016/j.taap.2010.02.002]

6   **el-Missiry MA**, Othman AI, Amer MA, Abd el-Aziz MA. Attenuation of the acute adriamycin-induced cardiac and hepatic oxidative toxicity by N-(2-mercaptopropionyl) glycine in rats. *Free Radic Res* 2001; **35**: 575-581 [PMID: 11767415 DOI: 10.1080/10715760100301581]

7   **Kalender Y**, Yel M, Kalender S. Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats. The effects of vitamin E and catechin. *Toxicology* 2005; **209**: 39-45 [PMID: 15725512 DOI: 10.1016/j.tox.2004.12.003]

8   **Henninger C**, Huelsenbeck J, Huelsenbeck S, Grösch S, Schad A, Lackner KJ, Kaina B, Fritz G. The lipid lowering drug lovastatin protects against doxorubicin-induced hepatotoxicity. *Toxicol Appl Pharmacol* 2012; **261**: 66-73 [PMID: 22712078 DOI: 10.1016/j.taap.2012.03.012]

9   **Yagmurca M**, Bas O, Mollaoglu H, Sahin O, Nacar A, Karaman O, Songur A. Protective effects of erdosteine on doxorubicin-induced hepatotoxicity in rats. *Arch Med Res* 2007; **38**: 380-385 [PMID: 17416283 DOI: 10.1016/j.arcmed.2007.01.007]

10 **Ladas EJ**, Jacobson JS, Kennedy DD, Teel K, Fleischauer A, Kelly KM. Antioxidants and cancer therapy: a systematic review. *J Clin Oncol* 2004; **22**: 517-528 [PMID: 14752075 DOI: 10.1200/JCO.2004.03.086]

11 **Kim SC**, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* 2006; **23**: 607-618 [PMID: 16916647 DOI: 10.1016/j.molcel.2006.06.026]

12 **Li Y**, Alam HB. Creating a pro-survival and anti-inflammatory phenotype by modulation of acetylation in models of

hemorrhagic and septic shock. *Adv Exp Med Biol* 2012; **710**: 107-133 [PMID: 22127890 DOI: 10.1007/978-1-4419-5638-5_11]

13   **Suzuki T**. 'Explorative Study on Isoform-Selective Histone Deacetylase Inhibitors'. *Chem Pharm Bull* (Tokyo) 2009; **57**: 897-906 [DOI: 10.1248/cpb.57.897]

14   **Chowdhury I**, Tharakan B, Bhat GK. Caspases - an update. *Comp Biochem Physiol B Biochem Mol Biol* 2008; **151**: 10-27 [PMID: 18602321 DOI: 10.1016/j.cbpb.2008.05.010]

15   **Freireich EJ**, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Rep* 1966; **50**: 219-244 [PMID: 4957125]

16   **Glass B**, Kloess M, Bentz M, Schlimok G, Berdel WE, Feller A, Trümper L, Loeffler M, Pfreundschuh M, Schmitz N. Dose-escalated CHOP plus etoposide (MegaCHOEP) followed by repeated stem cell transplantation for primary treatment of aggressive high-risk non-Hodgkin lymphoma. *Blood* 2006; **107**: 3058-3064 [PMID: 16384932 DOI: 10.1182/blood-2005-04-1570]

17   **Gilliam LA**, Ferreira LF, Bruton JD, Moylan JS, Westerblad H, St Clair DK, Reid MB. Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. *J Appl Physiol* (1985) 2009; **107**: 1935-1942 [PMID: 19779154 DOI: 10.1152/japplphysiol.00776.2009]

18   **Sorensen M**, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, Barja G. Effects of fasting on oxidative stress in rat liver mitochondria. *Free Radic Res* 2006; **40**: 339-347 [PMID: 16517498 DOI: 10.1080/10715760500250182]

19   **Marczuk-Krynicka D**, T Hryniewiecki, J Piatek, and J Paluszak. 'The Effect of Brief Food Withdrawal on the Level of Free Radicals and Other Parameters of Oxidative Status in the Liver'. *Med Sci Monit* 2003; **9**: 131-135

20   **Zhang YK**, Wu KC, Klaassen CD. Genetic activation of Nrf2 protects against fasting-induced oxidative stress in livers of mice. *PLoS One* 2013; **8**: e59122 [PMID: 23527105 DOI: 10.1371/journal.pone.0059122]

21   **Rambold AS**, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci USA* 2011; **108**: 10190-10195 [PMID: 21646527 DOI: 10.1073/pnas.1107402108]

22   **Romero-Calvo I**, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustin O, de Medina FS. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 2010; **401**: 318-320 [PMID: 20206115 DOI: 10.1016/j.ab.2010.02.036]

23   **Houtkooper RH**, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 2012; **13**: 225-238 [PMID: 22395773]

24   **Oppenheimer H**, Gabay O, Meir H, Haze A, Kandel L, Liebergall M, Gagarina V, Lee EJ, Dvir-Ginzberg M. 75-kd sirtuin 1 blocks tumor necrosis factor α-mediated apoptosis in human osteoarthritic chondrocytes. *Arthritis Rheum* 2012; **64**: 718-728 [PMID: 21987377 DOI: 10.1002/art.33407]

25   **Nakagawa T**, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000; **150**: 887-894 [PMID: 10953012 DOI: 10.1083/jcb.150.4.887]

26   **Nakagawa T**, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; **403**: 98-103 [PMID: 10638761 DOI: 10.1038/47513]

27   **Morishima N**, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002; **277**: 34287-34294 [PMID: 12097332 DOI: 10.1074/jbc.M204973200]

28   **Roy S**, Sharom JR, Houde C, Loisel TP, Vaillancourt JP, Shao W, Saleh M, Nicholson DW. Confinement of caspase-12 proteolytic activity to autoprocessing. *Proc Natl Acad Sci USA* 2008; **105**: 4133-4138 [PMID: 18332441 DOI: 10.1073/pnas.0706658105]

29   **Li Y**, Alam HB. Modulation of acetylation: creating a pro-survival and anti-inflammatory phenotype in lethal hemorrhagic and septic shock. *J Biomed Biotechnol* 2011; **2011**: 523481 [PMID: 21403879]

30   **Schwer B**, Eckersdorff M, Li Y, Silva JC, Fermin D, Kurtev MV, Giallourakis C, Comb MJ, Alt FW, Lombard DB. Calorie restriction alters mitochondrial protein acetylation. *Aging Cell* 2009; **8**: 604-606 [PMID: 19594485 DOI: 10.1111/j.1474-9726.2009.00503.x]

31   **Noriega LG**, Feige JN, Canto C, Yamamoto H, Yu J, Herman MA, Mataki C, Kahn BB, Auwerx J. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. *EMBO Rep* 2011; **12**: 1069-1076 [PMID: 21836635 DOI: 10.1038/embor.2011.151]

32   **Palacios OM**, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward JL, 3rd, L. J. Goodyear, and. Q. Tong, 'Diet and Exercise Signals Regulate Sirt3 and Activate Ampk and Pgc-1alpha in Skeletal Muscle'. *Aging* (Albany NY) 2009; **1**: 771-83

33   **Hayashida S**, Arimoto A, Kuramoto Y, Kozako T, Honda S, Shimeno H, Soeda S. Fasting promotes the expression of SIRT1, an NAD+ -dependent protein deacetylase, via activation of PPARalpha in mice. *Mol Cell Biochem* 2010; **339**: 285-292 [PMID: 20148352 DOI: 10.1007/s11010-010-0391-z]

34   **Dvir-Ginzberg M**, Gagarina V, Lee EJ, Booth R, Gabay O, Hall DJ. Tumor necrosis factor α-mediated cleavage and inactivation of SirT1 in human osteoarthritic chondrocytes. *Arthritis Rheum* 2011; **63**: 2363-2373 [PMID: 21305533 DOI: 10.1002/art.30279]

35   **Gabay O**, Oppenhiemer H, Meir H, Zaal K, Sanchez C, Dvir-Ginzberg M. Increased apoptotic chondrocytes in articular cartilage from adult heterozygous SirT1 mice. *Ann Rheum Dis* 2012; **71**: 613-616 [PMID: 22258484 DOI: 10.1136/ard.2011.200504]

36   **Lee HJ**, Lee SH, Park SH, Sharoar MG, Shin SY, Lee JS, Cho B, Park IS. Purification of catalytically active caspase-12 and its biochemical characterization. *Arch Biochem Biophys* 2010; **502**: 68-73 [PMID: 20646990 DOI: 10.1016/j.abb.2010.07.013]

37   **Labbé K**, Miu J, Yeretssian G, Serghides L, Tam M, Finney CA, Erdman LK, Goulet ML, Kain KC, Stevenson MM, Saleh M. Caspase-12 dampens the immune response to malaria independently of the inflammasome by targeting NF-kappaB signaling. *J Immunol* 2010; **185**: 5495-5502 [PMID: 20876354 DOI: 10.4049/jimmunol.1002517]

38   **Yang Y**, Xing D, Zhou F, Chen Q. Mitochondrial autophagy protects against heat shock-induced apoptosis through reducing cytosolic cytochrome c release and downstream caspase-3 activation. *Biochem Biophys Res Commun* 2010; **395**: 190-195 [PMID: 20361931 DOI: 10.1016/j.bbrc.2010.03.155]

39   **Alvarez AM**, Mukherjee D. Liver abnormalities in cardiac diseases and heart failure. *Int J Angiol* 2011; **20**: 135-142 [PMID: 22942628 DOI: 10.1055/s-0031-1284434]

40   **Kawaguchi T**, Takemura G, Kanamori H, Takeyama T, Watanabe T, Morishita K, Ogino A, Tsujimoto A, Goto K, Maruyama R, Kawasaki M, Mikami A, Fujiwara T, Fujiwara H, Minatoguchi S. Prior starvation mitigates acute doxorubicin cardiotoxicity through restoration of autophagy in affected cardiomyocytes. *Cardiovasc Res* 2012; **96**: 456-465 [PMID: 22952253 DOI: 10.1093/cvr/cvs282]

Apoptosis induced by Fas signaling does not alter hepatic hepcidin expression

Sizhao Lu, Emily Zmijewski, John Gollan, Duygu Dee Harrison-Findik

Sizhao Lu, Emily Zmijewski, John Gollan, Duygu Dee Harrison-Findik, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5820, United States
Sizhao Lu, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198-5820, United States
Correspondence to: Duygu Dee Harrison-Findik, Associate Professor, Department of Internal Medicine, University of Nebraska Medical Center, 95820 UNMC, Omaha, NE 68198-5820, United States. dharrisonfindik@unmc.edu
Telephone: +1-402-5596355 Fax: +1-402-5596494

Abstract

AIM: To determine the regulation of human hepcidin (*HAMP*) and mouse hepcidin (*hepcidin-1* and *hepcidin-2*) gene expression in the liver by apoptosis using *in vivo* and *in vitro* experimental models.

METHODS: For the induction of the extrinsic apoptotic pathway, HepG2 cells were treated with various concentrations of CH11, an activating antibody for human Fas receptor, for 12 h. Male C57BL/6NCR and C57BL/6J strains of mice were injected intraperitoneally with sublethal doses of an activating antibody for mouse Fas receptor, Jo2. The mice were anesthetized and sacrificed 1 or 6 h after the injection. The level of apoptosis was quantified by caspase-3 activity assay. Liver injury was assessed by measuring the levels of ALT/AST enzymes in the serum. The acute phase reaction in the liver was examined by determining the expression levels of *IL-6* and *SAA3* genes by SYBR green quantitative real-time PCR (qPCR). The phosphorylation of transcription factors, Stat3, Smad4 and NF-κB was determined by western blotting. Hepcidin gene expression was determined by Taqman qPCR. The binding of transcription factors to *hepcidin-1* promoter was studied using chromatin immunoprecipitation (ChIP) assays.

RESULTS: The treatment of HepG2 cells with CH11 induced apoptosis, as shown by the significant activation of caspase-3 ($P < 0.001$), but did not cause any significant changes in *HAMP* expression. Short-term (1 h) Jo2 treatment (0.2 μg/g *b.w.*) neither induced apoptosis and acute phase reaction nor altered mRNA expression of mouse *hepcidin-1* in the livers of C57BL/6NCR mice. In contrast, 6 h after Jo2 injection, the livers of C57BL/6NCR mice exhibited a significant level of apoptosis ($P < 0.001$) and an increase in SAA3 ($P < 0.023$) and IL-6 ($P < 0.005$) expression in the liver. However, mRNA expression of *hepcidin-1* in the liver was not significantly altered. Despite the Jo2-induced phosphorylation of Stat3, no occupancy of *hepcidin-1* promoter by Stat3 was observed, as shown by ChIP assays. Compared to C57BL/6NCR mice, Jo2 treatment (0.2 μg/g *b.w.*) of C57BL/6J strain mice for 6 h induced a more prominent activation of apoptosis, liver injury and acute phase reaction. Similar to C57BL/6NCR mice, the level of liver *hepcidin-1* mRNA expression in the livers of C57BL/6J mice injected with a sublethal dose of Jo2 (0.2 μg/g *b.w.*) remained unchanged. The injection of C57BL/6J mice with a higher dose of Jo2 (0.32 μg/g *b.w.*) did not also alter hepatic hepcidin expression.

CONCLUSION: Our findings suggest that human or mouse hepcidin gene expression is not regulated by apoptosis induced *via* Fas receptor activation in the liver.

apoptosis; Stat3

Core tip: Apoptosis and Fas signaling participate in the pathogenesis of various liver diseases. Iron also contributes to liver injury. Changes in the expression of hepcidin, a key iron regulatory hormone, has been reported in various liver diseases. Recently, apoptosis has been implicated in the regulation of hepcidin. This study investigates effector caspase activation and apoptosis induced by Fas receptor signaling and its relationship to hepatic hepcidin expression.

INTRODUCTION

Apoptosis is involved in the pathogenesis of various liver diseases[1]. Hepatocyte apoptosis can be activated *via* the extrinsic apoptotic pathway through the binding of ligands to death receptors such as Fas, TNF receptor 1 and TRAIL receptor 2. Upon ligand binding, the receptor will trimerize and the C-terminal death domain will recruit Fas-associated protein with death domain to form death-inducing signaling complex (DISC), which subsequently recruits procaspase-8 and induces its self-cleavage and activation. Activated caspase-8 can directly cleave and activate caspase-3, the executioner caspase, which is responsible for the cleavage of target proteins to execute apoptosis. Caspase-3 activation is frequently used as a marker for apoptosis. Flice-Inhibitory Protein Long form (FLIPL) blocks apoptosis by inhibiting the recruitment and autoproteolytic cleavage of procaspase-8. In addition, in hepatocytes, the signal from death receptor can be amplified through the mitochondrial (intrinsic) apoptotic pathway. Activated caspase-8 can cleave Bcl-2 family protein, Bid. Truncated Bid (tBid) activates proapoptotic Bcl-2 family proteins, and induces permeabilization of the mitochondrial outer membrane and the leakage of the mitochondrial content including cytochrome c. Cytochrome c forms a complex with apoptotic peptidase activating factor 1, recruits and activates caspase-9, which subsequently cleaves caspase-3 and executes apoptosis.

A role for apoptosis has been suggested in the regulation of hepcidin[2,3]. Hepcidin, an antimicrobial peptide synthesized primarily by the liver, is the central regulator of iron metabolism. It is synthesized as an 84 amino acid precursor peptide, which is then cleaved to its 25 amino acid biologically active circulatory form. Unlike humans, who have one copy of hepcidin gene (*HAMP*), mice have two hepcidin genes, *hepcidin-1* and *hepcidin-2*. Similar to *HAMP*, *hepcidin-1* is involved in the regulation of iron homeostasis but the function of *hepcidin-2* is unknown.

Hepcidin exerts its regulatory function by blocking the uptake and export of dietary iron from the intestine and the release of iron from macrophages. Hepcidin achieves this by binding to ferroportin, the only known iron exporter, and causing its internalization and degradation *via* the lysosomal pathway. The suppression of hepcidin expression in the liver therefore leads to systemic iron overload whereas its induction causes iron deficiency and anemia.

Weizer-Stern *et al*[4] have demonstrated that p53, a tumor suppressor and inducer of apoptosis, participates in the regulation of hepcidin. In their study, a putative p53 response element on hepcidin gene promoter has been identified and validated by chromatin immunoprecipitation assays. Over-expression of p53 in hepatoma cells has been shown to induce hepcidin gene transcription and conversely, the silencing of p53 resulted in down-regulation of hepcidin expression[4]. It is however unclear whether p53-mediated apoptosis is involved in the regulation of hepatic hepcidin expression[4]. On the other hand, Li *et al*[5] have suggested a role for Fas signaling in the regulation of hepcidin expression in tissue culture cells and female mouse livers. A lethal dose of anti-Fas activating antibody, Jo2 has been reported to exert an immediate stimulatory and a late suppression effect on hepcidin mRNA expression in the liver[5]. Although a relationship between FLIPL, IL-6, Stat3 and hepcidin expression has been shown, they did not however establish a direct correlation between apoptosis and hepcidin. Besides committing cell death, Fas induced DISC formation also participates in the activation of cell signaling pathways, including IL-6 and NF-κB[6]. Of note, hepcidin expression is regulated by various signaling pathways. As an acute phase protein, hepcidin is stimulated by endotoxin and inflammatory cytokine signaling[7-9]. The effect of IL-6 is mediated through the activation of Jak/Stat pathway and the binding of Stat3 to hepcidin gene promoter[10,11]. As an iron regulatory protein, hepcidin is also regulated by the signals from iron sensors, such as bone morphogenetic protein 6 (BMP6)[12-14]. The BMP receptor-specific Smad pathway (*via* the phosphorylation of transcription factors, Smad1/5/8) has been shown to be involved in the up-regulation of hepcidin transcription. BMP6 knockout mice exhibit iron overload and reduced hepcidin expression[15-17]. Similarly, mice lacking the expression of the common Smad protein, Smad4 exhibit iron overload and a dramatic decrease in the expression of hepcidin in the liver[18]. In addition, growth factors such as epidermal growth factor and hepatocyte growth factor suppress the expression of hepcidin by inhibiting the signaling of the BMP-Smad pathway[19].

The aim of this study is to investigate the causal relationship between Fas-signaling-induced effector caspase activation and apoptosis, and the regulation of human and mouse hepcidin gene transcription. These studies will help us to further understand the regulation and the role of hepcidin in liver diseases.

Table 1 Taqman probe and primer sequences for quantitative real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Taqman probe
HAMP	TGCCCATGTTCCAGAGGC	CCGCAGCAGAAAATGCAGAT	AAGGAGGCGAGACACCCACTTCCC
Human Gapdh	TGAAGGTCGGAGTCAACGG	AGAGTTAAAAGCAGCCCTGGTG	TTTGGTCGTATTGGGCGCCTGG
Hepcidin-1	TGCAGAAGAGAAGGAAGAGAGACA	CACACTGGGAATTGTTACAGCATT	CAACTTCCCCATCTGCATCTTCTGCTGT
Hepcidin-2	GCGATCCCAATGCAGAAGAG	TGTTACAGCACTGACAGCAGAATC	AGGAAGAGAGACATCAACTTCCCCATCTGC
Mouse Gapdh	TCACTGGCATGGCCTTCC	GGCGGCACGTCAGATCC	TTCCTACCCCCAATGTGTCCGTCG

Table 2 Primer sequences for SYBR green quantitative real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mouse SAA3	GCCTGGGCTGCTAAAGTCAT	TGCTCCATGTCCCGTGAAC
Mouse IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Mouse Gapdh	GTGGAGATTGTTGCCATCAACGA	CCCATTCTCGGCCTTGACTGT

MATERIALS AND METHODS

Cell culture experiments

HepG2 human hepatoma cells were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with glutamine and 10% fetal calf serum (Atlantic Biologicals). 1.3×10^6 or 3.9×10^6 cells were seeded in 25 cm^2 or 75 cm^2 flasks, respectively 24 h prior to experiments. Cells were exposed to an activating antibody, which is specific for human Fas (clone CH11, Millipore) at different concentrations for 12 h.

Animal experiments

Animal experiments were approved by the Animal Ethics Committee at the University of Nebraska Medical Center. C57BL/6J (the Jackson Laboratory) and C57BL/6NCR (NIH) strain male mice were maintained on a standard chow diet. 6 wk to 8 wk old male mice were injected intraperitoneally (*i.p.*) with an activating antibody, which is specific for mouse Fas (clone Jo2, BD Biosciences), at 0.2 μg or 0.32 μg per gram of body weight (*b.w.*). As control for *i.p.* injections, which per se might cause an acute phase reaction, a group of mice were injected with similar volume of 0.9% NaCl. All mice were sacrificed 1 or 6 h following injections.

RNA isolation, cDNA synthesis, and real-time quantitative PCR analysis

RNA isolation, cDNA synthesis and quantitative PCR (qPCR) were performed, as described previously[20]. The sequence of Taqman fluorescent probe [5'6-(FAM); 3' (TAMRA-Q)] and primers, and SYBR green primers are shown in Tables 1 and 2.

Detection of apoptosis

Caspase-3 activity assays were performed with Ac-DEVD-AMC caspase-3 fluorogenic substrate (BD Biosciences) as described previously[21].

Measurement of liver enzymes

Blood collected from the right atrium of the anesthetized animal before sacrifice and allowed to coagulate at room temperature for 30 min was centrifuged at $1300 \times g$ for 10 min to remove blood cells. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) liver enzyme levels in mice sera were measured at Clinical Chemistry Laboratory at University of Nebraska Medical Center.

Western Blotting

The isolation of nuclear lysates from the livers and western blotting were performed, as described previously[22]. Anti-phospo-Stat3, anti-Stat3, anti-phospho-Smad 1/5, anti-p65 and Gapdh primary antibodies, and secondary antibodies were obtained commercially (Santa Cruz, Cell Signaling).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed, as described previously[22,23]. Briefly, chromatin, isolated from formalin-fixed livers, was sheared by sonication and an aliquot was saved as total input DNA. Sheared chromatin was incubated with anti-Stat3, anti-Smad4 antibodies or control IgG and protein A-conjugated agarose beads (Santa Cruz, Cell signaling). Eluted DNA and total input DNA were subsequently analyzed by PCR using primers (sense: 5'- gccatactgaaggcactga -3'; antisense: 5'- gtgtggtggctgtctagg -3') to amplify a 358bp region of the mouse *hepcidin-1* promoter.

Statistical analysis

SPSS software was used for statistical analysis. The significance of difference between two groups was determined by Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS

Apoptosis and human hepcidin gene (HAMP) expression

CH11 antibody treatment for 12 h induced apoptosis in HepG2 cells in a concentration dependent manner, as confirmed with caspase-3 activity assay. A significant induction of caspase-3 activity was observed at 50
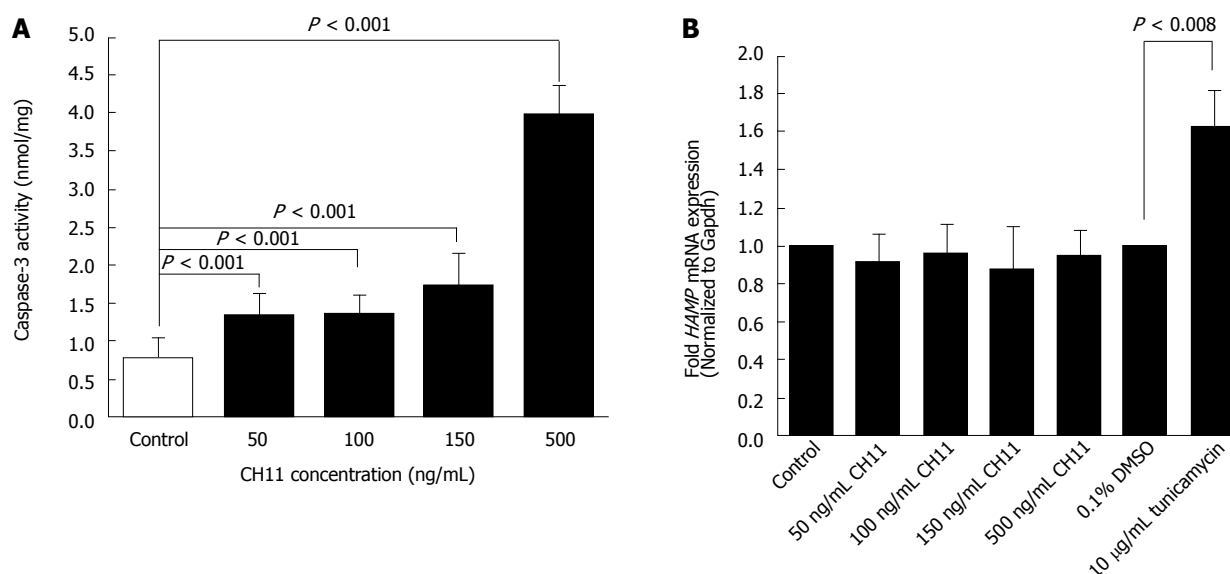
Figure 1 Caspase-3 activity and human hepcidin gene (*HAMP*) expression: HepG2 cells were treated with 50, 100, 150 and 500 ng/mL of CH11 antibody or solvent (control) for 12 h. A: Induction of apoptosis was confirmed by measuring caspase-3 activity, as described in the 'Materials and Methods' section. Caspase-3 activity was expressed as nanomole of flurogenic substrate cleaved per milligram of cell lysate protein; B: cDNA, synthesized from RNA isolated from CH11-treated, tunicamycin-treated and respective solvent-treated cells, was employed as a template in Taqman qPCR assays to determine *HAMP* mRNA expression, as described in Methods. *HAMP* expression in CH11-treated cells was expressed as fold expression of that in control cells.

ng/mL CH11 concentration, which increased four-fold with a 500 ng/mL concentration (Figure 1A). The level of *HAMP* mRNA expression in CH11-treated HepG2 cells was similar to that of control cells, as determined by qPCR (Figure 1B). HepG2 cells treated with 10 μg/mL of tunicamycin, a known inducer of hepcidin gene expression, for 8 h were used as the positive control (Figure 1B). These findings strongly suggest that the Fas-mediated apoptotic pathway does not alter *HAMP* transcription.

The effect of short-term (1 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6NCR mice

Since activation of human Fas did not alter human hepcidin gene expression, we performed experiments with activating antibody specific to mouse Fas, Jo2. C57BL/6NCR mice were injected with Jo2 antibody (0.2 μg/g *b.w.*) or NaCl (control), and sacrificed 1 h later. No significant increase in caspase-3 activity was observed in Jo2-injected mice compared to control mice (Figure 2A). The activation of acute phase reaction in the livers of these mice was evaluated by determining the levels of IL-6 and SAA3 mRNA expression by qPCR. Similar to caspase-3 activity, no significant changes were observed with the expression of these acute phase reaction genes (Figure 2B and C). The mRNA expression of *hepcidin-1* in mice livers was also unaltered by Jo2 exposure, as confirmed by qPCR (Figure 2D). However, 1 h Jo2 treatment induced a small but significant increase in *hepcidin-2* mRNA expression (Figure 2E).

The effect of longer (6 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6NCR mice

To further study the effect of Fas-mediated apoptosis,

C57BL/6NCR mice were sacrificed 6 h after Jo2 or NaCl injections. A significant increase in caspase-3 activity was observed in the livers of Jo2-injected mice (Figure 3A). Similarly, the expression of acute phase reaction markers, IL-6 and SAA3 were also elevated in mice exposed to Jo2 for 6 h compared to control mice (Figures 3B and C). The levels of *hepcidin-1* and *hepcidin-2* mRNA expression in mice treated with Jo2 for 6 h were not significantly different from that in control mice (Figures 3D and E).

Jo2-mediated Stat3, Smad1/5 activation and hepcidin-1 promoter occupancy

The cytokine, IL-6 is known to activate hepcidin transcription *via* the Jak/Stat3 signaling pathway[10,11].We therefore investigated the activation of Stat3 in mice injected with Jo2 antibody (0.2 μg/g *b.w.*) or NaCl and sacrificed 1 or 6 h later. Six hours, but not 1 h of Jo2 treatment, was sufficient to induce the phosphorylation of Stat3 in the livers of mice, compared to respective control mice (Figure 4). Despite the activation of Stat3, we did not observe any significant changes in Stat3 binding *to hepcidin-1* promoter in Jo2-treated mice compared to control mice, as determined by ChIP assays (Figure 5).

NF-κB is one of the important transcription factors activated by Fas ligand binding. NF-κB activates the transcription of inflammatory cytokines including IL-6[1]. We therefore investigated the phosphorylation of the p65 subunit of NF-κB in mice treated with Jo2 for 1 or 6 h time periods. In contrast to Stat3, Jo2 induced a fast and transient activation of NF-κB. The phosphorylation of p65 in the liver was observed within 1 h after Jo2 injection and was absent at 6 h after Jo2 exposure (Figure 4).

Besides Jak/Stat3 pathway, hepcidin is also regulated by bone morphogenetic protein 6 (BMP6) and Smad
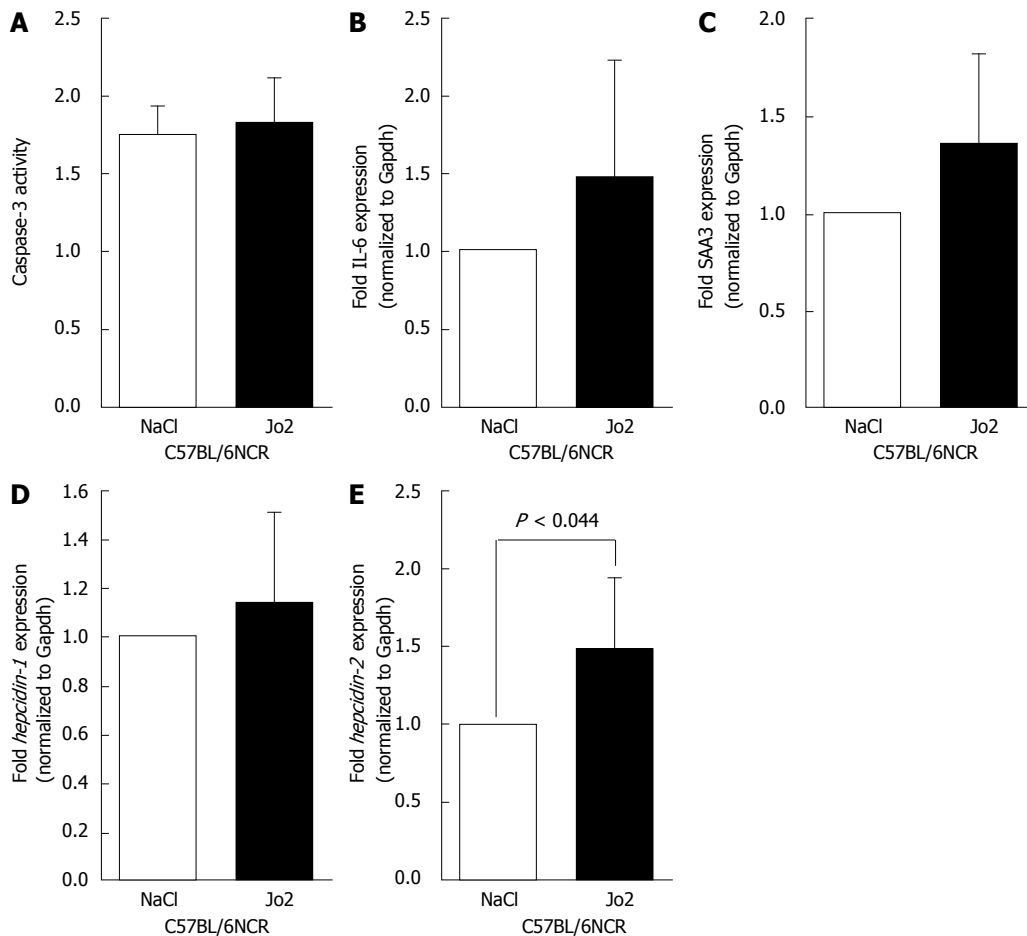
Figure 2 Caspase-3 activity, the expression of acute phase response and mouse hepcidin genes in the liver: C57BL/6NCR strain male mice, which were injected with Jo2 antibody (0.2 μg/g *b.w.*) or saline (as control), were sacrificed 1 h later. A: Caspase-3 activity was measured, as described in the 'Materials and Methods' section. B-E: *IL-6* (B), *SAA3* (C), *hepcidin-1* (D) and *hepcidin-2* (E) gene expression was determined by qPCR, and mRNA expression in Jo2-treated mice expressed as fold change of that in control mice.

pathway. This pathway has also been suggested to play a negative role in growth factor-induced regulation of hepcidin expression in the liver[19]. We therefore determined the activation of transcription factors, Smad1 and Smad5, which are activated downstream of BMP signaling pathway. Similar to NF-κB, Jo2 treatment induced an early and transient activation of Smad1/5 in the liver. The induction in Smad1/5 phosphorylation observed by 1 h Jo2 exposure was significantly weakened by 6 h after Jo2 injection (Figure 4). The binding of Smad4, the common mediator of Smad signaling, to mouse *hepcidin-1* promoter was also examined by ChIP assays. No significant increase in Smad4 occupancy of *hepcidin-1* promoter region harboring a Smad4 binding site was observed at 6 h after Jo2 injection, as compared to controls (Figure 5).
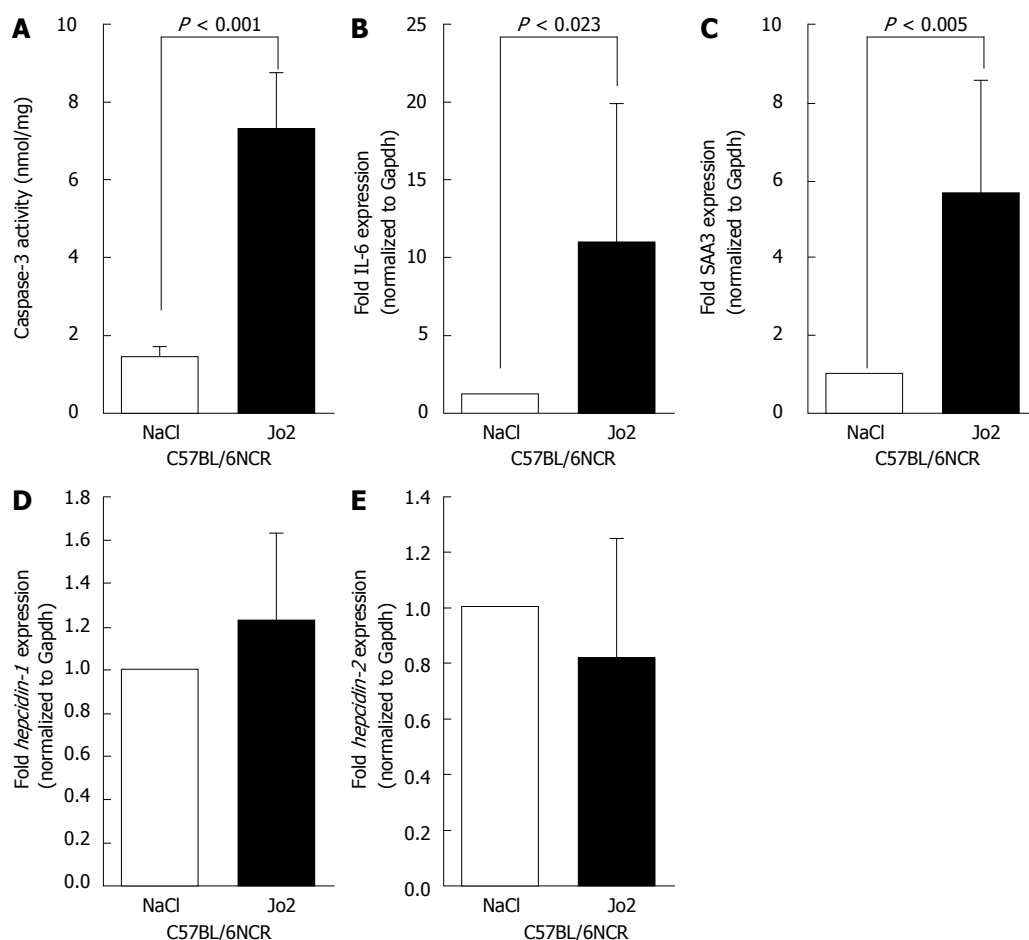
The effect of longer (6 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6J mice

In order to investigate the effect of Jo2 further, we employed a substrain of C57BL/6 mice. Of note, C57BL/6NCR and C57/BL/6J strains exhibit substantial genetic differences[24]. Compared to that observed with C57BL/

6NCR, C57BL/6J mice treated with Jo2 (0.2 μg/g *b.w.*) for 6 h exhibited a significantly higher elevation of caspase-3 activity (Figure 6A). Similar robust activation was also observed with the expression of acute phase marker genes, *IL-6* and *SAA3* (Figures 6B and C). However, despite stronger apoptosis and acute phase reactions, Jo2 treatment did not induce any significant changes in the expression of both *hepcidin-1* and *hepcidin-2* in the livers of C57BL/6J mice, as was the case with C57BL/6NCR mice (compare Figures 6D, E and 3D, E). Furthermore, the treatment of C57BL/6J mice with an even higher concentration of Jo2 (0.32 μg/g *b.w.*) did not induce any changes in the mRNA level of *hepcidin-1*. (Figure 6D). However, the treatment with 0.32 μg/g of Jo2 induced a significant suppression of *hepcidin-2* mRNA expression (Figure 6E).

The effect of Jo2 on liver enzymes in C57BL/6J and C57BL/6NCR mice

Jo2-induced apoptosis and acute phase reaction was stronger in the livers of C57BL/6J mice, compared to C57BL/6NCR mice. We therefore measured the serum levels of liver enzymes, ALT and AST, which is a com-

Figure 3 The effect of longer Jo2 treatment on the level of apoptosis, acute phase response and hepcidin gene expression: C57BL/6NCR male mice, which were injected with Jo2 (0.2 μg/g *b.w.*) or saline (control), were sacrificed 6 h later. A-C: Cell lysates and RNA isolated from the livers were employed in caspase-3 assays (A) or to synthesize cDNA as a template for SYB green qPCR assays to determine *IL-6* (B) or *SAA3* (C) gene expression; D, E: *Hepcidin-1* (D) and *hepcidin-2* (E) mRNA expression was determined by Taqman qPCR. Gene expression in Jo2-injected mice was expressed as fold change of that in control mice.
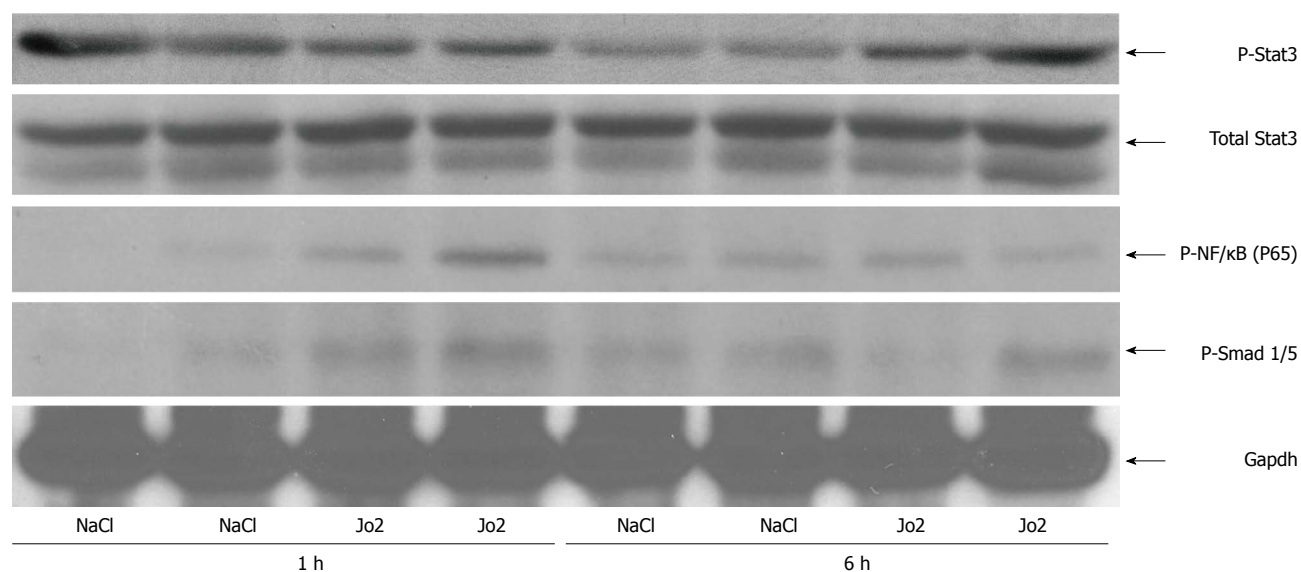
Figure 4 Phosphorylation of Stat3, NF-κB (P65) and Smad 1/5 in the liver. Whole cell lysates prepared from the livers of C57BL/6NCR mice injected with Jo2 (0.2 μg/g *b.w.*) and sacrificed after 1 or 6 h were employed for western blotting using anti-phospho--Stat3 (P-Stat3), anti-total Stat3, anti-phospho-P65 (P65) and anti-phospho-Smad1/5 antibodies, as described in Material and Methods. Anti-Gapdh antibody was used as protein loading control.
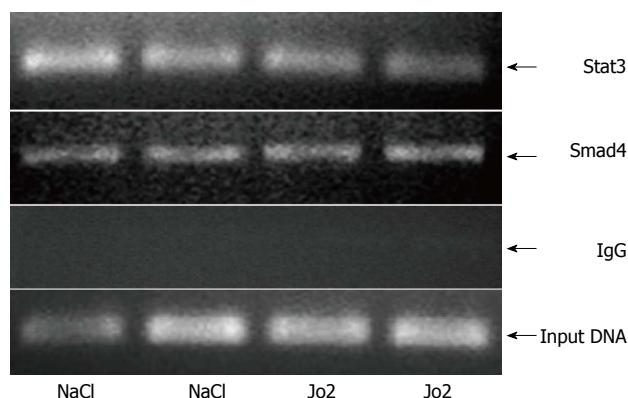
Figure 5 Chromatin immunoprecipitation assays. The binding of Stat3 or Smad4 to the *hepcidin-1* promoter in the livers of C57BL/6NCR mice, which were injected with Jo2 (0.2 µg/g *b.w.*) or saline (control) and sacrificed 6 h later, was determined by ChIP assays, as described in Material and Methods. Total input DNA was used as control to evaluate the amount of chromatin.

monly used diagnostic test to determine liver function and injury. Jo2 injected at a concentration of 0.2 µg/g did not cause a significant elevation of serum ALT or AST levels in C57BL6/NCR mice (Figures 7). However, the sera of C57BL/6J mice injected with 0.2 µg/g of Jo2, exhibited a dramatic increase in both ALT and AST levels, compared to controls (Figures 7). The injection of C57BL/6J mice with a higher dose of Jo2 (0.32 µg/g) induced further increase in serum ALT and AST levels (data not shown).

DISCUSSION

Apoptosis is one of the key factors which contribute to the pathogenesis of many liver diseases[1,25,26]. Apoptosis not only causes hepatocyte death directly but also induces inflammation and hepatic fibrosis[27,28]. The inhibition of caspase enzymes *via* known caspase inhibitors has been shown to effectively alleviate hepatocyte apoptosis and tissue damage in animal models of liver injury[29]. Due to its highly reactive nature and the liver serving as the major storage organ for it, iron is considered an important secondary risk factor in the progression of various liver diseases[30,31]. Therefore, it is of great importance to understand the interaction between apoptosis and iron metabolism. Since hepcidin is the central regulator of iron homeostasis and is primarily synthesized in the liver, this study investigated the effect of apoptosis on the regulation of hepcidin expression in the liver. Previously, Weizer-Stern *et al*[4] have elegantly demonstrated that p53, a tumor suppressor gene and an inducer of apoptosis, elevates human hepcidin gene transcription in HepG2 cells by binding to the corresponding response elements in hepcidin gene promoter. They have also reported that the overexpression of p53 blunts the stimulatory effect of IL-6 on hepcidin gene expression. Although indirect, these findings, for the first time, suggested a relationship between apoptosis and hepcidin and thereby the regulation of iron metabolism. However, due to various

reasons, the *in vivo* relevance of this potential interaction is unclear. First, apoptosis signaling in cancer cell lines is frequently distorted and secondly, forced overexpression of p53 might have caused artificial effects. However, in a recent study, Li *et al*[5] have investigated the relationship between Fas -activated apoptosis signaling and expression of hepcidin gene expression. Fas activation decreased both mouse and human hepcidin mRNA expression *in vitro*. They have also shown that Balb/C3 female mice injected with lethal dose of Jo2 antibody (which killed mice within 4 h) exhibit a biphasic regulation of mouse hepcidin mRNA expression in the liver, namely an immediate elevation (within 0.5-1 h) followed by a suppression (within 4 h). They suggested that these changes in hepcidin expression correlates with the changes in FLIPL and IL-6 expression as well as the activation of the transcription factors, NF-κB, and Stat3. The knock-down or over-expression of FLIPL exerted a negative and a positive effect, respectively, on hepcidin expression. Based on their data, Li *et al*[5] have proposed a model suggesting that the stimulatory effect of Fas on hepcidin expression is achieved *via* IL-6 and Stat3, which themselves are activated by FLIPL and NF-κB. However, Li *et al*[5] did not confirm the presence (and the level) of apoptosis in the livers of Jo2-injected mice. Hence, it is unclear whether Fas-mediated apoptosis is directly involved in the regulation of hepcidin gene expression in the liver.

In our current study, we examined the effect of Fas signaling on hepatic hepcidin gene expression both *in vivo* and *in vitro*. In *in vitro* studies, the effect of CH11, an activating antibody specific for human Fas, was evaluated on hepcidin expression in HepG2 hepatoma cells. Even though CH11 induced apoptosis in a concentration dependent manner, as confirmed by the increased caspase-3 activity, the expression of human hepcidin gene was not significantly altered in these cells. Although we cannot exclude the possibility that Fas-mediated signaling in HepG2 hepatoma cells might be different than primary human hepatocytes, our findings strongly suggest that hepcidin gene expression in hepatocytes does not correlate with the significant induction of caspase activation. Of note, the liver is composed of various cell types and it is therefore feasible that not only hepatocytes, but other cells such as Kupffer cells, might be involved in the regulation of hepcidin gene by apoptosis. Hence, an *in vivo* experimental model whereby male C57BL/6 mice are injected with Jo2 antibody to specifically activate Fas-mediated apoptosis was employed to study hepcidin gene expression in whole liver. Male mice were chosen for these studies because unlike humans, female mice express higher levels of hepcidin compared to male mice[32]. Sublethal concentrations of Jo2 antibody were chosen for our experiments because based on the reports in the literature, this dose of Jo2 is more suitable for studies, which investigate the activation of Fas-mediated apoptosis in liver diseases[33]. Accordingly, we observed no lethality under our experimental conditions. Mice with short-term (1 h) Jo2 treatment did not display any significant
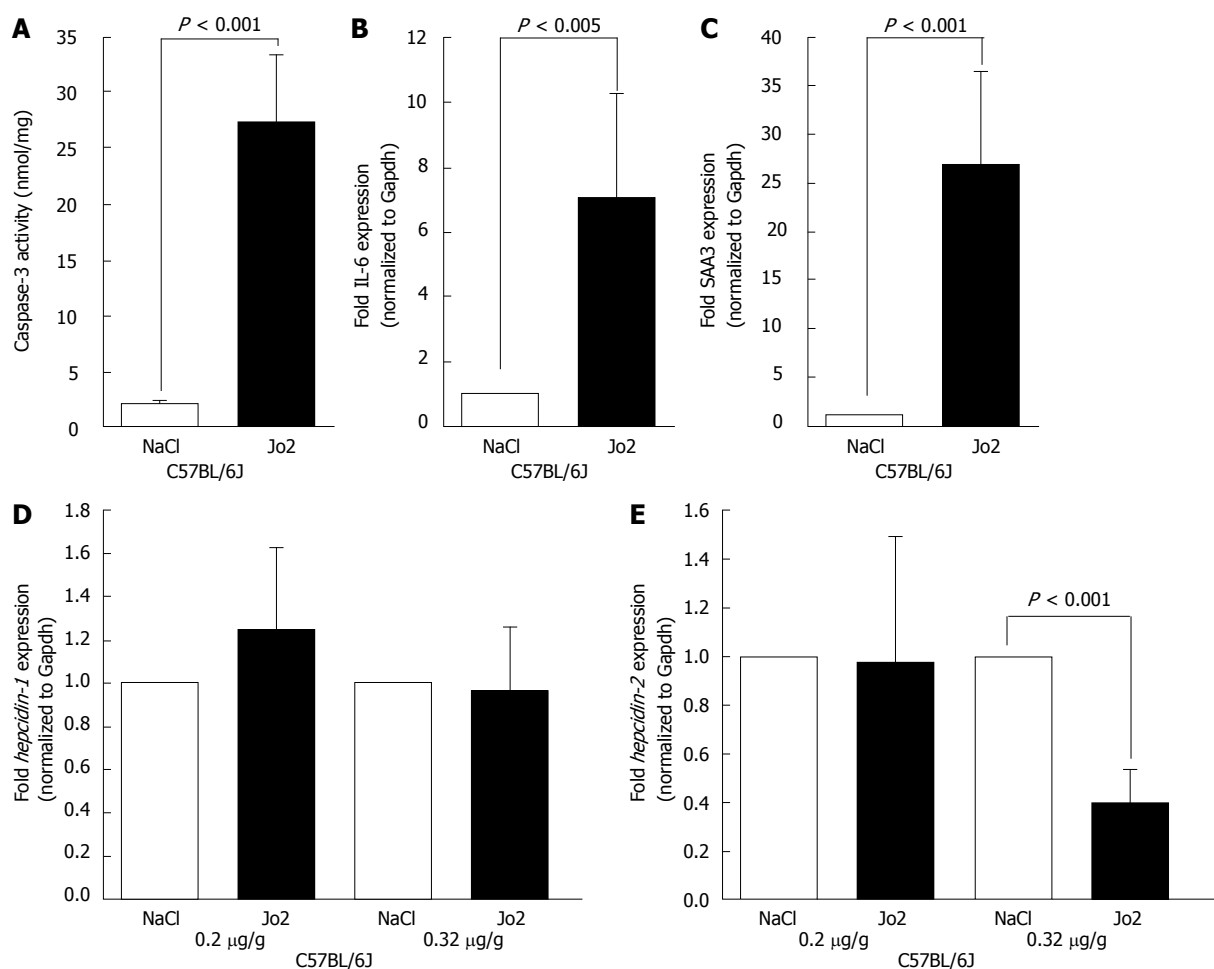
Figure 6 The effect of Jo2 on apoptosis, acute phase response and hepcidin gene expression in C57BL/6J mice. C57BL/6J male mice (*n* = 21) were injected with 0.2 μg/g *b.w.* and 0.32 μg/g *b.w.* Jo2 or saline and sacrificed 6 h later. Cell lysates and RNA isolated from the livers were employed in caspase-3 assays (A) or to synthesize cDNA as a template for SYB green qPCR assays to determine *IL-6* (B) or *SAA3* (C) gene expression. *Hepcidin-1* (D) and *hepcidin-2* (E) mRNA expression was determined by Taqman qPCR. Gene expression in Jo-2 injected mice was expressed as fold change of that in control mice.
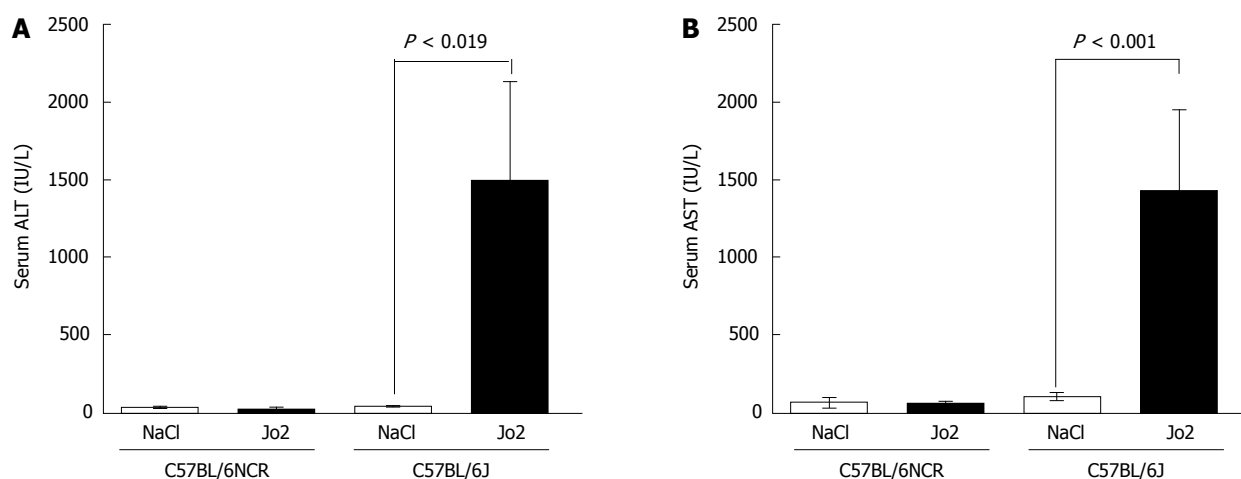
Figure 7 Comparison of serum liver enzyme levels in Jo2-injected C57BL/6J and C57BL/6NCR mice. Mice injected with Jo2 (0.2 μg/g *b.w.*) were sacrificed 6 h later. Serum ALT (A) and AST (B) enzymes were measured, as described in "Materials and Methods".

changes both in the activity of caspase-3 enzyme and the expression of acute phase marker genes, *IL-6* and *SAA3*, strongly suggesting the absence of apoptosis and acute phase responses in the livers of these mice. The mac-roscopic appearance of the livers from short-term Jo2-injected mice was also similar to those in control mice (data not shown). Further, the expression level of mouse *hepcidin-1* was not affected in the livers of these mice. In

contrast, longer treatment with Jo2 (6 h) significantly induced apoptosis and acute phase reaction. Concurrently, the livers of these mice displayed macroscopic differences such as a darker color suggesting the presence of hepatic hemorrhage and liver injury (data not shown). Interestingly, these changes did not correlate with the level of *hepcidin-1* mRNA expression in the liver. Similar to short-term, the longer treatment of mice with Jo2 did not alter the level of hepcidin gene expression.

It is well known that hepcidin gene transcription is strongly stimulated by IL-6 and Stat3 pathway. Since, we have shown that long-term, but not short-term, Jo2 treatment can induce acute phase reactions in the liver, the phosphorylation status of Stat3 was investigated to confirm its activation. In accordance with our acute phase gene expression findings, we observed Stat3 phosphorylation following 6 h, but not 1 h of Jo2 treatment. Taken together, our findings show that the activation of IL-6/Stat3 axis by Fas is not sufficient to induce *hepcidin-1* transcription and strongly suggest the presence of inhibitory mechanisms. This is also supported by our ChIP findings, which show that the occupancy of *hepcidin-1* promoter by Stat3 is similar in the livers of both Jo2-treated and control mice despite the differences in the activation status of Stat3. In contrast to Stat3, the phosphorylation of NF-κB was observed with short-term, but not long-term, Jo2 treatment. NF-κB is involved in inflammatory cytokine production in the liver including IL-6[26]. It is therefore possible that Jo-2-mediated early phase activation of NF-κB subsequently facilitates the induction of IL-6 transcription and consequent activation of Stat3, which was observed in the livers of mice with longer (6 h) Jo2 treatment.

Hepcidin is also activated by BMP/Smad pathway and Smad4 knockout mice display reduced hepcidin expression[18]. However, 6 h Jo2 administration did not significantly alter the phosphorylation of Smad1/5 proteins, which are transcription factors known to be activated by BMP pathway. Growth factors have been shown to suppress the signaling of BMP/Smad pathway and its stimulatory effect on hepcidin gene expression in the liver[19]. Of note, liver injury is known to stimulate the expression of growth factors as part of the liver regeneration process. It is therefore feasible that Fas-induced liver injury might suppress Smad activation and thereby counteract the stimulation of *hepcidin-1* transcription by Stat3. However, it should also be noted that despite significant differences in the level of liver injury, acute phase response and apoptosis, both C57BL/6J and C57BL/6NCR mice (under similar experimental conditions) did not display any significant changes in liver *hepcidin-1* expression. This suggests that mechanisms other than growth factors and inhibitory Smads might play a role in this process. Jo2-induced liver damage accompanied by DNA damage and the activation of p53 might be involved since p53 has been shown to suppress the stimulatory effect of IL-6 on hepcidin gene expression[4]. Furthermore, we observed differential regulation of *hepcidin-2* expression by Jo2 in

the liver. Since the function of *hepcidin-2* is unknown, the significance of this finding and its potential role in liver injury and disease will be addressed in future studies.

In conclusion, Fas ligand-induced signaling and apoptosis do not play a significant role in the regulation of human (*HAMP*) or mouse (*hepcidin-1*) hepatic hepcidin gene expression, as shown by *in vitro* and *in vivo* experimental systems using human or mouse Fas receptor-activating antibodies, CH11 or Jo2 , respectively. The induction of extrinsic apoptotic pathway *via* Fas receptor signaling, as confirmed by effector caspase activation, significantly induced the phosphorylation and activation of the transcription factor, Stat3 in the liver. Stat3 is well known to be involved in inflammation-mediated elevation of hepcidin expression. However, no significant binding of Stat3 to hepcidin gene promoter was observed, as confirmed by chromatin immunoprecipitation studies. Our findings strongly suggest that the activation of Stat3 by Fas signaling-mediated apoptosis is not sufficient to stimulate hepcidin transcription in the liver. Using different strains of mice, we were also able to confirm that the severity of Fas-induced apoptosis (acute phase reaction or tissue injury) does not correlate with its effect on hepcidin gene expression in the liver. Interestingly, Jo2 treatment induced changes in *hepcidin-2* expression in a time-dependent manner but the function of this mouse gene is as yet unknown.

COMMENTS

Background
Apoptosis is widely observed and participates in the pathogenesis of various liver diseases. Iron, due to its highly reactive nature as a transitional metal, acts as a secondary risk factor in various liver diseases. Hepcidin, a small peptide primarily synthesized in the liver, is the central regulator of iron metabolism. Hepcidin maintains iron homeostasis by inhibiting iron absorption by the enterocytes in the duodenum and iron release by the macrophages of reticuloendothelial system. Hepcidin expression in the liver has been shown to be modulated by iron, inflammation and hypoxia but a direct role of apoptosis in hepcidin regulation has not been elucidated.

Research frontiers
Iron plays a role in liver injury and the transcriptional regulation of hepcidin gene in liver diseases has been highlighted by recent studies from various laboratories. A better understanding of the regulation of hepcidin and thereby iron homeostasis in the pathogenesis of liver diseases might facilitate the development of novel diagnosis and treatment strategies.

Innovations and breakthroughs
P53, a tumor suppressor and inducer of apoptosis, has been reported to promote hepcidin gene transcription through direct binding to its promoter. An independent study has recently suggested the involvement of Fas signaling in the regulation of liver hepcidin expression. However, a causal relationship between Fas-mediated effector caspase activation and apoptosis, and the regulation of hepcidin gene transcription has not been demonstrated. This study addressed this question by quantifying the level of Fas-induced apoptosis by caspase-3 activity assays and correlating it to both human and mouse hepcidin gene expression. In addition, different strains of mice with significant variances in their response to Fas treatment were also employed. Collectively, the authors' findings clearly demonstrate the lack of correlation between Fas-mediated apoptosis and hepatic hepcidin gene transcription.

Applications
The findings of this study, which strongly suggest that Fas-mediated apoptosis is not involved in the regulation of hepcidin expression, will further our understanding of the pathogenesis of liver diseases associated with increases in he-

patic iron content. Furthermore, the authors' findings, which demonstrate strain-specific responses to anti-Fas treatment, highlights the importance of choosing optimal mice strains for studies with Fas signaling in the liver.

Terminology

Apoptosis, also known as programmed cell death, is characterized by the sequential activation of a series of caspases, which also serve as markers for apoptosis. The pathogenesis of many liver diseases involve apoptotic pathways. Hepcidin is a small antimicrobial peptide synthesized mainly by the hepatocytes. It regulates iron homeostasis by binding to and inducing the degradation of the only known iron exporter, ferroportin.

Peer review

In this paper, Lu *et al* aimed to determine the regulation of human hepcidin (*HAMP*) and mouse hepcidin (*hepcidin-1* and *hepcidin-2*) gene expression in the liver by apoptosis using *in vivo* and *in vitro* experimental models. The role of hepcidin in liver fibrosis, *via* apoptosis, has emerged in recent years. Given the goal of achieving an explanation about the role of hepcidin is a growing concern, the analysis is justified and the aim of the study is clinically relevant. It is a well-designed study and the conclusions are consistent with the results.

REFERENCES

1   **Schattenberg JM**, Galle PR, Schuchmann M. Apoptosis in liver disease. *Liver Int* 2006; **26**: 904-911 [PMID: 16953829 DOI: 10.1111/j.1478-3231.2006.01324.x]

2   **Ganz T**. Hepcidin and iron regulation, 10 years later. *Blood* 2011; **117**: 4425-4433 [PMID: 21346250 DOI: 10.1182/blood-2011-01-258467]

3   **Ganz T**, Nemeth E. Hepcidin and disorders of iron metabolism. *Annu Rev Med* 2011; **62**: 347-360 [PMID: 20887198 DOI: 10.1146/annurev-med-050109-142444]

4   **Weizer-Stern O**, Adamsky K, Margalit O, Ashur-Fabian O, Givol D, Amariglio N, Rechavi G. Hepcidin, a key regulator of iron metabolism, is transcriptionally activated by p53. *Br J Haematol* 2007; **138**: 253-262 [PMID: 17593032 DOI: 10.1111/j.1365-2141.2007.06638.x]

5   **Li X**, Xu F, Karoopongse E, Marcondes AM, Lee K, Kowdley KV, Miao CH, Trobridge GD, Campbell JS, Deeg HJ. Allogeneic transplantation, Fas signaling, and dysregulation of hepcidin. *Biol Blood Marrow Transplant* 2013; **19**: 1210-1219 [PMID: 23707854 DOI: 10.1016/j.bbmt.2013.05.012]

6   **Guicciardi ME**, Gores GJ. Life and death by death receptors. *FASEB J* 2009; **23**: 1625-1637 [PMID: 19141537 DOI: 10.1096/fj.08-111005]

7   **Villarroel P**, Le Blanc S, Arredondo M. Interleukin-6 and lipopolysaccharide modulate hepcidin mRNA expression by HepG2 cells. *Biol Trace Elem Res* 2012; **150**: 496-501 [PMID: 23065424 DOI: 10.1007/s12011-012-9522-6]

8   **Nemeth E**, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* 2003; **101**: 2461-2463 [PMID: 12433676 DOI: 10.1182/blood-2002-10-3235]

9   **Nemeth E**, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004; **113**: 1271-1276 [PMID: 15124018 DOI: 10.1172/JCI200420945]

10 **Verga Falzacappa MV**, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood* 2007; **109**: 353-358 [PMID: 16946298 DOI: 10.1182/blood-2006-07-033969]

11 **Pietrangelo A**, Dierssen U, Valli L, Garuti C, Rump A, Corradini E, Ernst M, Klein C, Trautwein C. STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; **132**: 294-300 [PMID: 17241879 DOI: 10.1053/j.gastro.2006.10.018]

12 **Babitt JL**, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. Bone morphogenetic protein signaling

13 **Truksa J**, Peng H, Lee P, Beutler E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc Natl Acad Sci USA* 2006; **103**: 10289-10293 [PMID: 16801541 DOI: 10.1073/pnas.0603124103]

14 **Lin L**, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. *Blood* 2007; **110**: 2182-2189 [PMID: 17540841 DOI: 10.1182/blood-2007-04-087593]

15 **Shanmugam NK**, Cherayil BJ. Serum-induced up-regulation of hepcidin expression involves the bone morphogenetic protein signaling pathway. *Biochem Biophys Res Commun* 2013; **441**: 383-386 [PMID: 24157792 DOI: 10.1016/j.bbrc.2013.10.065]

16 **Andriopoulos B**, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet* 2009; **41**: 482-487 [PMID: 19252486 DOI: 10.1038/ng.335]

17 **Meynard D**, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet* 2009; **41**: 478-481 [PMID: 19252488 DOI: 10.1038/ng.320]

18 **Wang RH**, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab* 2005; **2**: 399-409 [PMID: 16330325 DOI: 10.1016/j.cmet.2005.10.010]

19 **Goodnough JB**, Ramos E, Nemeth E, Ganz T. Inhibition of hepcidin transcription by growth factors. *Hepatology* 2012; **56**: 291-299 [PMID: 22278715 DOI: 10.1002/hep.25615]

20 **Harrison-Findik DD**, Klein E, Crist C, Evans J, Timchenko N, Gollan J. Iron-mediated regulation of liver hepcidin expression in rats and mice is abolished by alcohol. *Hepatology* 2007; **46**: 1979-1985 [PMID: 17763462 DOI: 10.1002/hep.21895]

21 **Harrison-Findik DD**, Lu S, Zmijewski EM, Jones J, Zimmerman MC. Effect of alcohol exposure on hepatic superoxide generation and hepcidin expression. *World J Biol Chem* 2013; **4**: 119-130 [PMID: 24340135 DOI: 10.4331/wjbc.v4.i4.119]

22 **Gerjevic LN**, Liu N, Lu S, Harrison-Findik DD. Alcohol Activates TGF-Beta but Inhibits BMP Receptor-Mediated Smad Signaling and Smad4 Binding to Hepcidin Promoter in the Liver. *Int J Hepatol* 2012; **2012**: 459278 [PMID: 22121494 DOI: 10.1155/2012/459278]

23 **Weinmann AS**, Bartley SM, Zhang T, Zhang MQ, Farnham PJ. Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol Cell Biol* 2001; **21**: 6820-6832 [PMID: 11564866 DOI: 10.1128/MCB.21.20.6820-6832.2001]

24 **Mekada K**, Abe K, Murakami A, Nakamura S, Nakata H, Moriwaki K, Obata Y, Yoshiki A. Genetic differences among C57BL/6 substrains. *Exp Anim* 2009; **58**: 141-149 [PMID: 19448337 DOI: 10.1538/expanim.58.141]

25 **Rust C**, Gores GJ. Apoptosis and liver disease. *Am J Med* 2000; **108**: 567-574 [PMID: 10806286 DOI: 10.1016/S0002-9343(00)00370-3]

26 **Chakraborty JB**, Oakley F, Walsh MJ. Mechanisms and biomarkers of apoptosis in liver disease and fibrosis. *Int J Hepatol* 2012; **2012**: 648915 [PMID: 22567408 DOI: 10.1155/2012/648915]

27 **Canbay A**, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. Apoptotic body engulfment by a human stellate cell line is profibrogenic. *Lab Invest* 2003; **83**: 655-663 [PMID: 12746475 DOI: 10.1097/01.LAB.0000069036.63405.5C]

28 **Canbay A**, Feldstein A, Baskin-Bey E, Bronk SF, Gores GJ. The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. *J Pharmacol Exp*

Ther 2004; **308**: 1191-1196 [PMID: 14617689 DOI: 10.1124/jpet.103.060129]

29   **Natori S**, Higuchi H, Contreras P, Gores GJ. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. *Liver Transpl* 2003; **9**: 278-284 [PMID: 12619025 DOI: 10.1053/jlts.2003.50019]

30   **Fargion S**, Valenti L, Fracanzani AL. Beyond hereditary hemochromatosis: new insights into the relationship between iron overload and chronic liver diseases. *Dig Liver Dis* 2011; **43**: 89-95 [PMID: 20739232 DOI: 10.1016/j.dld.2010.07.006]

31   **Datz C**, Felder TK, Niederseer D, Aigner E. Iron homeo-

stasis in the metabolic syndrome. *Eur J Clin Invest* 2013; **43**: 215-224 [PMID: 23289518 DOI: 10.1111/eci.12032]

32   **Vujic Spasic M**, Kiss J, Herrmann T, Kessler R, Stolte J, Galy B, Rathkolb B, Wolf E, Stremmel W, Hentze MW, Muckenthaler MU. Physiologic systemic iron metabolism in mice deficient for duodenal Hfe. *Blood* 2007; **109**: 4511-4517 [PMID: 17264297 DOI: 10.1182/blood-2006-07-036186]

33   **Haga S**, Terui K, Zhang HQ, Enosawa S, Ogawa W, Inoue H, Okuyama T, Takeda K, Akira S, Ogino T, Irani K, Ozaki M. Stat3 protects against Fas-induced liver injury by redox-dependent and -independent mechanisms. *J Clin Invest* 2003; **112**: 989-998 [PMID: 14523036 DOI: 10.1172/JCI17970]

INSTRUCTIONS TO AUTHORS

GENERAL INFORMATION

World Journal of Biological Chemistry (*World J Biol Chem*, *WJBC*, online ISSN 1949-8454, DOI: 10.4331), is a peer-reviewed open access (OA) academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

Aims and scope

WJBC is to rapidly report the most recent developments in the research by the close collaboration of biologists and chemists in area of biochemistry and molecular biology, including: general biochemistry, pathobiochemistry, molecular and cellular biology, molecular medicine, experimental methodologies and the diagnosis, therapy, and monitoring of human disease.

We encourage authors to submit their manuscripts to *WJBC*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

WJBC is edited and published by Baishideng Publishing Group (BPG). BPG has a strong professional editorial team composed of science editors, language editors and electronic editors. BPG currently publishes 43 OA clinical medical journals, including 42 in English, has a total of 15471 editorial borad members or peer reivewers, and is a world first-class publisher.

Columns

The columns in the issues of *WJBC* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These

commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in biological chemistry; (12) Brief Articles: To briefly report the novel and innovative findings in biological chemistry; (13) Meta-Analysis: To evaluate the clinical effectiveness in biological chemistry by using data from two or more randomised control trials; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJBC*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of biological chemistry; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

Editor-in-chief
Jingfang Ju, PhD, Associate Professor, Director, Department of Pathology and Stony Brook University Cancer Center, Stony Brook University-SUNY, Stony Brook, NY 11794, United States

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director

SPECIAL STATEMENT

All articles published in journals owned by the BPG represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated..

Biostatistical editing

Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including t-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (n). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the P value (if it indicates statistical significance).

Conflict-of-interest statement

In the interests of transparency and to help reviewers assess any potential bias, *WJBC* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights

When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of BPG, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://www.wjgnet.com/1949-8454office. Authors are

highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/1949-8454/g_info_20100316155305.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to bpgoffice@wjgnet.com, or by telephone: +86-10-85381892. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece

Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, e.g. Telephone: +86-10-85381892 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJBC*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

There are unstructured abstracts (no less than 200 words) and structured abstracts. The specific requirements for structured abstracts are as follows:

An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present P values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: …*etc*. It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any

footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. [a]$P < 0.05$, [b]$P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, [c]$P < 0.05$ and [d]$P < 0.01$ are used. A third series of P values can be expressed as [e]$P < 0.05$ and [f]$P < 0.01$. Other notes in tables or under illustrations should be expressed as [1]F, [2]F, [3]F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1   **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2   **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic

effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3   **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4   **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5   **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6   21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7   **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8   **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9   Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10   **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11   **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12   **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13   **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14   **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15   Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16   **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning

tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data
Write as mean ± SD or mean ± SE.

Statistical expression
Express t test as t (in italics), F test as F (in italics), chi square test as χ^2 (in Greek), related coefficient as r (in italics), degree of freedom as υ (in Greek), sample number as n (in italics), and probability as P (in italics).

Units
Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1949-8454/g_info_20100309232449.htm.

Abbreviations
Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics
Quantities: t time or temperature, c concentration, A area, l length, m mass, V volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc*.
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc*.
Biology: *H. pylori*, *E coli*, *etc*.

Examples for paper writing
All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15

RESUBMISSION OF THE REVISED MANUSCRIPTS
Authors must revise their manuscript carefully according to the revision policies of BPG. The revised version, along with the signed copyright transfer agreement, responses to the reviewers, and English language Grade B certificate (for non-native speakers of English), should be submitted to the online system via the link contained in the e-mail sent by the editor. If you have any questions about the revision, please send e-mail to esps@wjgnet.com.

Language evaluation
The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A.

Copyright assignment form
Please download a Copyright assignment form from http://www.wjgnet.com/1949-8454/g_info_20100309233100.htm.

Responses to reviewers
Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/1949-8454/g_info_20100309232833.htm.

Proof of financial support
For paper supported by a foundation, authors should provide a copy of the document and serial number of the foundation.

STATEMENT ABOUT ANONYMOUS PUBLICATION OF THE PEER REVIEWERS' COMMENTS
In order to increase the quality of peer review, push authors to carefully revise their manuscripts based on the peer reviewers' comments, and promote academic interactions among peer reviewers, authors and readers, we decide to anonymously publish the reviewers' comments and author's responses at the same time the manuscript is published online.

PUBLICATION FEE
WJBC is an international, peer-reviewed, OA online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium and format, provided the original work is properly cited. The use is non-commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. Publication fee: 698 USD per article. All invited articles are published free of charge.

Baishideng®