ISSN 1881-7815 Online ISSN 1881-7823

BST BioScience Trends

Volume 16, Number 5
October, 2022

IRCA BSSA

IACMHR

www.biosciencetrends.com

BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group. It is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

BioScience Trends devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

BioScience Trends publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Communications, Editorials, News, and Letters on all aspects of the field of life science. All contributions should seek to promote international collaboration.

Editorial Board

Editorial and Head Office

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan
E-mail: office@biosciencetrends.com

BioScience Trends

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

E-mail: office@biosciencetrends.com
URL: www.biosciencetrends.com

Editorial Board Members

CONTENTS

Volume 16, Number 5, 2022

Review

Original Article

Correspondence

Comment

Perspective

Review

DOI: 10.5582/bst.2022.01278

Contemporary oral anticoagulant therapy of patients with atrial fibrillation in China: Status, obstacles, and strategies for improvement

Luxiang Shang[1], Yujiao Zhang[1], Yang Zhao[2], Baopeng Tang[2], Yinglong Hou[1,*]

[1] Department of Cardiology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Shandong Medicine and Health Key Laboratory of Cardiac Electrophysiology and Arrhythmia, Ji'nan, China;
[2] Department of Pacing and Electrophysiology, Xinjiang Key Laboratory of Cardiac Electrophysiology and Remodeling, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China.

SUMMARY Atrial fibrillation (AF) and subsequent stroke and death have become major public health problems in China. Oral anticoagulant (OAC) forms the backbone of prevention of AF-related stroke. However, the quality of OAC use in AF patients in China is not clear. The focus of this narrative review is to summarize the current status of OAC therapy in China and compare it with the studies conducted internationally. In general, most data of OAC use in China were reported around 10-50%, with an increasing proportion of high-risk patients receiving OACs, however, still much lower than those in other countries and regions. Moreover, the phenomenon of inappropriate OAC prescribing and poor long-term persistence and adherence with OAC therapy in AF patients in China have also been noted. The 1-year adherence and persistence of OACs are as low as 50%. Multiple factors from the physicians, patients, and OAC drugs contribute to these phenomena. The management of OACs in AF patients in China needs to be further improved by the joint efforts of healthcare administration (policy makers) and health systems including medical associations, hospitals, and physicians.

Keywords atrial fibrillation, oral anticoagulation, stroke, temporal trends, China.

1. Introduction

Atrial fibrillation (AF) is the most prevalent arrhythmia in clinical practice (*1*). It is well known that one of the major hazards of AF is the significantly increased risk of ischemic stroke, disability, and mortality (*2*). Hence, stroke prevention is central in the management of AF.

Oral anticoagulation (OAC) therapy, involving the use of vitamin K antagonists (VKA, mostly warfarin) and non-VKA oral anticoagulants (NOAC), could reduce thromboembolic events and improve prognosis in patients with AF who have a high stroke risk. Currently, OAC therapy is recommended as a standard treatment for stroke prevention by international guidelines for AF (*3-5*). However, OAC usage is inconsistent on the global and national levels. Although numerous data on OAC use in AF patients in China have been published, a systematic overview and discussion are still lacking. In the present review, we intend to summarize the contemporary OAC use for patients with AF in China and compare it with the studies conducted internationally to help identify which aspects need refining. We also analyzed potential causes of the deficiencies, and explored improvement strategies. Furthermore, we present some recommendations on OAC management during the COVID-19 pandemic.

2. Burden of AF in China

With the aging trends in global population, the incidence and prevalence of AF are increasing (*6*). The Global Burden of Diseases study in 204 countries found that in 2019, there were about 59.7 million (95% uncertainty interval: 45.7 to 75.3 million) prevalent cases of AF and atrial flutter, nearly doubled to the prevalent cases in 1990 (Figure 1A) (*7*). The problem seems to be even worse in China. On one hand, the rapid aging of the population and the increased industrialization and urbanization have resulted in a rapid increase in the prevalence and absolute number of patients with AF in China (Table 1). Two community-based studies showed the weighted prevalence rates of 0.65% (*8*) and 0.77% (*9*) for AF in Chinese adults nearly twenty years ago. A subsequent data from the China Hypertension Survey Group found that prevalence of AF in the

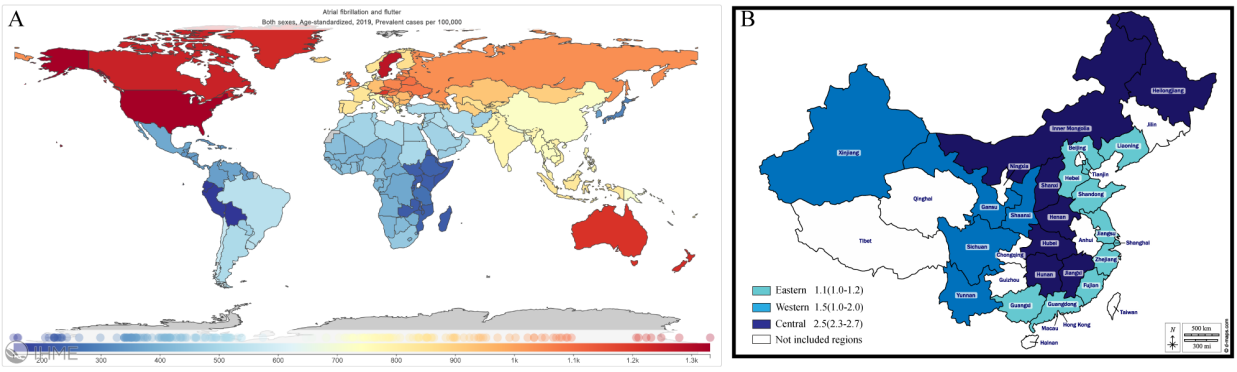

Figure 1. The map of age-standardized AF prevalence in the world (1A) and China (1B). Data in panel A are derived from GBD 2019, and are presented as cases of AF per 100,000 population in both sexes (available from *http://vizhub.healthdata.org/gbd-compare*); data in panel B are from the latest national epidemiology survey of China (Data from Shi S, et al. Lancet Reg Health West Pac. 2022, 11;23:100439. Map data is copyrighted and available from *https://d-maps.com/carte.php?num_car=17503*).

Table 1. Overview of the prevalence of AF in the general population of China

First author	Period	Population (age in years)	Sample size	Age	Male (%)	Num. of AF	Prevalence of AF	*Ref.*
Zhou Z	2003	Adult (≥ 30)	29,079	52.5 ± 22.4	46.6	224	0.65	*(8)*
Li Y	2004	Adult (≥ 35)	19,363	NA	44.6	199	0.77	*(9)*
Wang Z	2012	Adult (≥ 35)	31,230	52 (51 - 53)	50.0	357	0.71	*(10)*
Du X	2014 to 2016	Adult (≥ 45)	47,841	NA	NA	932	1.8	*(11)*
Shi S	2020 to 2021	Adult (≥ 18)	114,039	55±17	47.9	2,604	1.6	*(12)*

Abbreviations: AF, atrial fibrillation; Num., number; Ref., reference; NA, not available.

Chinese population above 35 years of age was 0.71% (*10*). A national survey of middle-aged and older adults conducted between 2014 and 2016 indicated the weighted AF prevalence was 1.8% (*11*). A most recent epidemiological survey showed that the prevalence of AF in Chinese adults was 1.6% (Figure 1B), and it is estimated that currently approximately 20 million patients have AF in China, which is significantly higher than previously reported and the estimation (*12*). We anticipate that with an aging population, the growing trend in the causes of AF (such as ischemic heart disease), and the higher detection of AF with advanced technology and screening, the healthcare burden of AF will continue to increase in China.

On the other hand, AF-related stroke is becoming an alarmingly increasing burden on the national healthcare system. Based on data from medical insurance database, the incidence of AF-related stroke has increased by more than 13-fold in the southwest of China from 2001 to 2012 (*13*). Another study from Hong Kong illustrated a 2.5-fold increase of AF-related stroke and transient ischemic attack over a 15-year period in Chinese populations, affecting all age groups and mostly non-anticoagulated patients (*14*). Thus, more effort is needed to improve health care for AF in China.

3. OAC use in patients with AF in China

In general, epidemiological studies investigating OAC use in patients with AF in China have been uneven. Few nationally representative surveys have been conducted, and provincial-level studies are often conducted in economically developed regions. The vast majority of studies showed that the proportion of patients with AF prescribed OACs ranged from 10% to 50% in China, which is much lower than that in European and American countries and many Asian countries such as Japan and South Korea (*15-18*). These data provide deep insight into current practice and opportunities for quality improvement in AF management.

3.1. Unsatisfied OAC use in hospital-based studies

Early nationwide, multicenter studies were mostly subgroups of global registries, as listed in Table 2. Two large-scale, worldwide, prospective registries of AF, including Global Anticoagulant Registry in the Field-Atrial Fibrillation (GARFIELD-AF) and Global Registry on Long-Term Oral Antithrombotic Treatment in Patients with Atrial Fibrillation (GLORIA-AF) cohort have both shed light on the lowest OAC use in China compared with other countries or regions (Figure 2) (*15,17-19*). Among the cohorts, OAC therapy was administered to only 20% to 30% of patients in China, which was paralleled by up to 80% usage in patients from Japan. Gratifyingly, follow up studies identified promising trends in OAC use for AF in China (Figure 3). Two latter national investigations, performed in 2012 and 2014, respectively, showed that OAC use had increased to 31.7% and 43.7% in AF patients in China (*20,21*). The most recent data from the China AF Center (which involved 362 hospitals and is the

Table 2. The OAC use in patients with AF from multicenter or international studies in China

First author	Data source	Period	Study location	Numbers of patients	OAC use	*Ref.*
Sun Y	GARFIELD-AF	Dec 2009 to Oct 2011	29 tertiary hospitals	805 NVAF	28.7%	*(19)*
Huisman MV	GLORIA-AF Phase I	May 2011 to Jan 2013	NA	713 NVAF	20.3%	*(17)*
Mazurek M	GLORIA-AF Phase II	Nov 2011 to Dec 2014	NA	1,018 NVAF	21.0%	*(15)*
Sun Y	China Registry of Atrial Fibrillation	Jul 2012 to Dec 2012	111 hospitals	3,562 NVAF and 599 VAF	31.7%	*(20)*
Guo Y	ChiOTEAF registry	Oct 2014 to Dec 2018	44 hospitals	6,420 AF	43.7%	*(21)*
Zhao QY	China AF Center	Nov 2017 to Oct 2018	362 tertiary hospitals	137,181 AF	64.8%	*(22)*

Abbreviations: OAC, oral anticoagulation; NVAF, non-valvular atrial fibrillation; AF, atrial fibrillation; Ref., reference; GARFIELD, Global Anticoagulant Registry in the Field-Atrial Fibrillation; GLORIA-AF, Global Registry on Long-Term Oral Antithrombotic Treatment in Patients with Atrial Fibrillation; NA, not available; ChiOTEAF, Optimal Thromboprophylaxis in Elderly Chinese Patients with Atrial Fibrillation.

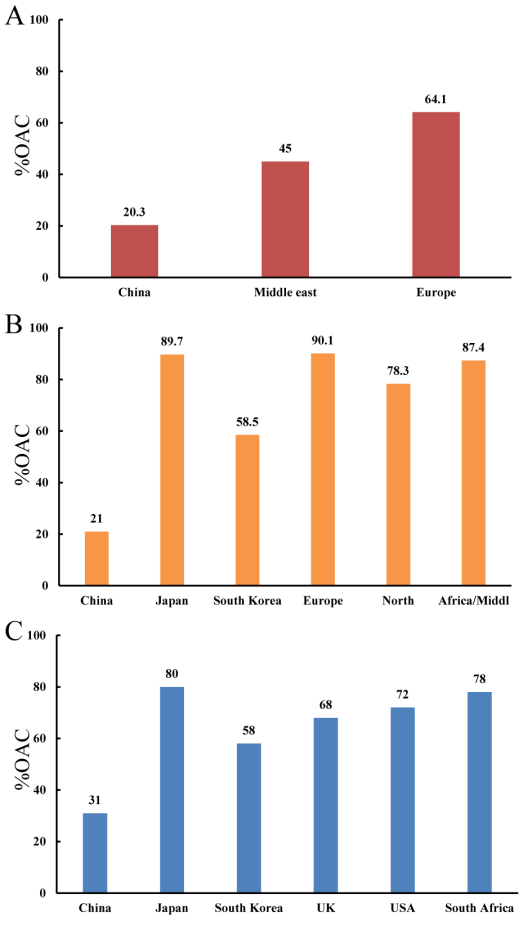

Figure 2. The OAC use in patients with AF in China compared with other countries or regions. Data in panel A are derived from GLORIA-AF Phase I; data in panel B are from GLORIA-AF Phase II; data in panel C are from GARFIELD-AF.

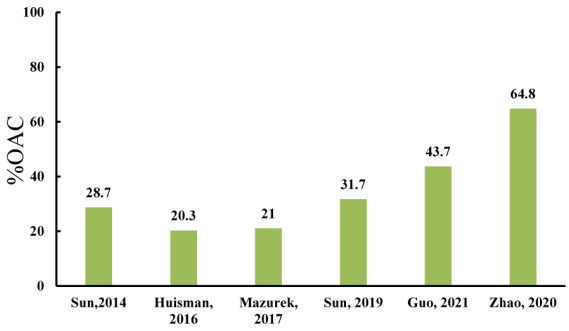

Figure 3. Secular trends of OAC use in patients with AF in China. The OAC use gradually increased in national studies over time.

largest sample size analyzed to date) demonstrated that among 137,181 hospitalized patients with AF in reporting information systems from November 2017 to October 2018, two-thirds had a CHA_2DS_2-VASc score of ≥ 2, 79.1% (72,176/91,246) of whom were treated with OAC (*22*). However, the data from the China AF Center were manually entered and collected only from tertiary general medical centers; these might have led to an overestimation of the true OAC use in AF patients in China.

Regarding regional studies from different provinces, widespread but extremely variable OAC underuse for AF could be observed. However, it is gradually improving. As early as 2006, there have been reports of only 18.2% of high-risk patients with NVAF on OAC medication in Beijing, the capital city of China (*23*). Two registry-based studies thereafter indicated that the use of OAC in patients with AF admitted into tertiary hospitals in Beijing increased from 35% in 2011 to 49% in 2015 (*24*). Similarly, a recent report on the medical insurance database covering up to 30 million people in Shanghai, the most developed city in China, showed a substantial increase in OAC use among patients with AF, in which more than half (56.57%) were taking OAC in 2020, compared to 19.46% in 2015 (*25*). Two cross-sectional survey studies conducted in 2013 and 2018, respectively, showed OAC use of inpatients with NVAF had increased from 11.5% to 36.2% in Chongqing area (*26,27*).

In addition, the presence of OAC underuse in AF patients with ischemic stroke has also been noted. OAC therapy should be made mandatory in these patients in the absence of contraindications according to the current guidelines. Approximately ten years ago, there were reports of a 20% of OAC use in patients with AF-associated stroke in northwestern China (*28*). In another study from Beijing, this rate was reported to be 40% in patients with NVAF within 3 months of new-onset acute stroke (*29*). Similarly, the use of OAC in stroke patients with AF and/or rheumatic heart disease at discharge

increased between 2010 and 2011 and 2016 and 2017 (26.4% to 45.1%, *P* for trend < 0.001) in West China Hospital, one of the top comprehensive hospitals in China (*30*). Data from the most authoritative survey data source of China Stroke Center Alliance, involving 35,767 patients from 1,430 hospitals, showed OAC use was almost double in the five years from 2015 to 2019 (pre-hospital: 14.3% to 21.1%, at discharge: 23.2% to 47.1%) (*31*).

It appears from the above studies (either national or regional) that although OAC use has gradually increased over time in China, it has remained less than satisfactory. Another notable fact is that most previous studies focused exclusively on patients in tertiary-grade hospitals. Further research is needed on OAC use in patients with AF in hospitals at the lower end of the distribution and economically underdeveloped areas.

3.2. Gross OAC underuse in population- or community-based samples

Although OAC undertreatment was found in hospital-based studies in China, it is important to acknowledge this problem is not limited to that. Typically, the OAC intake rate of in-hospital patients is often higher than that of patients in the community. In fact, statistics have shown more serious OAC underuse in population-based studies in China.

Data from the China National Stroke Screening Survey, collected between 2013 and 2014, a representative nationwide sample of 1,252,703 individuals showed that as low as 2.2% of AF patients with stroke were taking OAC medication (*32*). Two later large-scale AF epidemiology surveys found that 4.1% and 6.0% of high-risk patients with AF received OAC, respectively (*33,34*). This situation is also not optimistic even in economically developed regions. A cross-sectional survey conducted in rural Shanghai in 2015 showed that 5.9% of patients with AF were on warfarin, while up to 61.1% were off any antithrombotic medication (*35*). In another community-based study recently conducted in Shanghai in 2017, Chen *et al*. (*36*) found that the OAC use had risen to 20%. Thus, the lack of awareness and undertreatment of community patients is already a serious public health problem in China that needs to be addressed using a whole-of-system approach.

3.3. Temporal trends of OAC prescription patterns

For almost a decade, the change in the OAC use away from VKA towards NOACs for thromboembolic prevention in AF worldwide is evident from studies across various countries (*15,37*). However, NOACs use varied substantially across countries because of the different timing of approval and variations in the attitudes of physicians/patients. In 2017, about 86.1% to 93.1% of patients with AF initiated on NOACs in Europe and the United States through electronic health records or administrative claims databases (*38*). The Fushimi AF Registry indicated NOACs overtook warfarin in 2016 in Japan (*39*). In China, NOAC use has increased rapidly in parallel with global trends and became the most common OAC in 2019, 3-7 years later than other main countries. Through the database of the Hospital Prescription Analysis Corporation Program of China, the prescription of NOACs has rapidly increased in visits and costs in five major, well-developed economic cities (Beijing, Shanghai, Hangzhou, Guangzhou, and Chengdu) from 2012 to 2019 (*40, 41*). And that the defined daily doses of rivaroxaban exceeded those of warfarin in patients with NVAF since 2019 (*42*). Similarly, data from the China Stroke Center Alliance also showed that the combined use of dabigatran and rivaroxaban exceeded the use of warfarin for AF patients with stroke in the third quarter of 2019 (*31*). In fact, the costs of NOACs decreased markedly after they were included in the National Health Insurance in 2017 and had similar cost-effectiveness to warfarin in patients with NVAF (*42*). The overall use rate of OAC has increased in China, partly driven by an increase in the availability of NOACs.

Although it is evident from time-series analyses that the OAC use in patients with AF in China is consistently gradually improving. However, these studies also showed that overall OAC use remained suboptimal and that there is still much room for improvement. Further concerted and comprehensive efforts by society as a whole are needed to improve the quantity and quality of OAC therapy for patients with AF in China.

4. Other roadblocks to high-quality OAC usage in China

It should be emphasized that the use of OAC is the first step in preventing ischemic stroke in patients with AF. In clinical practice, however, other issues also remain. In addition to low OAC use, the presence of inappropriate OAC prescribing and poor adherence and persistence with OAC therapy in AF patients in China have also been noted.

4.1. High prevalence of inappropriate OAC prescribing

A reasonable dose of OAC can maximize its effect and reduce adverse events. However, studies have shown that less than 50% of patients on OAC medication were prescribed with appropriate OAC in China according to the recommended guidelines. In an early analysis of the China National Stroke Registry, among 96 NVAF patients with stroke who were taking warfarin, only one was admitted with an international normalized ratio between 2 and 3 (*43*). A later multicenter study conducted in Xinjiang province showed that up to 86.3% and 3.3% of patients with a CHA_2DS_2-VASc score ≥ 2 received insufficient and excessive OAC,

respectively (*44*). Two recent single-center studies from Fuwai Yunnan Cardiovascular Hospital and West China Hospital reported the prevalence of inappropriate OAC prescription was 46.3% and 56.1% in 2020, respectively (*45,46*). By contrast, the rate of inappropriate OAC prescription was found to be 13.7% to 26.3% in elderly Japanese AF patients from ANAFIE Registry (*47*), and 17% in AF patients from Swiss-AF and BEAT-AF registers (*48*).

Despite the magnitude of the problem, research in this field is not active in China. To uncover the frequency and factors that may contribute to inappropriate NOACs use, two ongoing, national, multicenter studies were investigating real-world data on the rationality of NOACs prescription in AF patients in China (*49,50*). The results of these studies are keenly awaited and will be of importance in guiding rational OAC therapy in Chinese patients.

4.2. Poor adherence and persistence with OAC therapy in Chinses patients

The optimal benefit of OAC therapy requires good medication-taking behavior. Both non-adherence and non-persistence of OAC are associated with increased stroke risk in patients with AF. Long-term adherence and persistence with OAC therapy are global challenges (*51*). However, significant area disparities were observed. Although studies investigated the adherence and persistence of OAC in patients with AF in China are few with small sample sizes. The published data revealed a relatively lower adherence and persistence to OAC among AF patients in China compared with American, Western European, and major Asian cities (Figure 4) (*16,52-54*). In addition, during the literature review, we found a noteworthy issue in that many of the studies from China conflated the concepts of "adherence" and "persistence". Indeed, adherence or compliance refers to patients actually taking the prescribed medication, while persistence or continuation indicates a patient staying on therapy, regardless of drug dosing and schedule (*55*).

A recent meta-analysis showed that 1 in 3 patients with AF adhered to NOAC < 80% of the time at the global scale, whereas the good adherence rate at 1 year can be up to 90% in many Western countries (*54*). One study involving 315 patients with AF in 11 hospitals across China showed that adherence (defined as the mean proportion of days covered ≥ 0.80) declined steadily to 73.7% after the first month after admission and 36.4% in the 10-month follow-up (*56*).

Data from several studies conducted in Beijing showed a considerable variation in the proportion of OAC persistence (*57-59*). In one study, only 40.4% of patients who received warfarin completed the first repeat prescription within 3 months (*57*). Similar findings have been reported in another multicenter study, in which 44.4% of NVAF patients discontinued warfarin within one year (*58*). However, researchers of the China-AF Registry reported that the 3-year persistence to warfarin and NOACs were 87.2% and 81.3% over a similar period, respectively (*59*). The authors believed that the China-AF Registry was created by cardiologists; therefore, patients enrolled in this registry could have actively or passively gained knowledge about OAC.

5. Potential reasons for unsatisfactory OAC therapy in China

Multiple possible factors (Figure 5) may be responsible for the unsatisfactory use of OAC in China. First, the gaps in medical technical level and service quality in this middle-income country compared to Western countries cannot be ignored. Second, the difficulty in detecting AF and screening universal high-risk populations influences the awareness and further treatment of patients with AF. Third, OAC use in patients with a known AF may be influenced by several physician-, patient-, and drug-related factors such as physician specialization, patient health literacy, and patient economic status (Table 3).

5.1. Physician-related factors

Physicians' knowledge of anticoagulation and correct assessment of thromboembolic risk in AF patients is the premise of OAC therapy. However, multiple studies have shown that Chinese doctors' knowledge of OAC is

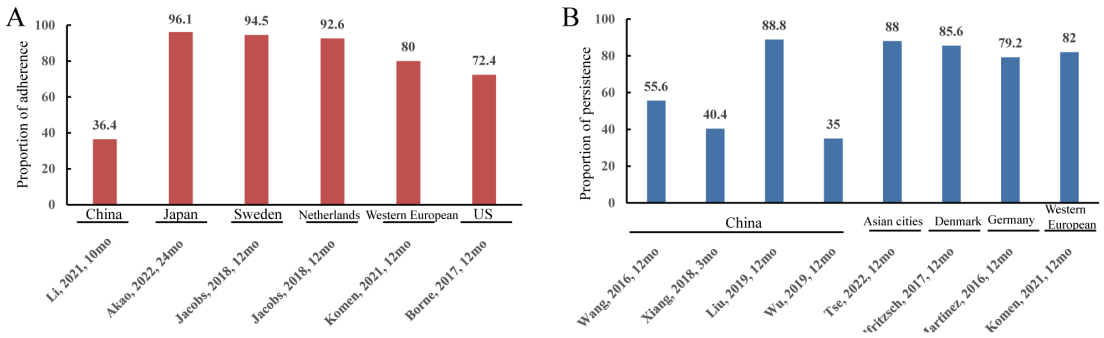

Figure 4. The proportion of adherence (4A) and persistence (4B) to OAC therapy of AF patients in China compared with other countries or regions.

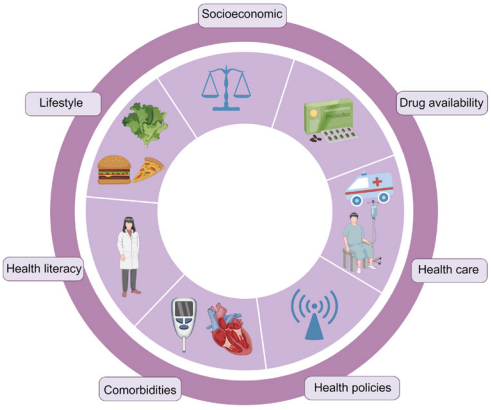

Figure 5. Illustration of the multifactorial etiology of OAC underuse in China. Although there is overlap among these factors, their co-existence induces unsatisfactory OAC therapy in China.

Table 3. Common factors associated with suboptimal OAC use in AF patients in China

Physician-related factors
Medical services vary markedly by hospitals
Lack of knowledge about oral anticoagulants among doctors
Synchronization of previous experience with evolving guidelines
Insufficient focus on the quality of OAC therapy
Patient-related factors
Unaware of AF-related stroke risk
Medication concerns
Multiple comorbidities
Elderly
Lower health literacy
Low socioeconomic status
Drug-related factors
Drug price, costs and availability
Health policy on OAC drugs
Multiple medication

Abbreviations: OAC, oral anticoagulation; AF, atrial fibrillation.

insufficient and that there are huge gaps between regions, hospitals, and departments, which suggests opportunities for improvement at the hospital level.

A national survey of Chinese doctors showed approximately 70% of emergency physicians and general practitioners were unfamiliar with the CHA_2DS_2-VASc score, while cardiologists and clinical pharmacists performed better (*60*). Another survey of neurologists in Hubei Province showed that education level, professional title, working years, hospital grade, and hospital location influenced doctors' knowledge of AF and the options for OAC (*61,62*). Several studies performed in different areas, such as Shanghai (*63*), Guangdong (*64*), and western provinces (*65*), reported similar results: up to 80% of doctors in primary hospitals or communities had insufficient knowledge of OAC use for patients with AF. It seems that grassroots physicians are the weak link in the management of AF in China. Therefore, it is necessary to improve the practice of primary care medicine.

In addition, possessing knowledge does not translate into action. Based on data from the Improving Care for Cardiovascular Disease in China (CCC)-Atrial Fibrillation (AF) project, the proportion of hospitalized patients with NVAF who underwent embolization risk assessment increased from 16.2% in the first quarter of 2015 to 67.1% in the fourth quarter of 2019 ($P < 0.001$) in tertiary hospitals in China (*66*). This suggests that the evaluation of thromboembolism risk in patients with AF is not ideal, even in a batch of top hospitals in China. There is a need for more training on anticoagulant therapy and for clinicians to standardize their clinical practice.

5.2. Patient-related factors

Patient cognition and preferences are important reasons for the initiation, adherence, and persistence of OAC therapy. A cross-sectional survey conducted in the People's Hospital of Henan Province showed that 32.6% and 15.6% of patients with AF passively forgot and voluntarily discontinued warfarin, respectively (*67*). The concerns arising from the belief that OAC therapy is harmful are important reasons for non-adherence to OAC (*67*). A cohort study from five hospitals in China showed that 66.3% of the patients discontinued OAC between 3 and 14 months after AF ablation because of concerns over the risk of bleeding (*68*). A discrete choice experiment investigated the attribute preference for OAC therapy in patients with AF from Beijing and Shenzhen, and the results showed that patients were most concerned about the risks of myocardial infarction, stroke, and major bleeding (*69*). These findings reflect concerns about the possible adverse health effects of OAC. Therefore, it is necessary to educate patients with AF on the efficacy and safety of anticoagulation therapy to encourage them to adhere to OAC therapy.

In addition to intrinsic patient factors such as health literacy and knowledge regarding AF, many clinical patient-level factors could also influence OAC therapy in patients with AF. Several studies have investigated the characteristics of patients who did not take, adhere to, or persist with OAC therapy (*31,46,70-73*). Common patient factors integrated from the findings of previous studies were older age, non-urban residence, lower education level, lower income, history of stroke, multiple comorbidities, and antiplatelet therapy (*14*). In fact, it is important to note that the risk factors for poor compliance to OAC in AF patients in China were usually not the same as those in patients from other countries. For example, older patients often had better adherence and persistence to OAC therapy in other countries (*74*), but not in China. Therefore, we should focus on identifying the risk factors and characteristics associated with OAC adherence and persistence in Chinese patients, which may be helpful in identifying patients with non-adherence and non-persistence. The next step is to translate these findings into incentives to help patients take OAC appropriately.

5.3. Drug-related factors

Compared with warfarin, NOACs have the advantages of having a rapid onset of action, no need for unnecessary routine coagulation monitoring, no dosage adjustment, and fewer food-drug interactions. The long-term persistence of NOACs is better than that of warfarin (*75*). However, domestic studies have shown that the rate of discontinuation of NOACs was higher than that of warfarin in AF patients in China (*59,76*). According to a questionnaire survey from the China-AF registry, dabigatran therapy in patients with NVAF was associated with no improvement in satisfaction and a higher discontinuation rate compared with warfarin therapy, which was thought to be largely due to the increased economic burden of NOACs (176.78 ± 9.15 *vs*. 2.49 ± 0.76 USD/month, $P < 0.001$) (*77*). Indeed, the level of NOACs use also partly depends on government subsidies. However, with the patent expired status of many NOACs, the costs of China's generic drugs have markedly dropped, and we speculate that circumstances might change and drug costs would no longer be a decisive factor governing the use of NOACs. Further studies on this topic are required. In addition, studies have shown that other drug-related factors, such as dosing frequency, affect OAC use and deserve attention.

6. Strategies to improve OAC therapy in AF patients in China

How can we overcome these obstacles? We believe that the collaboration of our healthcare administration, medical associations, hospitals, and physicians is needed to promote OAC use in AF patients in China and further improve the overall prognosis of patients with AF in China (Table 4).

6.1. Measures from the government departments

In fact, the government can do much to ameliorate the reality of OAC underuse in AF patients in China; first of all, they could reduce the economic burden on patients. In 2019, the Chinese government launched a centralized medicine procurement policy together with the resident medical insurance reimbursement policy for chronic diseases, both of which substantially reduced the price and improved the cost-effectiveness of drugs (*78*). A cost-benefit analysis showed that NOACs have comparable and even better cost-effectiveness than warfarin in Chinese patients with NVAF since the inclusion of NOACs in the catalog of medication reimbursement (*42,79*). A welcome fact is that there has been an increase in overall OAC prescriptions since NOACs became available in China (*80*). At present, dabigatran and rivaroxaban have entered the fifth batch of national centralized drug procurement, and it is expected that implementing low central government prices would benefit more patients with AF.

Second, most published data regarding OAC use in AF patients in China were obtained from hospital digital information systems or community sampling surveys, although there have been a small number of reports using regional medical insurance databases (*81*). In contrast, many national cardiovascular registries in other countries are widely used to improve the quality of healthcare services, assess the effectiveness and safety of new therapies, and conduct research (*82*). Thus, we suggest that the national medical insurance database and medicine agency monitoring data be developed and made open access to facilitate better evaluation of the actual OAC usage in China, which will lead to the proposal of new strategies (*83*).

Third, quality control should be further strengthened through medical administration. Currently, the National Health Commission of China has formulated five management indicators for admitted patients with AF: the evaluation rates of stroke and bleeding risk, the rate of OAC use in patients with valvular and non-valvular AF, and the incidence of complications during left atrial appendage closure (*84*). It is hoped that national-level interventions will be more effective than calls from doctors and that medical institutions will continue to improve the OAC management of patients with AF.

Fourth, the Ministry of Health of China established

Table 4. Strategies to combat poor OAC use of patients with AF in China

Topic	Strategies	Scope/Location
Poor knowledge of AF patients	1. Improving residents' health literacy.	Government, public policies.
	2. Promoting patient education campaigns on AF and OAC.	Hospitals and physicians.
	3. More convenient tools such as multimedia, electronic communications, and networking technology.	Government, public policies, related enterprises, physicians.
Insufficient capacity of physicians	1. Promotion and introduction of the AF guideline.	Medical associations and large hospitals.
	2. Build hierarchical diagnosis and treatment system.	Government, medical associations, and hospitals.
	3. More home-grown clinical trials to offer more clinical evidence.	Medical associations and hospitals.
High cost of NOACs	1. When the patent expires, the drug price decreases through competition with generic drugs.	Pharmaceutical companies in China.
	2. NOACs include in medical insurance reimbursement.	Government and national health insurance system.

Abbreviations: OAC, oral anticoagulation; AF, atrial fibrillation; NOAC, non-vitamin K antagonist oral anticoagulant.

a hierarchical diagnosis and treatment system for AF management. We hope that the efficiency of AF therapy can be improved through this hierarchical system. The form of "up and down assistance" is supported by tertiary hospitals and participated in by secondary hospitals and community hospitals, which enables grassroots patients with AF to obtain homogeneous, standardized, and efficient management. However, the establishment of the AF management system in such a large setting is a lengthy and challenging process and requires the cooperation of specialist cardiologists, pharmacist teams, and community general practitioners. Several top hospitals have established integrated management models based on local characteristics (*85*). These hospitals have developed a local network for sharing clinical decision making; they have used the alliance platform to link the management of AF in top and primary hospitals, which is worthy of widespread promotion.

In addition, the government should support regular AF screening for those at high risk of AF in the context of an aging population and support original research on anticoagulation therapy in the Chinese population. We strongly believe that these administrative interventions under China's unique medical model may have distinct advantages in improving the quality of OAC use in patients with AF.

6.2. Measures from the cardiovascular medical associations

The dedicated efforts of many cardiologists have greatly improved anticoagulation therapy in AF patients in China. Experts formed the Atrial Fibrillation Center Union of China (AFCUC), which created the China Atrial Fibrillation Day (June 6th) and developed a flurry of activities such as advocating for education of inhabitants on AF and helping with the construction of the AF management system. Nevertheless, additional efforts are needed to maintain and further improve OAC use in AF patients in China.

First, studies have shown that doctors in primary and community hospitals and non-cardiologists, such as emergency department physicians, are the weak link in the management of AF. However, these doctors are in close contact with patients with AF. Therefore, it is necessary to guide and train these medical staff to improve their knowledge and strengthen their management capacity. We believe that the goal of medical associations and top hospitals is by no means solely to foster experienced electrophysiologists. Training primary healthcare physicians will be driven by medical associations, such as AFCUC, so that the primary healthcare facilities can strengthen their gatekeeper role.

Second, most of the studies cited in the current AF guidelines of China were conducted abroad. Management strategies and guidelines for AF need to be modified using data obtained from Chinese patients. However, single-center studies have often provided inadequate clinical evidence. Hence, medical associations should prompt high-quality, multicenter clinical research to fill the evidence gap for OAC therapy in AF patients in China, such as the comparison of the clinical net benefit of different NOACs and the efficacy and safety of OAC therapy in high bleeding risk groups, the elderly, and other special populations.

Third, medical associations should launch more quality improvement programs, such as the CCC-AF project (*86*). A major strength of medical associations is that they can recruit hospitals to participate in one program to improve the quality of national medical care. Such projects will involve collection of clinical information from patients with AF, analysis, feedback, and process improvement to eventually help guide the development of the national health quality improvement system (*86*).

Overall, medical associations should leverage this strength to achieve the most impact in this area of national health. The associations could provide detailed national information and discover the gaps and barriers to effective OAC use in AF patients in China, which is valuable for better OAC usage across China and to explore more reasonable management pathways suitable for the national conditions in China (*87*).

6.3. Measures from the hospitals and physicians

Medical treatment can be characterized by a series of interactions between patients and doctors. The first step in solving current problems is recognizing that collaboration must occur between health care practitioners and patients. Poor knowledge about AF and OAC among patients with AF is associated with adverse cardiovascular events (*88*). Educating patients is an essential part of the clinical management of AF. Propagating and educating patients about their disease conditions and about the efficacy, and advantages and disadvantages of OAC use during outpatient visits, hospitalization, and after discharge is an important basis for improving drug compliance and fostering mutual trust between physicians and patients. An international cluster-randomized trial conducted in developing countries showed that a multifaceted educational intervention could increase the proportion of patients with AF treated with OACs (*89*).

Growing evidence-based research findings support the Atrial Fibrillation Better Care (ABC) pathway as a structured approach for the management of AF, endorsed in the 2020 European Society of Cardiology guidelines (*3,90,91*). In fact, the ABC pathway emphasizes the importance of integrated care, which requires a multidisciplinary team (from specialists to caregivers) in AF management. First, patient participation should be encouraged in the process of deciding whether anticoagulation should be used as this can improve

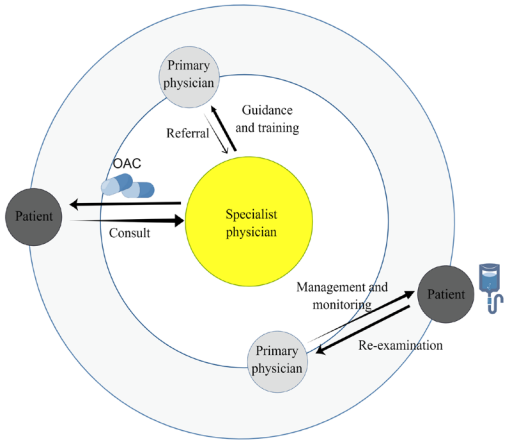

Figure 6. A central-radiation model for OAC therapy in AF patients in China. Specialist physician in the center and doctors in peripheral hospitals should assume corresponding responsibilities to better communicate with patients and improve their OAC use.

patient understanding, trust, satisfaction, and OAC compliance (*92*). Secondly, clinical pharmacists can assist clinicians in prescribing rational doses of OAC and avoiding adverse drug interactions. Studies from Shanghai (*93*) and Chongqing (*94*) showed that patients' OAC compliance can be improved through comprehensive evaluation and medication education by clinical pharmacists and physicians. Therefore, for some qualified top hospitals, clinical pharmacists should be encouraged to participate in OAC treatment and the whole-process management of patients with AF (Figure 6). Third, with the development of modern technology, many medical tools such as mobile phone software have been developed. A pilot study developed a WeChat mini-program integrating decision support and patient follow-up, which was shown to improve medication compliance and satisfaction in patients with AF (*95*). The mobile Atrial Fibrillation App trial managed patients with AF using mobile phone software that integrates clinical decision support, health education, patient participation, and structured follow-up. The results showed that mobile health management can improve patients' disease cognition, medication adherence, quality of life, anticoagulation satisfaction (*96*), and long-term outcomes (*97*). In addition, another computer-assisted platform was also shown to improve the medical compliance of patients with AF (*98*). However, more efficient and easy-to-accept management platforms should be explored to improve OAC compliance in patients with AF. We also hope that artificial intelligence-assisted tools could help doctors in primary care to manage patients with AF (*99*).

To conclude, the integrated care approach to AF management is not only for prompting OAC use but also for improving patient prognosis and reducing the risk of mortality and hospitalization. This requires consistent efforts and the involvement of the medical team. Education and changing ideas in health care need to be increased in hospitals and physicians in China.

7. Management of OACs during the COVID-19 pandemic

Today, the global epidemic situation remains sobering, and China is no exception. The increase of AF-related mortality during the COVID-19 pandemic in US has been observed (*100*). Several issues should be taken into consideration in order to protect AF patients in China. First, the epidemic has reduced the frequency of patients seeking medical treatment, especially those in grassroots areas and far from hospitals. Thus, it is necessary to encourage primary physicians to be responsible for the OAC management and follow-up visit. Second, NOACs is especially suitable during the epidemic since it does not require frequent blood monitoring. Third, personalized smartphone Apps with user-friendly design that involve medical professionals are needed for improving antithrombotic therapy safety.

8. Summary and perspective

AF-related stroke incurs a heavy burden to patients, doctors, and the healthcare system in China. Over the past two decades, clinical management of AF has gradually improved in China through updates in AF management guidelines, various practice incentives, the introduction of NOACs, and the strong marketing of biopharmaceutical manufacturers. However, at present, OAC therapy in AF patients in China is far from ideal, with obstacles such as low OAC use, frequent inappropriate dose prescription of OAC drugs, and poor long-term medication adherence and persistence in patients with AF. We have come a long way, but we know that there is yet far to go.

Our present review highlights important gaps in the current clinical management of AF patients in China, which has important implications for healthcare policy making to achieve the Healthy China 2030 goal. Overall, these aspects are timely and thought-provoking. The information may provide further insights for more effective surveillance of AF and stroke prevention for improved clinical outcomes. Joint efforts of the government and medical communities are needed to minimize the burden of stroke and death caused by poor OAC use, adherence, and persistence.

Acknowledgements

We sincerely thank "HOME for Researchers" and "Figdraw" for linguistic and graphic assistance.

Funding: Natural Science Foundation of China (82100343, 81970281, 81873488), Scientific Research Foundation of Beijing Key Laboratory of Metabolic

Disorders Related Cardiovascular Disease (DXWL2022-04).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Rahman F, Kwan GF, Benjamin EJ. Global epidemiology of atrial fibrillation. Nat Rev Cardiol. 2014; 11:639-654.
2. Chugh SS, Roth GA, Gillum RF, Mensah GA. Global burden of atrial fibrillation in developed and developing nations. Glob Heart. 2014; 9:113-119.
3. Hindricks G, Potpara T, Dagres N, *et al*. 2020 ESC Guidelines for the diagnosis and management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS): The Task Force for the diagnosis and management of atrial fibrillation of the European Society of Cardiology (ESC) Developed with the special contribution of the European Heart Rhythm Association (EHRA) of the ESC. Eur Heart J. 2021; 42:373-498.
4. January CT, Wann LS, Calkins H, *et al*. 2019 AHA/ACC/HRS Focused Update of the 2014 AHA/ACC/HRS Guideline for the Management of Patients With Atrial Fibrillation: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Rhythm Society in Collaboration With the Society of Thoracic Surgeons. Circulation. 2019; 140:e125-e151.
5. Chao TF, Joung B, Takahashi Y, Lim TW, Choi EK, Chan YH, Guo Y, Sriratanasathavorn C, Oh S, Okumura K, Lip GYH. 2021 Focused update of the 2017 consensus guidelines of the Asia Pacific Heart Rhythm Society (APHRS) on stroke prevention in atrial fibrillation. J Arrhythm. 2021; 37:1389-1426.
6. Chugh SS, Havmoeller R, Narayanan K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, Kim YH, McAnulty JH Jr, Zheng ZJ, Forouzanfar MH, Naghavi M, Mensah GA, Ezzati M, Murray CJ. Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. Circulation. 2014; 129:837-847.
7. Roth GA, Mensah GA, Johnson CO, *et al*. Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. J Am Coll Cardiol. 2020; 76:2982-3021.
8. Zhou Z, Hu D. An epidemiological study on the prevalence of atrial fibrillation in the Chinese population of mainland China. J Epidemiol. 2008; 18:209-216.
9. Li Y, Wu YF, Chen KP, Li X, Zhang X, Xie GQ, Wang FZ, Zhang S. Prevalence of atrial fibrillation in China and its risk factors. Biomed Environ Sci. 2013; 26:709-716.
10. Wang Z, Chen Z, Wang X, Zhang L, Li S, Tian Y, Shao L, Hu H, Gao R. The Disease Burden of Atrial Fibrillation in China from a National Cross-sectional Survey. Am J Cardiol. 2018; 122:793-798.
11. Du X, Guo L, Xia S, *et al*. Atrial fibrillation prevalence, awareness and management in a nationwide survey of adults in China. Heart. 2021; 107:535-541.
12. Shi S, Tang Y, Zhao Q, *et al*. Prevalence and risk of atrial fibrillation in China: A national cross-sectional epidemiological study. The Lancet Regional Health - Western Pacific. 2022; 100439.
13. Guo Y, Tian Y, Wang H, Si Q, Wang Y, Lip GYH. Prevalence, incidence, and lifetime risk of atrial fibrillation in China: new insights into the global burden of atrial fibrillation. Chest. 2015; 147:109-119.
14. Soo Y, Chan N, Leung KT, Chen XY, Mok V, Wong L, Leung T. Age-specific trends of atrial fibrillation-related ischaemic stroke and transient ischaemic attack, anticoagulant use and risk factor profile in Chinese population: a 15-year study. J Neurol Neurosurg Psychiatry. 2017; 88:744-748.
15. Mazurek M, Huisman MV, Rothman KJ, Paquette M, Teutsch C, Diener HC, Dubner SJ, Halperin JL, Ma CS, Zint K, Elsaesser A, Lu S, Lip GYH. Regional Differences in Antithrombotic Treatment for Atrial Fibrillation: Insights from the GLORIA-AF Phase II Registry. Thromb Haemost. 2017; 117:2376-2388.
16. Tse HF, Teo WS, Siu CW, Chao TF, Park HW, Shimizu W, Wong YK, Lip GYH. Prognosis and treatment of atrial fibrillation in Asian cities: 1-year review of the Asia-Pacific Heart Rhythm Society Atrial Fibrillation Registry. Europace. 2022.
17. Huisman MV, Ma CS, Diener HC, Dubner SJ, Halperin JL, Rothman KJ, Teutsch C, Schoof N, Kleine E, Bartels DB, Lip GY. Antithrombotic therapy use in patients with atrial fibrillation before the era of non-vitamin K antagonist oral anticoagulants: the Global Registry on Long-Term Oral Antithrombotic Treatment in Patients with Atrial Fibrillation (GLORIA-AF) Phase I cohort. Europace. 2016; 18:1308-1318.
18. Steinberg BA, Gao H, Shrader P, *et al*. International trends in clinical characteristics and oral anticoagulation treatment for patients with atrial fibrillation: Results from the GARFIELD-AF, ORBIT-AF I, and ORBIT-AF II registries. Am Heart J. 2017; 194:132-140.
19. Sun Y, Hu D. Chinese Investigators of GARFIELD. Chinese subgroup analysis of the global anticoagulant registry in the FIELD (GARFIELD) registry in the patients with non-valvular atrial fibrillation. Zhonghua Xin Xue Guan Bing Za Zhi. 2014; 42:846-850. (in Chinese)
20. Sun Y, Zhu J, Ma C, Liu S, Yang Y, Hu D. Stroke Risk Status, Anticoagulation Treatment, and Quality-of-Life in Chinese Patients with Atrial Fibrillation: China Registry of Atrial Fibrillation (CRAF). Cardiovasc Ther. 2019; 2019:7372129.
21. Guo Y, Kotalczyk A, Imberti JF, Wang Y, Lip GYH. Quality indicators in the management of elderly Chinese patients with atrial fibrillation: A report from the Optimal Thromboprophylaxis in Elderly Chinese Patients with Atrial Fibrillation (ChiOTEAF) registry. European heart journal Quality of care & clinical outcomes. 2021.
22. Zhao QY, Shi SB, Huang H, *et al*. Contemporary characteristics, management, and outcomes of patients hospitalized for atrial fibrillation in China: results from the real-world study of Chinese atrial fibrillation registry. Chin Med J (Engl). 2020; 133:2883-2884.
23. Zuo HJ, Su JL, Zeng H, Yuan BH, Yao CH. [Anticoagulation treatment in real-life practice of patient with nonvalvular atrial fibrillation in Beijing city]. Zhonghua Yi Xue Za Zhi. 2007; 87:2328-2331. (in Chinese)
24. Yu HR, Ma CS, Du X, Chang SS, Dong JZ. Evaluation of medical quality and treatment trends of nonvalvular atrial fibrillation in Beijing inpatients. Ann Palliat Med. 2021; 10:5270-5279.
25. Chen M, Li C, Liao P, Cui X, Tian W, Wang Q, Sun J, Yang M, Luo L, Wu H, Li YG. Epidemiology,

management, and outcomes of atrial fibrillation among 30 million citizens in Shanghai, China from 2015 to 2020: A medical insurance database study. Lancet Reg Health West Pac. 2022; 23:100470.
26. Zheng HJ, Ouyang SK, Zhao Y, Lu K, Luo SX, Xiao H. The use status of anticoagulation drugs for inpatients with nonvalvular atrial fibrillation in Southwest China. Int J Gen Med. 2017; 10:69-77.
27. Xia Z, Lu K, Chen X, Xie S, Hu H, Xiao H. Anticoagulation status of elderly patients with non-valvular atrial fibrillation in secondary and tertiary hospitals of Chongqing. Journal of third military medical university. 2021; 43:347-353. (in Chinese)
28. Zhang J, Yang XA, Zhang Y, Wei JY, Yang F, Gao H, Jiao WW, Sun XL, Gao Q, Jiang W. Oral Anticoagulant Use in Atrial Fibrillation-Associated Ischemic Stroke: A Retrospective, Multicenter Survey in Northwestern China. J Stroke Cerebrovasc Dis. 2017; 26:125-131.
29. Wang JR, Du X, Dong JZ, Chang SS, Jiang C, Sang CH, Long DY, Tang RB, Zhang HB, Guo JC, Wen YM, He L, Ma CS. Use of oral anticoagulants and its associated factors among nonvalvular atrial fibrillation patients with new-onset acute ischemic stroke: A report from the China Atrial Fibrillation Registry study. Clin Cardiol. 2022; 45:60-67.
30. Liu J, Wang Y, Guo W, Cheng Y, Zhang S, Wu B, Liu M. Temporal trends of atrial fibrillation and/or rheumatic heart disease-related ischemic stroke, and anticoagulant use in Chinese population: An 8-year study. Int J Cardiol. 2021; 322:258-264.
31. Gu HQ, Yang X, Wang CJ, *et al*. Assessment of Trends in Guideline-Based Oral Anticoagulant Prescription for Patients With Ischemic Stroke and Atrial Fibrillation in China. JAMA Netw Open. 2021; 4:e2118816.
32. Guo J, Guan T, Fan S, Chao B, Wang L, Liu Y. Underuse of Oral Anticoagulants in Patients With Ischemic Stroke and Atrial Fibrillation in China. Am J Cardiol. 2018; 122:2055-2061.
33. Xing L, Lin M, Du Z, Jing L, Tian Y, Yan H, Ren G, Dong Y, Sun Q, Dai D, Shi L, Chen H, Liu S. Epidemiology of atrial fibrillation in northeast China: a cross-sectional study, 2017-2019. Heart. 2020; 106:590-595.
34. Du X, Guo L, Xia S, *et al*. Atrial fibrillation prevalence, awareness and management in a nationwide survey of adults in China. Heart (British Cardiac Society). 2021; 107:535-541.
35. Wei Y, Xu J, Wu H, Zhou G, Chen S, Wang C, Shen Y, Yang S, Wang B, He Z, Sun J, Sun W, Ouyang P, Liu S. Survey of Antithrombotic Treatment in Rural Patients (>60 years) with Atrial Fibrillation in East China. Sci Rep. 2018; 8:6830.
36. Chen Y, Huang QF, Sheng CS, Zhang W, Shao S, Wang D, Cheng YB, Wang Y, Guo QH, Zhang DY, Li Y, Lowres N, Freedman B, Wang JG. Detection rate and treatment gap for atrial fibrillation identified through screening in community health centers in China (AF-CATCH): A prospective multicenter study. PLoS Med. 2020; 17:e1003146.
37. Grymonprez M, Simoens C, Steurbaut S, De Backer TL, Lahousse L. Worldwide trends in oral anticoagulant use in patients with atrial fibrillation from 2010 to 2018: a systematic review and meta-analysis. Europace. 2021.
38. Vora P, Morgan Stewart H, Russell B, Asiimwe A, Brobert G. Time Trends and Treatment Pathways in Prescribing Individual Oral Anticoagulants in Patients with Nonvalvular Atrial Fibrillation: An Observational Study of More than Three Million Patients from Europe and the United States. Int J Clin Pract. 2022; 2022:6707985.
39. Akao M, Ogawa H, Masunaga N, *et al*. 10-Year Trends of Antithrombotic Therapy Status and Outcomes in Japanese Atrial Fibrillation Patients - The Fushimi AF Registry. Circ J. 2022; 86:726-736.
40. Yu Z, Yu L, Shan C. Trends of ambulatory oral anticoagulant prescription in five major cities of China, 2012-2017. BMC health services research. 2020; 20:209-209.
41. Gong X, He Q, Yan J, Chen J, Chen X, Huang M, Li J, Chen P. A drug utilization study of oral anticoagulants in five representative cities of China between 2015 and 2019. J Clin Pharm Ther. 2022; 47:38-45.
42. Zhou H, Nie X, Jiang M, Dong W. Cost-effectiveness of anticoagulants for preventing stroke in patients with non-valvular atrial fibrillation in mainland China. J Clin Pharm Ther. 2022; 47:523-530.
43. Wang C, Yang Z, Wang C, Wang Y, Zhao X, Liu L, Wang DZ, Li H, Wang Y. Significant underuse of warfarin in patients with nonvalvular atrial fibrillation: results from the China national stroke registry. J Stroke Cerebrovasc Dis. 2014; 23:1157-1163.
44. Cheng X, Zhou X, Song S, Wu M, Baolatejiang R, Lu Y, Li Y, Zhang W, Lv W, Ye Y, Zhou Q, Wang H, Zhang J, Xing Q, Tang B. Ethnicity and anticoagulation management of hospitalized patients with atrial fibrillation in northwest China. Sci Rep. 2017; 7:45884.
45. Qian Y, Zhang J, Li J, Weng Z. A retrospective study on the evaluation of the appropriateness of oral anticoagulant therapy for patients with atrial fibrillation. PLoS One. 2021; 16:e0259199.
46. Xia X, Wang L, Lin T, Yue J, Yang Z, Mi C, Liao Z, Chen Y, Ge N, Wu C. Barriers to prescribing oral anticoagulants to inpatients aged 80 years and older with nonvalvular atrial fibrillation: a cross-sectional study. BMC Geriatr. 2022; 22:263.
47. Akao M, Shimizu W, Atarashi H, *et al.* Oral Anticoagulant Use in Elderly Japanese Patients With Non-Valvular Atrial Fibrillation - Subanalysis of the ANAFIE Registry. Circ Rep. 2020; 2:552-559.
48. Montrasio G, Reiner MF, Wiencierz A, *et al*. Prevalence and risk of inappropriate dosing of direct oral anticoagulants in two Swiss atrial fibrillation registries. Vascul Pharmacol. 2022; 147:107120.
49. Ding Z, Zhang C, Qian YY, Wang N, Gu ZC, Xu H, Zheng YL. Rationale and design of a prospective, multicenter, cross-sectional study of appropriateness evaluation of the prescription of non-vitamin K antagonist oral anticoagulants for Chinese atrial fibrillation patients (Chi-NOACs-AF trial). Ann Transl Med. 2021; 9:580.
50. Zhao SJ, Chen BY, Hong XJ, Liu YP, Cai HX, Du S, Gu ZC, Ma PZ. Prevalence, risk factors, and prediction of inappropriate use of non-vitamin K antagonist oral anticoagulants in elderly Chinese patients with atrial fibrillation: A study protocol. Front Cardiovasc Med. 2022; 9:951695.
51. Komen JJ, Heerdink ER, Klungel OH, Mantel-Teeuwisse AK, Forslund T, Wettermark B, Hjemdahl P. Long-term persistence and adherence with non-vitamin K oral anticoagulants in patients with atrial fibrillation and their associations with stroke risk. Eur Heart J Cardiovasc Pharmacother. 2021; 7:f72-f80.
52. Komen JJ, Pottegård A, Mantel-Teeuwisse AK, *et al*.

Persistence and adherence to non-vitamin K antagonist oral anticoagulant treatment in patients with atrial fibrillation across five Western European countries. Europace. 2021; 23:1722-1730.
53. Shang L, Shao M, Zhou X, Tang B. Non-adherence of NOACs therapy as a serious phenomenon in patients with non-valvular atrial fibrillation in China. Int J Cardiol. 2019; 294:42.
54. Ozaki AF, Choi AS, Le QT, Ko DT, Han JK, Park SS, Jackevicius CA. Real-World Adherence and Persistence to Direct Oral Anticoagulants in Patients With Atrial Fibrillation: A Systematic Review and Meta-Analysis. Circ Cardiovasc Qual Outcomes. 2020; 13:e005969.
55. Obamiro KO, Chalmers L, Bereznicki LR. A Summary of the Literature Evaluating Adherence and Persistence with Oral Anticoagulants in Atrial Fibrillation. Am J Cardiovasc Drugs. 2016; 16:349-363.
56. Li Y, Chen R, Lu X, Zhang Y, Tian Y, Liu X. Adherence of oral anticoagulants in patients with atrial fibrillation in 11 hospitals: Current state and influential factors. Chinese Journal of Cardiac Pacing and Electrophysiology. 2021; 35:245-248. (in Chinese)
57. Xiang X, Cao Y, Sun K, Song J, Tian Y, Yin Q, Juan J, Hu Y. Real world adherence to oral anticoagulant in non-valvular atrial fibrillation patients in China. Curr Med Res Opin. 2018; 34:255-261.
58. Wang ZZ, Du X, Wang W, *et al*. Long-Term Persistence of Newly Initiated Warfarin Therapy in Chinese Patients With Nonvalvular Atrial Fibrillation. Circ Cardiovasc Qual Outcomes. 2016; 9:380-387.
59. Liu C, Du X, Jiang C, He L, Chang SS, Guo XY, Yu RH, Long DY, Bai R, Liu N, Sang CH, Jiang CX, Dong JZ, Lip GYH, Ma CS.. Long-Term Persistence with Newly-Initiated Warfarin or Non-VKA Oral Anticoagulant (NOAC) in Patients with Non-Valvular Atrial Fibrillation: Insights from the Prospective China-AF Registry. Med Sci Monit. 2019; 25:2649-2657.
60. Zhang C, Shen L, Pan MM, Zheng YL, Gu ZC, Lin HW. Perceptions and knowledge gaps on CHA(2)DS(2)-VASc score components: a joint survey of Chinese clinicians and clinical pharmacists. Postgrad Med. 2022; 134:64-77.
61. Shen J, Xia Y, Cao S, Lu Z, He Q, Li M, Wang H, Bi Y, Chen S, Hu B, Cao F. Knowledge, attitude, and practice regarding atrial fibrillation among neurologists in central China: A cross-sectional study. Clin Cardiol. 2020; 43:639-646.
62. Shen J, Li M, Cao S, Lu Z, Xia Y, Chen S, Bi Y, Cai Z, Hu B, Cao F. Neurologists' attitudes and options for anticoagulation therapy in central China. Int J Clin Pract. 2021; 75:e14305.
63. Ye S, Wang T, Liu A, Yu Y, Pan Z, Gu J. A study of knowledge, attitudes, and practices of primary care physicians toward anticoagulant therapy in patients with non-valvular atrial fibrillation in Shanghai, China. BMC Fam Pract. 2020; 21:165.
64. Bo L, Hong Y, Feng Y, Fei H, Chongzhou Z. Investigation and analysis about atrial fibrillation related knowledge for grassroots medical staff. Chinese Journal of Cardiac Pacing and Electrophysiology. 2021; 35:41-43. (in Chinese)
65. Lu Y, Tang B, Shang L, *et al*. Investigation on knowledge of anticoagulant and use of anticoagulant drugs by physicians in western China. Chinese Journal of Cardiac Arrhythmias. 2019; 23:424-429. (in Chinese)
66. Yang N, Zhao D, Liu J, Hao YC, Zeng YY, Hu DQ, Sun ZQ, Yang YQ, Li HW, Liu TX, Wang Y, Liu J. Current status of thromboembolism risk assessment in patients hospitalized with non-valvular atrial fibrillation in tertiary hospitals in China. Zhonghua Xin Xue Guan Bing Za Zhi. 2021; 49:856-865. (in Chinese)
67. Zhao S, Zhao H, Wang X, Gao C, Qin Y, Cai H, Chen B, Cao J. Factors influencing medication knowledge and beliefs on warfarin adherence among patients with atrial fibrillation in China. Patient Prefer Adherence. 2017; 11:213-220.
68. Yu R, Xi H, Lu J, Xu F, Shi L, Kong Q, Hu Y, Zhao X, Liu N. Real-world investigation on discontinuation of oral anticoagulation after paroxysmal atrial fibrillation catheter ablation in China. Ann Palliat Med. 2020; 9:940-946.
69. Zhao J, Wang H, Li X, Hu Y, Yan VKC, Wong CKH, Guo Y, Cheung MKH, Lip GYH, Siu CW, Tse HF, Chan EW. Importance of attributes and willingness to pay for oral anticoagulant therapy in patients with atrial fibrillation in China: A discrete choice experiment. PLoS Med. 2021; 18:e1003730.
70. Zhao S, Zhao H, Wang X, Gao C, Qin Y, Cai H, Chen B, Cao J. A prospective study investigating the causes of warfarin under-utilization in Chinese patients. Int J Clin Pharm. 2016; 38:1286-1293.
71. Zhu L, Zhang X, Yang J. Analysis of Influencing Factors of Compliance with Non-Vitamin K Antagonist Oral Anticoagulant in Patients with Nonvalvular Atrial Fibrillation and Correlation with the Severity of Ischemic Stroke. Evid Based Complement Alternat Med. 2021; 2021:1021127.
72. Song T, Xin X, Cui P, Zong M, Li X. Factors Associated with Anticoagulation Adherence in Chinese Patients with Non-Valvular Atrial Fibrillation. Patient Prefer Adherence. 2021; 15:493-500.
73. Gong X, Chen H, Wang J, Zhong W, Chen L, Yan S, Lou M. Undertreatment of Anticoagulant Therapy in Hospitalized Acute Ischemic Stroke Patients With Atrial Fibrillation. Front Cardiovasc Med. 2022; 9:841020.
74. Lowres N, Giskes K, Hespe C, Freedman B. Reducing Stroke Risk in Atrial Fibrillation: Adherence to Guidelines Has Improved, but Patient Persistence with Anticoagulant Therapy Remains Suboptimal. Korean Circ J. 2019; 49:883-907.
75. Simons LA, Ortiz M, Freedman SB, Waterhouse BJ, Colquhoun D, Thomas G. Improved persistence with non-vitamin-K oral anticoagulants compared with warfarin in patients with atrial fibrillation: recent Australian experience. Curr Med Res Opin. 2016; 32:1857-1861.
76. Wu S, Xie S, Xu Y, Que D, Yau TO, Wang L, Huang Y. Persistence and outcomes of non-vitamin K antagonist oral anticoagulants versus warfarin in patients with non-valvular atrial fibrillation. J Clin Nurs. 2019; 28:1839-1846.
77. Geng YP, Lan DH, Liu N, *et al*. Patient-Reported Treatment Satisfaction with Dabigatran versus Warfarin in Patients with Non-Valvular Atrial Fibrillation in China. Thromb Haemost. 2018; 118:1815-1822.
78. Wang M, Liu J, Bellows BK, Qi Y, Sun J, Liu J, Moran AE, Zhao D. Impact of China's Low Centralized Medicine Procurement Prices on the Cost-Effectiveness of Statins for the Primary Prevention of Atherosclerotic Cardiovascular Disease. Glob Heart. 2020; 15:43.
79. Wei H, Cui C, Cui X, Liu Y, Li D. Cost-effectiveness analysis of dabigatran, rivaroxaban and warfarin in the prevention of stroke in patients with atrial fibrillation in

China. BMC Health Serv Res. 2021; 21:96.
80. Ma C, Riou França L, Lu S, Diener HC, Dubner SJ, Halperin JL, Li Q, Paquette M, Teutsch C, Huisman MV, Lip GYH, Rothman KJ. Stroke prevention in atrial fibrillation changes after dabigatran availability in China: The GLORIA-AF registry. J Arrhythm. 2020; 36:408-416.
81. Guo Y, Wang H, Tian Y, Wang Y, Lip GYH. Time Trends of Aspirin and Warfarin Use on Stroke and Bleeding Events in Chinese Patients With New-Onset Atrial Fibrillation. Chest. 2015; 148:62-72.
82. Malenka DJ, Bhatt DL, Bradley SM, Shahian DM, Draoui J, Segawa CA, Koutras C, Abbott JD, Blankenship JC, Vincent R, Windle J, Tsai TT, Curtis J, Roe M, Masoudi FA.. The National Cardiovascular Data Registry Data Quality Program 2020: JACC State-of-the-Art Review. J Am Coll Cardiol. 2022; 79:1704-1712.
83. Chao TF, Chiang CE, Lin YJ, Chang SL, Lo LW, Hu YF, Tuan TC, Liao JN, Chung FP, Chen TJ, Lip GYH, Chen SA. Evolving Changes of the Use of Oral Anticoagulants and Outcomes in Patients With Newly Diagnosed Atrial Fibrillation in Taiwan. Circulation. 2018; 138:1485-1487.
84. China NHCoPsRo. Cardiovascular disease-related professional medical quality control indicators (2021 edition). Chinese Circulation Journal. 2021; 36:733-742. (in Chinese)
85. Hui S, Zheng L, Xinhua W, Long S, Zhichun G, Wei Z, Chi Z, Mangmang P, Lingcong K, Tian S, Qian Z, Yining Y, Liqing Z, Jun P. A plateform for management of atrial fibrillation based on internet and medical concortium. Chinese Journal of General Practitioners. 2021; 20:1100-1104. (in Chinese)
86. Hao Y, Liu J, Smith SC, Huo Y, Fonarow GC, Ge J, Liu J, Taubert KA, Morgan L, Guo Y, Zhou M, Zhao D, Ma C. Rationale and design of the improving Care for Cardiovascular Disease in China (CCC) project: a national registry to improve management of atrial fibrillation. BMJ Open. 2018; 8:e020968.
87. Li M, Shi J, Chu M, Shen Y, Zhang S, Sun X, Zhang H, Yan Q, Gong J, Lip GYH, Chen M. Screening for Atrial Fibrillation by Village Doctors in Rural Areas of China: The Jiangsu Province Rural Community AF Project. Vasc Health Risk Manag. 2022; 18:757-766.
88. Konieczyńska M, Bijak P, Malinowski KP, Undas A. Knowledge about atrial fibrillation and anticoagulation affects the risk of clinical outcomes. Thromb Res. 2022; 213:105-112.
89. Vinereanu D, Lopes RD, Bahit MC, *et al*. A multifaceted intervention to improve treatment with oral anticoagulants in atrial fibrillation (IMPACT-AF): an international, cluster-randomised trial. Lancet. 2017; 390:1737-1746.
90. Stevens D, Harrison SL, Kolamunnage-Dona R, Lip GYH, Lane DA. The Atrial Fibrillation Better Care pathway for managing atrial fibrillation: A review. Europace. 2021; 23:1511-1527.
91. Romiti GF, Proietti M, Vitolo M, Bonini N, Fawzy AM, Ding WY, Fauchier L, Marin F, Nabauer M, Dan GA, Potpara TS, Boriani G, Lip GYH. Clinical complexity and impact of the ABC (Atrial fibrillation Better Care) pathway in patients with atrial fibrillation: a report from the ESC-EHRA EURObservational Research Programme in AF General Long-Term Registry. BMC Med. 2022; 20:326.
92. Song D, Zhou J, Fan T, Chang J, Qiu Y, Zhuang Z, Ma J, Zeng L. Decision aids for shared decision-making and appropriate anticoagulation therapy in patients with atrial fibrillation: a systematic review and meta-analysis. Eur J Cardiovasc Nurs. 2022; 21:97-106.
93. Chen QQ, Li L, Liao Y, Wang LP. Study on warfarin medication checklist for improving patient compliance. Zhonghua Yu Fang Yi Xue Za Zhi. 2021; 55:1133-1138. (in Chinese)
94. Qiu S, Wang N, Zhang C, Gu ZC, Qian Y. Anticoagulation Quality of Warfarin and the Role of Physician-Pharmacist Collaborative Clinics in the Treatment of Patients Receiving Warfarin: A Retrospective, Observational, Single-Center Study. Front Pharmacol. 2020; 11:605353.
95. Zhang C, Pan MM, Wang N, Wang WW, Li Z, Gu ZC, Lin HW. Feasibility and usability of a mobile health tool on anticoagulation management for patients with atrial fibrillation: a pilot study. Eur J Clin Pharmacol. 2022; 78:293-304.
96. Guo Y, Chen Y, Lane DA, Liu L, Wang Y, Lip GYH. Mobile Health Technology for Atrial Fibrillation Management Integrating Decision Support, Education, and Patient Involvement: mAF App Trial. Am J Med. 2017; 130:1388-1396.e1386.
97. Guo Y, Guo J, Shi X, Yao Y, Sun Y, Xia Y, Yu B, Liu T, Chen Y, Lip GYH. Mobile health technology-supported atrial fibrillation screening and integrated care: A report from the mAFA-II trial Long-term Extension Cohort. Eur J Intern Med. 2020; 82:105-111.
98. Liu W, Zhou P, Wu B, Xiong N, Xie K, Bao L, Gao X, Wang Y, Shi H, Luo X, Li J. Effect of information management platformon the follow-up and anticoagulant therapy of 6 923 patients with atrial fibrillation. Chin J Cardiac Arrhyth. 2020; 24:293-297.
99. Ru X, Zhu L, Ma Y, Wang T, Pan Z. Effect of an artificial intelligence-assisted tool on non-valvular atrial fibrillation anticoagulation management in primary care: protocol for a cluster randomized controlled trial. Trials. 2022; 23:316.
100. Zuin M, Bilato C. Increase in United States atrial fibrillation-related mortality during the COVID-19 pandemic. Heart Rhythm. 2022.

Received August 15, 2022; Revised October 25, 2022; Accepted October 28, 2022.

**Address correspondence to:*
Yinglong Hou, Department of Cardiology, The First Affiliated Hospital of Shandong First Medical University, No. 16766, Jingshi Road, Lixia Zone, Ji'nan 250014, China.
E-mail: yinglonghou@hotmail.com

Released online in J-STAGE as advance publication October 30, 2022.

Review

DOI: 10.5582/bst.2022.01304

Current status and perspectives of non-coding RNA and phase separation interactions

Yue Gao[1,§], Chunhui Liu[2,§], Tiange Wu[1,§], Ruiji Liu[1], Weipu Mao[1], Xinqiang Gan[1], Xun Lu[1], Yifan Liu[1], Lilin Wan[1], Bin Xu[2,*], Ming Chen[2,*]

[1] Surgical Research Center, Institute of Urology, Medical School of Southeast University, Nanjing, Jiangsu, China;
[2] Department of Urology, Affiliated Zhongda Hospital of Southeast University, Nanjing, Jiangsu, China.

SUMMARY Phase separation refers to a phenomenon in which different components of a cell collide and fuse with each other to form droplets such that some components are encapsulated within the droplet and some are blocked outside. It is prevalent in eukaryotic cells and is closely related to genome assembly and transcriptional regulation, enabling multiple biological functions. With the development of high-throughput sequencing technologies, several non-coding RNAs (ncRNAs) have been shown to play an important role in epigenetic regulation of gene expression in addition to their roles at the transcriptional and post-transcriptional levels. In addition, some ncRNAs are involved in the formation of membraneless organelles (MLOs), the regulation of genomic stability and stress response through phase separation. Notably, phase separation can also affect the biogenesis, processing and maturation of ncRNAs. This review summarizes recent discoveries related to the relationship between ncRNAs and phase separation, providing new perspectives to guide future interventions.

Keywords phase separation, non-coding RNA (ncRNA), membraneless organelle (MLO), genomic stability, stress response

1. Introduction

Phase separation between intracellular biomolecules usually refers to the separation of eukaryotic proteins or nucleic acids (mostly RNA) through protein-protein, protein-nucleic acid and nucleic acid-nucleic acid multivalent binding aggregates from the surrounding liquid (cytoplasm or nucleoplasm) to form a semi-liquid (*1*), thereby maintaining order in the crowded chaos of cells. Study of phase separation began in 2009 when Tony Hyman and Cliff Brangwynne stuck worm gonads filled with P particles between two thin glass plates and slid the plates against each other. Under the shear stress of the sliding plates, the solids should have fallen off, however, the particles merged like raindrops, dropping and beading together (*2*). In 2021, Michael Rosen and Steven McKnight discovered a weak force between RNA and protein molecules in test tubes, which came close to each other and formed droplet-like substances (*3*). In 2015, Julie Forman-Kay discovered that a protein that affects sperm function forms droplets within human cells; this study accelerated the research into phase separation (*4*).

Many intracellular biological processes involve phase separation (Figure 1). Accumulating evidence has revealed that multiple membraneless organelles (MLOs) within cells, such as paraspeckles, Cajal bodies and stress granules, are condensates formed *via* phase separation of specific proteins/RNAs. These dynamic MLOs, along with the normative membranous organelles, maintain the spatial order within the cell, thus ensuring that continuous or independent biochemical reactions and regulatory processes are performed in an orderly manner. In response to stimulation by cellular stressors such as heat shock, hypoxia, nutritional starvation and DNA damage, ncRNAs can control the stability of the intracellular environment by forming biological condensates through phase separation (*5*). SERRATE-mediated phase separation forms D-bodies, which are essential for miRNA production (*6*). In addition, phase separation can lead to the formation of physicochemical and mechanical filters, such as nuclear pores (*7*). Meanwhile, ncRNA-mediated phase separation is required for genomic stability (*8*). Phase separation can locally concentrate molecules in cohesive condensates to activate cytoskeletal structural responses, signaling processes and nucleation (*9*). The understanding that phase separation underlies the formation of membrane-

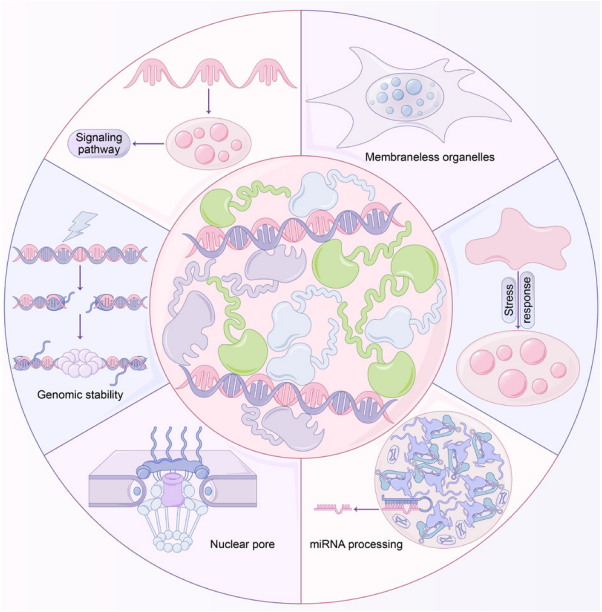

Figure 1. Summary of functions of phase separation. i. Formation of MLOs for spatial and temporal control. ii. Perceives external stimuli such as heat shock, hypoxia, nutritional starvation and DNA damage, and responds to them quickly. iii. Mediate miRNA production. iv. Formation of physicochemical and mechanical filters such as nuclear pore. v. Maintain genomic stability by repairing broken DNA duplexes. vi. Concentrate molecules in condensates to activate signaling process.

free compartments in cells has led to significant efforts to characterize the function of biomolecular condensates in neurodegenerative diseases and tumors (*7*).

The genomes of higher eukaryotes are commonly transcribed to produce large amounts of non-coding RNAs (ncRNAs) (*10*), which function in many ways and can interact with proteins, DNAs and RNAs to participate in various cellular activities, including but not limited to gene activation and silencing, RNA splicing, modification and editing and protein translation. Since the 1950s, various types of ncRNAs have been identified in eukaryotic cells, including the most abundant transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), microRNA (miRNA), linear long ncRNA (lncRNA), circular RNA (circRNA), etc. ncRNAs can be classified according to their length: small (about 20 bp), intermediate (less than 200 bp) and long (longer than 200 bp). Small ncRNAs have attracted many studies, such as: piwi-interacting RNAs (piRNAs) and miRNAs [14]. Intermediate ncRNAs include snRNAs involved in splicing during protein synthesis, and the remaining ncRNAs larger than 200 bp are classified as lncRNAs, which are involved in epigenetic regulation of transcripts and inactivation of the X chromosome.

ncRNAs, especially miRNAs and lncRNAs, have been shown to play an important role in the pathogenesis of many diseases. miRNA-132/212 promotes axonal evolution, neural migration, plasticity, and has very promising applications in neurodegenerative diseases such as Alzheimer's, Parkinson's, and epilepsy (*11*). Zhixin Ling reported miR-193a inhibited prostate cancer cell growth, suppressed migration and invasion, and significantly reduced prostate cancer xenograft tumor growth (*12*). lncRNA ITGB8-AS1 is highly expressed in colon cancer cells and tissues, and promotes proliferation, colony formation and tumor growth of colon cancer cells through integrin-mediated focal adhesion signaling (*13*). lncRNA SNHG1 regulates Treg cell differentiation and thus immune escape from breast cancer by regulating the miR-448/IDO signaling axis (*14*). A growing body of research indicates that ncRNAs can not only serve as biomarkers for disease diagnosis and prognosis, but they are also expected to be targets for novel therapeutic strategies.

Although ncRNAs were initially defined as a class of RNA transcripts without coding capacity, it has been determined that some ncRNAs actually contain open reading frames that can to be translated into micropeptides or microproteins with regulatory functions in a variety of biological and oncological processes. ncRNA performs biological functions in the form of encoded peptides, suggesting it as a new tumor marker and anti-cancer drug target with high clinical translational value.

Recent studies have revealed different effects of RNA on the occurrence of phase separation in cells. Some RNAs can facilitate protein phase separation by decreasing the critical saturation concentration. For example, the RNA-binding protein PTB can bind to RNA in the absence of intrinsically disordered regions (IDRs) and undergo phase separation in vitro. In addition, RNA can influence the properties of intracellular phase separation products. For example, short poly(A) RNA (50 nt) has a minimal effect on the critical saturation concentration for phase separation of the P-granule protein LAF-1 but causes dramatic changes in the internal physical properties of LAF-1 phase separation droplets, thus reducing their viscosity (*15*). Furthermore, including lncRNAs, miRNAs, circRNAs and eRNAs, can be involved in the regulation of biological processes *via* phase separation. In this review, we have summarized recent studies on the regulatory functions of phase separation and discussed its research progress in various biological processes in recent years, beginning from the relationship between ncRNAs and phase separation (Figure 2).

2. Molecular characterization and environmental conditions of phase separation

Phase separation is a process in which large molecules are separated from a large volume of solvent into different liquid phases, which is affected by a variety of factors. First, it is driven by multivalent interactions and prion-like structural domains (PrLDs), and IDRs and low-complexity regions (LCRs) of the protein are essential for the formation of phase separation (*16*). IDRs

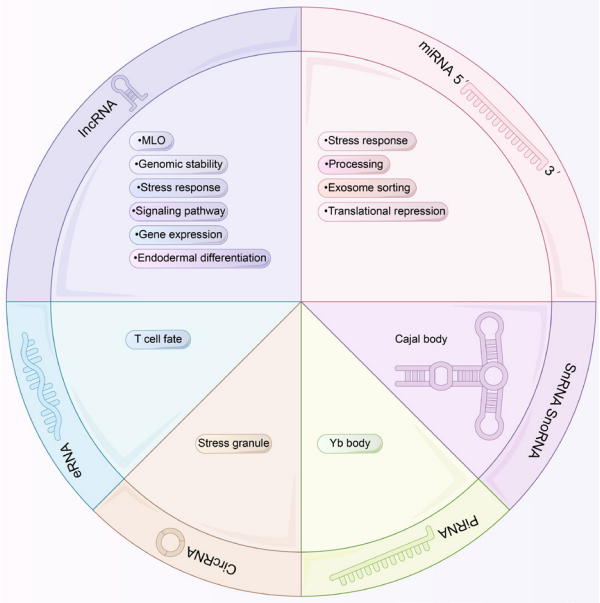

Figure 2. Interactions between ncRNAs and phase separation. i. lncRNAs mediate formation of MLOs, genomic stability, stress response, activation of signaling pathways, gene expression and endodermal differentiation via phase separation. ii. miRNAs regulate stress response by phase separation while phase separation contributes to miRNA processing, sorting miRNAs into exosomes and miRNA-mediated translational repression. iii. snRNA and snoRNA act as a scaffold for protein recruitment. iv. Yb bodies are multivalent condensates formed by phase separation and are responsible for piRNA biogenesis in Drosophila. v. circVAMP3 contributes to the formation of stress granules through phase separation. vi. ThymoD induces Bcl11b enhancer and promoter to form a loop that controls T cell development.

are usually free of the more aromatic and aliphatic amino acids that often construct the core of a folded structural domain. LCRs are regions with a higher proportion of a specific amino acid compared with other amino acids. In addition, the linear crosstalk of multiple identical/similar structural domains, such as SH2 and SH3 repeats of proteins such as NCK, and oligomerization of proteins can specifically mediate phase separation (*17*). During phase separation, solutions containing proteins and other biomolecules automatically form two phases, one enriched in proteins and one deficient in proteins. The dense phase formed is surrounded by a membrane that is selectively permeable to certain macromolecules and acts as a relative isolator, which ensures that various components of the cell are assembled at the right time and space to perform appropriate functions. The compartment formed *via* phase separation is similar to a liquid droplet and allows rapid exchange of substances with the surroundings (*18*).

Second, phase separation of certain proteins can also be regulated by other related proteins, DNAs and RNAs. If a nucleic acid molecule has a strong and specific multivalent interaction with a protein molecule, the nucleic acid molecule promotes protein phase separation. If the strongly charged nature of the nucleic acid molecule acts mainly as a salt ion-like electrostatic shield, weakening protein interactions, addition of the nucleic acid molecule inhibits phase separation; if both nucleic acid-protein interaction and electrostatic shielding are present but weak, phase separation is usually promoted at low nucleic acid concentrations and inhibited at high nucleic acid concentrations. This phenomenon provides novel insights into the relationship between ncRNA and phase separation and its mode of regulation in biological processes.

Third, the occurrence of phase separation is highly dependent on the proteins and nucleic acids in solution, but also on the environment and physicochemical properties of the solution such as temperature, pH, salt ion concentration, salt ion type, and the presence of other biomolecules in solution (*19*). Once phase separation occurs, the biomolecule is present in two forms, one at a low concentration in the solution and one at a higher concentration in the 'droplet'. The two forms can be transformed into each other as the relevant conditions change. In other words, phase separation is a highly dynamic process.

3. Experimental approaches and vital roles of phase separation

Various experimental approaches continue to enhance our understanding of phase separation (Table 1). The existence of a phase separation mechanism was demonstrated by purifying the target substance expression and reconstituting phase separation in vitro (*20*). Microscopic imaging plays an indispensable role as the main technique for observing phase separation. Phase separation in vitro can be observed very simply with an ordinary light microscope (*21*). The occurrence of phase separation is characterized by a cloudy solution and the presence of droplets in the solution such as oil droplets in water. The use of PEG or lipid-coated slides allows for better observation and documentation of phase separation. Time-lapse imaging, fluorescence recovery after photobleaching (FRAP) and 1,6-hexanediol sensitivity assays have been used to verify the dynamics and reversibility of phase-separated droplets (*7*). In addition, phase separation can be detected using a method that detects turbidity of a solution, or *via* centrifugal precipitation (*17*). Electrophoretic mobility shift assay (EMSA) and thermal shift measurement are also used to verify protein and nucleic acid interactions.

The role of phase separation in various physiological processes confers its importance in clinical translation. YAP mediates the pro-tumor effects of IFN-γ *via* phase separation and can be used as a predictive biomarker and target for anti-PD-1 combination therapy (*22*). Wang Shuai *et al*. demonstrated that the phase separation activity of SARS2-NP protein can be a possible therapeutic target for neo-coronavirus infection, providing new possibilities for related drug development (*23*). Lu Bing *et al*. proposed a small-molecule compound called GSK-J4, which regulates

Table 1. A set of experiments used to verify phase separation

Event	EWebsite/Method	Description
Phase-separated	MetaDisorder (*http://iimcb.genesilico.pl/metadisorder/*)	▪ Integrating results from multiple individual predictors
Protein prediction	MobiDB (*http://mobidb.bio.unipd.it/about*)	▪ Combining annotations from external databases, indirect evidence from experimental data
	PLAAC (*http://plaac.wi.mit.edu/*)	▪ Predicting PrLDs
	ZipperDB (*https://services.mbi.ucla.edu/zipperdb/*)	▪ Predicting fibril-forming segments
	FoldUnfold (*http://bioinfo.protres.ru/ogu/*)	▪ Predicting IDRs
	DISOPRED (*http://bioinf.cs.ucl.ac.uk/psipred/?disopred=1*)	▪ Providing information on non-structural regions of protein
	PONDR (*http://www.pondr.com/*)	▪ Predicting IDRs
	Iupred2a (*https://iupred2a.elte.hu/*)	▪ Predicting disordered and binding sites
	D2P2 (*http://d2p2.pro*)	▪ Providing the specific location of IDRs
Function	Microscopic detection	▪ Finding the presence of liquid droplets under the microscope (Incubation time and imaging parameters should be kept constant)
	Turbidity measurements	▪ Being detected by optical density measurement or direct static light scattering (Combined with microscope)
	Centrifugal grading	▪ Precipitating dense phase by centrifugation
	Inverse capillary velocity measurement	▪ Using fluorescence or transmitted light microscopy to photograph a molten droplet and measuring the time it takes for two droplets to fully fuse into a single droplet
	FRAP	▪ Assessing changes in the state of matter over time
	Fluorescence correlation spectroscopy	▪ Evaluating the diffusivity of individual molecules within the liquid state and the dilution of droplets
	1,6-hexanediol treatment	▪ Interfering with phase separation of many IDRs

phase separation of core regulatory circuitry factors, for therapeutic intervention in patients with chemoresistant and metastatic osteosarcoma (*24*). Ali Miserez *et al*. reported a coupled peptide that can form pH- and redox-responsive cohesive microdroplets through phase separation, thus facilitating delivery across cell membranes (*25*). In addition, stimulation of chaperone mechanisms to disassemble MLOs, induction of pathways that may inhibit aberrant phase separation, and development of antisense oligonucleotides to knock down RNAs can be evaluated as new strategies for the treatment of human diseases characterized by abnormal phase separation.

With the gradual increase in the study of phase separation, researchers have found that phase separation may lead to diseases such as neurodegenerative diseases, tumors, and aging. FUS aggregation is a pathological hallmark in patients with amyotrophic lateral sclerosis (ALS). FUS protein and hnRNPA1 form droplets in ALS, which become progressively more viscous and eventually form fibrous solids that are abnormally deposited in cells (*26*). Phosphorylation of Tau repeats under cellular protein conditions leads to phase separation of Tau and promotes its abnormal deposition in the brains of Alzheimer's disease patients (*27*). EWSR1 is a protein with a PrLD that is a fusion partner with transcription factors leading to oncogenicity. The abnormal 'phase separation' of EWSR1 promotes its accumulation near the genome associated with tumorigenesis, which is important for the oncogenicity of Ewing sarcoma (*28*). Super enhancers can induce the activation of classical oncogenes and other genes associated with tumorigenesis. Recent studies have found that transcription factors containing IDRs, transcriptional cofactors and RNA pol II form phase-separated condensates at super enhancers (*29*). APC phase separation was observed in colorectal cancer, resulting in excessive activation of the Wnt pathway, affecting cell differentiation and promoting rapid proliferation, playing a key role in the initiation of tumorigenesis (*30*). The tumor suppressor gene SPOP forms phase-separated membrane-free clusters in nuclear speckles, and these droplets play a central role in suppressing the development of a variety of human malignancies such as gastric, hepatocellular and prostate cancers (*31*). Biomolecular condensates are intracellular assemblies without membranes that are usually formed by phase separation. Its formation and dissolution can lead to protein misfolding and aggregation, which is often the cause of aging-related diseases (*32*).

4. Interaction between lncRNAs and phase separation

lncRNAs are ncRNAs that are > 200 nt in length. They play an important role in various life processes including a dosage compensation effect, epigenetic regulation, cell cycle regulation, and cell differentiation regulation. Like mRNAs, lncRNAs are transcribed from corresponding

genes with 5' caps and polyA tails, and are formed into mature lncRNAs by splicing. lncRNAs show a strong tissue-specific expression pattern, suggesting their integral role in cell type-specific processes. Abnormal expression or function of lncRNAs is closely related to the development of human diseases, including cancer and degenerative neurological diseases, and other serious human health risks (*33*). In recent years, it has been demonstrated that lncRNAs can mediate phase separation involved in various physiological processes such as the formation of membrane-free compartments and maintenance of genomic stability (Table 2).

4.1. lncRNA mediates the formation of MLOs through phase separation

The eukaryotic nucleus is not a homogeneous monospace organelle but a highly compartmentalized organelle separated by various types of membrane-free structures. In living organisms, cells ensure accurate spatial and temporal control of complex biochemical reactions through independent subcellular compartments. Although these compartments differ in composition, localization, and function, they have extremely similar morphological features, kinetic properties, and assembly (*34*). Understanding the mechanism and function of membrane-free compartments in detail has been a challenge because of their protein and RNA complexity. The interaction between proteins and RNAs leads to the formation of MLOs, which are involved in various biological functions including but not limited to response to environmental changes. The liquid condensates formed *via* lncRNA-related phase separation include nucleolus, paraspeckles, nuclear speckles, Cajal bodies, amyloid bodies (A-bodies), stress bodies and Omega speckles (Figure 3). These compartments are composed of specific lncRNAs, which play a role in their organization and function.

alluRNA

The nucleolus is the most evident structure in the interphase nucleus of eukaryotic cells, and its main function is to synthesize rRNA. It consists of three major components as follows: a fiber center (FC), dense fibrillar component (DFC) and granular component (GC). The lncRNA alluRNA, an RNA polymerase II transcript derived from the intron Alu element, plays an important role in the assembly and function of the nucleolus. Both NCL and NPM contain IDRs and LCRs that form protein structural domains. alluRNA acts as a scaffold to regulate nucleolus formation by promoting the self-organization of NCL/NPM-containing 'droplets' into domains that efficiently associate with rDNA and support Pol I transcriptional activity within the nucleolus, leading to nucleolus structure regulation and rRNA production (*35*).

Table 2. List of lncRNAs interacting with phase separation

lncRNA	Localization	Structure	Interaction	Function	References
alluRNA	Nucleus	Not known	NCL, NPM	Maintain nucleolar structure and function	*35*
NEAT1	Nucleus	tRNA-like small RNA at its 3' end	Paraspeckle components	Formation of paraspeckles	*36-39*
MALAT1	Nucleus	tRNA-like small RNA at its 3' end	SRSF1, SRSF2, SPOP	Formation of nuclear speckles	*42*
rIGSRNA	Nucleus	Not known	Short cationic peptidedomains	Formation of Amyloid bodies	*44*
HSATIII	Nucleus	Not known	SAFB, SR proteins, transcription factors	Formation of unclear stress bodies	*45-46*
hsr-omega	Nucleus	Not known	hnRNPs	Formation of omega speckles	*47*
TNBL	Nucleus	Not known	NPM1, SAM68	Formation of aggregates	*48*
dilncRNA	Nucleus	Not known	DNA damage responses RNAs and proteins	DNA damage responses	*52*
BGL3	Nucleus	Not known	BARD1	Homologous recombination	*53*
LINP1	Nucleus	Not known	Ku70/80	Non-homologous end joining	*54*
NORAD	Cytoplasm	Enriched with Pumilio response elements	PUM	Nucleation of PUM condensates	*8*
TERRA	Nucleus	Not known	TRF2	Telomere higher-ordered structure	*55*
Sme2	Nucleus	Not known	Smp	Recombination-independent pairing	*56-57*
SNHG8	Nucleus	Not known	Histone H1 proteins	Regulation of chromatin condensation	*58*
Xist	Nucleus	Repetitive RNA Subdomains	Repressive proteins complexes	Inactivation of X chromosome	*59-60*
GIRGL	Cytoplasm	Not known	CAPRIN1	Mediating glutamine deprivation stress	*63*
SNHG9	Cytoplasm	Six loops	LATS1	Progression of breast cancer	*64*
PNCTR	Nucleus	Not known	PTBP1	Inhibiting PTBP1 splicing activity	*65*
SLERT	Nucleus	Not known	DDX21	Facilitating Pol I transcription	*66*
DIGIT	Nucleus	Not known	BRD3	Regulating endoderm differentiation	*68*

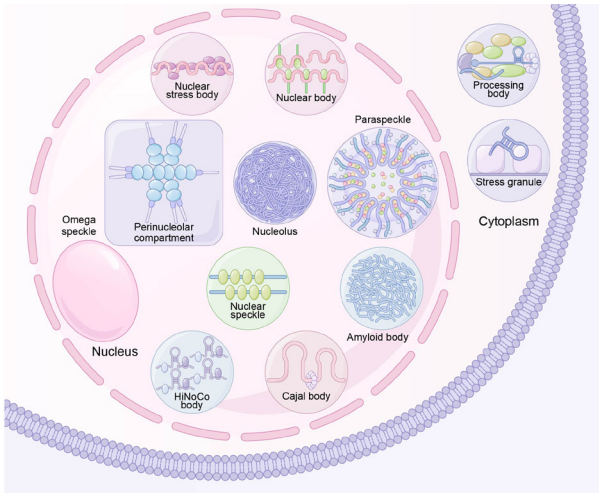

Figure 3. Biomolecular condensates formed by ncRNAs mediated phase separation. Nucleolus, Nuclear stress body, Perinucleolar compartment, Omega speckle, Nuclear body, Paraspeckle, Nuclear speckle, Amyloid body, HiNoCo body and Cajal body are found in the nucleus while processing body and stress granule are found in the cytoplasm. Not all compartments are present in each cell type, but are shown here for the sake of completeness. For example, Cajal bodies are only observed in a limited number of human cell types such as neurons and cancer cells.

NEAT1

Paraspeckles are composed of component proteins and RNA scaffolds that are essential for gene expression and intracellular homeostasis. They are subnucleosomes that are found near speckles, contain splicing factors and may regulate gene expression by sequestering mRNAs and proteins (*36*). The number of paraspeckles increases in many situations, such as when cells change from one state to another, are infected by viruses and bacteria, begin to degenerate and are in a state of inflammation, aging and cancer (*37*). Many paraspeckle proteins contain prion-like IDR structural domains that contribute to the formation of large phase-separated condensates in cell nuclei and hydrogels in vitro. Recent studies have shown that lncRNA NEAT1 can act as a scaffold to recruit specific component proteins to be enriched at high local concentrations. Subsequently, these component proteins recruit other proteins that mediate phase separation and the formation of paraspeckles through LCR. NEAT1 has three distinct RNA structural domains (A, B and C) that play a role in stabilization (A), isoform conversion (B) and scattering assembly (C) (*38*). NEAT1 has two transcripts, NEAT1-1 and NEAT1-2, both of which share the same 5' terminus. The longer NEAT1_2 isoform (22.7 kb in humans) is an important component of paraspeckles, whereas the shorter NEAT1_1 isoform (3.7 kb in humans) is dispensable. NEAT1-2 provides a structural scaffold for paraspeckles and has high-affinity constitutive sites for the core paraspeckle proteins NONO and SFPQ, which rapidly bind to NEAT1-2 ribonucleoproteins, which are intermediates in para-plaque formation. Subsequently, FUS and RBM14 are recruited and mediate the onset of phase separation, resulting in the formation of mature paraspeckles (*39*).

MALAT1

Nuclear speckles are prominent membrane-free compartments in the nucleus, which control different steps in gene expression, including transcription, splicing and mRNA export (*40*). An important function of nuclear speckles is to harbor spliceosomal small nuclear ribonucleoproteins (RNPs) and enable them to catalyze the removal of introns from transcriptionally active genes located at the periphery of the speckle. lncRNA MALAT1 is a major component of this droplet. It is localized in the periphery of nuclear speckles and in the centrally located pre-RNA splicing factor (*41*). Most proteins within nuclear speckles can directly bind to RNA, including SRSF1, SRSF2 and SPOP, leading to the recruitment of polyadenylated mRNA and uridine-rich small nuclear RNA within nuclear speckles and eventually phase separation to initiate droplet formation (*42*). m6A-modified MALAT1 acts as a scaffold to recruit YTHDC1 to nuclear speckles and regulate the expression of key oncogenes (*43*).

rIGSRNA

A-bodies are a prime example of the widespread use of architectural RNA in the construction of membrane-free compartments. They are non-dynamic MLOs, and their non-dynamic nature is attributed to proteins that adopt a reversible amyloid conformation. Assembly of A-bodies requires the expression of rIGSRNA derived from stimulus-specific sites of rDNA intergenic spacers. Low-complexity rIGSRNA sequences interact with short cationic peptides to induce a unique liquid-like phase in the nucleolus region. rIGSRNA is a determinant of A-body protein recruitment because its silencing reduces the number of liquid-like nucleolus foci and impairs the formation of mature A-bodies during heat shock (*44*).

HSATIII

HSATIII is transcriptionally repressed under physiological conditions, and its transcription is induced by HSF1 under heat shock stress conditions. Several repetitive sequences of HSATIII can bind to scaffold attachment factor B (SAFB), SR proteins and transcription factors to assemble nuclear stress bodies. Downregulation of HSATIII significantly affects the recruitment of RNA processing factors to nuclear stress bodies without altering the association of HSF-1 with these structures or the presence of acetylated histones in the nuclear stress bodies (*45*). Comprehensive HSATIII identification of RNA-binding proteins *via* mass spectrometry has helped to identify multiple splicing

factors in nuclear stress bodies, including SRSFs, whose phosphorylation status affects the splicing pattern. SRSFs rapidly dephosphorylate upon heat stress exposure. During stress recovery, CDC recruits substances such as CLK1 to nuclear stress bodies and accelerates the rephosphorylation of SRSF9, thereby promoting target intron retention (*46*).

hsr-omega

Omega speckles are scaffolded by hsr-omega and distributed in the interchromatin space, close to chromatin. In situ immunocytochemical staining using antibodies against heterogeneous nuclear RNA binding proteins (hnRNPs) such as HRB87F, Hrp40, Hrb57A and S5 has shown that all hnRNPs resulted in diffuse staining of chromatin regions in all cell types, in addition to the presence of a large number of spots. In addition, studies have revealed absolute co-localization of hnRNPs and omega speckles. Immunoprecipitation studies using hnRNP antibodies have demonstrated the physical association between hnRNPs and hsr omega. Therefore, hsr-omega plays an important structural and functional role in the organization and establishment of hnRNP-containing omega speckles, thereby regulating the transport and availability of hnRNPs and other associated RNA-binding proteins in the nucleus (*47*).

TNBL

Somatic global genomic hypomethylation is common in almost all cancer types, including colon cancer. Following DNA hypomethylation and histone acetylation, NBL2 repeat sequences are transcribed in colon cancer cell lines, exhibit promoter activity, and are contained in a novel non-polyA antisense lncRNA named TNBL. TNBL is stable throughout the mitotic cycle and forms perinuclear aggregates preferentially in the interphase nucleus near a subpopulation of NBL2 sites (*48*).

In addition, processing bodies (PBs) are RNPs formed *via* RNA-dependent phase separation, are enriched with various enzymes required for RNA processing and degradation and play a key role in the spatial regulation of gene expression. Super-resolution single-molecule fluorescence microscopy has shown that most miRNAs are stably anchored to the core or shell layer of PBs, whereas lncRNAs are temporarily bound to the PB shell, indicating that the localization of RNAs in PBs is closely related to the RNA species (*49*). Examining the effects of RNA assemblies on particle formation, and the effects of particle formation on RNA assemblies, may help to reconstruct at least some aspects of RNA particles in vitro.

4.2. lncRNA affects genomic stability *via* phase separation

Although proteins and DNA were previously considered to be the major components of chromatin, scholars have recently recognized that RNA occupies a large amount of chromatin and acts as a regulator of nuclear structure (*50*). Many lncRNAs tend to remain in the nucleus and cooperate with protein complexes to regulate epigenetic regulation, which is essential for gene expression and genomic stability (*51*).

dilncRNA and BGL3

DNA damage can increase genomic instability, which can lead to cellular senescence and death. DNA damage response (DDR) identifies the sites of DNA damage and repairs them, which can be regulated by RNAs. In DDR, 53BP1 protein is recruited to form foci in DNA double-strand breaks (DSBs). These foci have recently been identified as biomolecular condensates with droplet-like behavior. Homologous recombination and non-homologous end-joining are two repair modes of DSBs. DDSR1, BGL3, PRLH1, and TERRA promote DNA break repair through homologous recombination, whereas HITT, LINP1 and SNHG promote DNA break repair through non-homologous end-joining.

Once a DSB has occurred, the MRE11-RAD50-NBS1 (MRN) complex recognizes the exposed DNA ends and recruits RNA polymerase II (RNAPII) to synthesize damage-induced long non-coding RNA (dilncRNA). dilncRNA promotes DDR proteins, such as 53BP1, to undergo phase separation, causing DDR lesions to exhibit fluid-like behavior, which in turn completes the process of DNA damage repair (*52*). LncRNA BGL3 acts as a molecular scaffold during homologous recombination by binding BARD1 and enhancing the binding of BARD1 to other repair proteins (*53*).

LINP1

Studies have confirmed that some RNAs can form phase-separated condensates through RNA-RNA interaction, such as the lncRNA LINP1. LINP1 is highly conserved among species and is involved in DNA repair in triple-negative breast cancer; its expression is closely associated with tumorigenesis. In the presence of 5% PEG400, full-length LINP1 is expressed as droplets ranging in size from 10 to 24 nm. Non-homologous end-joining does not depend on DNA homology but avoids the retention of DNA or chromosome breaks and the resulting DNA degradation or impact on viability by connecting two broken DNA ends to each other via Ku proteins. Ku is a heterodimer composed of 70 and 80 kDa polypeptides (Ku70/80) of heterodimers that bind to LINP1, which in turn multimerizes to stabilize the initial synaptic event, thus enabling efficient non-homologous end joining (*54*).

NORAD

NORAD is a highly conserved and cytoplasmically enriched lncRNA that is activated by DNA damage and is required for mammalian genomic stability. It drives efficient condensation and sequestration of PUM through multivalent PUM-NORAD RNA binding interactions and IDR-driven PUM-PUM interactions to form phase-separated PUM condensates that competitively repress other PUM-binding transcripts. NORAD deficiency leads to PUM hyperactivity, resulting in the suppression of PUM target mRNAs, which includes important regulators of mitosis, eventually leading to a significant genomic instability phenotype in NORAD-deficient cells (*8*).

TERRA

Telomeres are nuclear protein structures formed at the end of chromosomes, which are essential for chromosome integrity and stability. One of the hallmarks of pluripotent telomeres is high TERRA levels, and TERRA depletion induces telomere dysfunction. In humans, TERRA is transcribed from subtelomeric promoters at the ends of most chromosomes and is associated with telomere maintenance. In mice, TERRA originates primarily from the pseudoautosomal PAR locus; however, TERRA derived from chromosomes 18q, 2 and X has also been identified. TERRA is enriched in telomeric regions and scaffolds the nucleation of telomere-associated proteins. Phase separation provides an attractive model for coordinating various biochemical reactions occurring in genomic compartments and the nucleus and is fundamental for maintaining genomic integrity (*55*).

Sme2

Meiosis is the basic process of sexual reproduction in eukaryotes and plays an important role in the inheritance and variation of organisms. During this process, homologous chromosomes are selectively aligned and paired. Sme2 RNA is a meiosis-specific 1,500 nt lncRNA that accumulates at its locus, plays an active role in recombination-independent pairing, and is essential for recombination-independent pairing of homologous chromosomes in *Schizosaccharomyces pombe*. A portion of Sme2 RNA is present in the nucleus and co-localizes with Mei2p sites during the prophase of meiosis, forming Mei2p dots with droplet-like morphological features, which are smaller and clearer than the nucleolus and appear very compact (*56*). Smp proteins are conserved RNA-binding proteins that are associated with meiosis-specific lncRNAs that co-accumulate at sme2 and two other chromosomal loci. In addition, Smp proteins co-localize with Mei2-mCherry, a known protein located at the sme2 locus, and are required for robust pairing at the sme2 locus. Smp proteins contain IDRs necessary for phase separation. 1,6-hexanediol treatment reversibly disassembles these complexes and disrupts pairing at the relevant loci, suggesting that lncRNA-protein complexes mediate homologous chromosome recognition through phase separation, thereby mediating the pairing of homologous chromosomes (*57*).

SNHG8

The binding of histone H1 to ribosomes stabilizes the condensed state of chromatin and allows for the relative structural isolation of DNA. lncRNA SNHG8 is localized to chromatin and can interact with and promote the phase separation of histone H1. Overexpression of SNHG8 increases the amount of H1 bound to chromatin, promotes chromatin condensation, and induces gene expression patterns associated with epithelial differentiation (*58*).

lncRNA XIST mediates the silencing of gene transcription on the X chromosome during female mammalian development by recruiting repressive protein complexes (*59*). During early development, one of the two X chromosomes in female mammals is suppressed for dosage compensation. During this process, approximately 17 kb of XIST is produced from a region of the inactive X chromosome called the X inactivation center. It subsequently spreads along the entire chromosome, forming a cloud of XIST RNA on it, and compresses and inhibits it. In the nucleus, XIST RNA can act as a platform to create a microenvironment for repression of genes on inactive X chromosomes. For example, XIST recruits proteins such as HNRNPU (with a phase separation propensity of 2.5) and MATR3 (with a phase separation propensity of 1.5) to recruit the polycomb repression complex 2 (PRC2), which contributes to chromosome compaction and gene repression. Similarly, Andrea Cerase hypothesized that during X chromosome inactivation, XIST acts as a scaffold to recruit repressor proteins, which in turn recruit other IDR-containing proteins to bind to them, forming phase separation agglutination from which inactive X chromosomes are separated, and the peripheral localization of inactive X chromosomes helps to maintain phase separation. However, further experiments are warranted to support this hypothesis (*60*).

4.3. lncRNA mediates cellular stress response through phase separation

Stress is the adaptive response of an organism to environmental changes. Various factors such as hormones, neuroendocrine mediators, peptides and neurotransmitters are involved in maintaining the homeostasis of the central nervous system, digestive system, cardiovascular system and endocrine system. In recent years, it has been shown that membrane-free compartments formed via the phase separation of specific proteins are involved in the stress response. lncRNAs are

one of the important regulators of cellular stress. Some lncRNAs interact with proteins, resulting in the partition of molecules between dense cohesive and dilute liquid phases. Upregulation or translocation of lncRNAs may lead to changes in the local concentration of lncRNAs, which may trigger the nucleation of RNPs under stressful conditions (*5*).

MALAT1

MALAT1 is primarily localized to nuclear speckles and regulates selective splicing in nuclear speckles by interacting with serine- and arginine-rich family proteins. It is released from nuclear speckles shortly after heat shock via the PCBP-mediated pathway and localizes to HiNoCo bodies in an activity-dependent transcriptional manner. MALAT1 translocates from nuclear speckles to the nucleoplasm and is predominantly translocated to the nucleus after 10-15 min. HiNoCo bodies remain in the nucleus as long as 3 h after heat shock. They are disrupted after treatment with 1,6-hexanediol, indicating that they may be formed via phase separation. In addition, cell proliferation is reduced under heat shock stress after MALAT1 knockdown, suggesting that MALAT1/HiNoCo bodies are important for the heat shock response (*61*).

NEAT1

TDP-43 is a highly conserved RNP that is currently considered a pathological marker protein for neurodegenerative diseases such as ALS. The TDP-43 protein contains a nuclear localization signal, a nuclear export signal, and three potential Caspase-3 recognition sites. In recent years, TDP-43 has been shown to undergo phase separation in vitro. Nuclear bodies (NBs) are dynamic, membrane-free structures that contain specific nuclear proteins and RNAs to regulate nuclear function and homeostasis. Various cellular stresses trigger the dynamic, reversible formation of TDP-43 NBs, which are partially co-localized with paraspeckles, and their scaffold lncRNA. NEAT1 is dramatically upregulated in stressed neurons and mediates their nucleation. Because NEAT1 is > 20 kb in length and has a complex secondary structure, it can provide multiple binding sites for TDP-43 molecules. This provision may increase multivalent interactions, leading to the co-phase separation of TDP-43 and NEAT1, thus forming the first line of defense against stress and diseases (*62*).

GIRGL

Glutamine is the most abundant circulating amino acid, and glutaminase-1 (GLS1) is the rate-limiting enzyme for glutamine catabolism. Under glutamine-deficient conditions, GLS1 expression is inhibited. lncRNA GIRGL inhibits GLS1 translation via phase separation. Under physiological conditions, HuR affects the stability of GIRGL transcripts via an AGO2-mediated RNA-induced silencing complexes (RISCs) mechanism. In glutamine-deficient cells, GIRGL levels are upregulated owing to both a c-Jun-mediated increase in transcription and significantly longer half-lives, thus inducing the complex formation of CAPRIN1 and GLS1 dimers. This phenomenon helps to promote phase separation of CAPRIN1 and induces the formation of stress granules, which are produced by various factors, including heat shock, osmotic stress, oxidative stress, and nutrient starvation (*63*).

4.4. lncRNA regulates signaling pathways through phase separation

Some lncRNAs regulate signal transduction and cancer progression through phase separation. One of the core kinases of the Hippo pathway, LATS1, contains a PrLD in its N-terminal fragment, indicating that LATS1 can undergo phase separation. In addition, exogenously expressed LATS1-GFP forms puncta in the cytoplasm, whereas PrLD-deficient LATS1 mutants cannot form puncta. When the Hippo pathway is activated, MST, MAP4K, TAOK and other upstream regulators phosphorylate and activate LATS1. Activated LATS1 promotes YAP phosphorylation and isolates YAP in the cytoplasm, leading to YAP degradation. SNHG9, a conservative lncRNA in humans, rhesus monkeys and mice, is up-regulated in breast cancer and interacts directly with LATS1 to promote the formation of heavy particles or potential droplets containing LATS1. SNHG9 reduces LATS1 phosphorylation and its kinase activity, inhibiting the Hippo pathway and leading to the activation and translocation of YAP to the nucleus, thereby promoting breast cancer progression (*64*).

4.5. lncRNA regulates gene expression through phase separation

The function of many lncRNAs depends on their ability to interact with multiple copies of specific RNA binding proteins (RBPs). Such lncRNAs can mediate gene expression in various ways via phase separation.

PNCTR

lncRNA PNCTR is upregulated in multiple cancers and is associated with programmed cell death, which is encoded as a short tandem repeat in the rDNA intergenic spacer and contains many PTBP1-specific motifs. PTBP1 has been shown to stimulate the expression of several apoptosis activators by altering the splicing of its pre-mRNA or increasing its translation efficiency. It has recently been shown that PNCTR contains hundreds of PTBP1-specific motifs, acting as a scaffold structure to recruit PTBP1 and form relatively isolated perinuclear

compartments to control alternative splicing and promote cell survival (*65*).

SLERT

The nucleolus is a membrane-free nuclear condensate driven by phase separation, in which FC/DFC units are the site of RNA polymerase I (RNAPI)-mediated transcription of ribosomal DNA (rDNA) and pre-processing of rRNA. Rapid rRNA transcription occurs at the boundary of FC and DFC units, which is formed by the RNA helicase DDX21. lncRNA SLERT facilitates the transition of DDX21 from an open to a closed conformation through phase separation. DDX21 forms loose clusters in the closed conformation, providing the FC/DFC units with sufficient mobility and space required for RNAPI synthesis, thus regulating the multilayer nucleolus structure and enabling rapid RNAPI synthesis (*66*).

4.6. lncRNA regulates definitive endodermal differentiation through phase separation

The liver, lungs, pancreas and digestive tract originate from the endoderm, and the optimization and specification of endodermal differentiation are essential for generating the cell types of these organs. lncRNA DIGIT is induced during endodermal differentiation, and the loss of its expression results in defective endodermal differentiation (*67*). Mass spectrometry and immunoblotting have validated the interaction between DIGIT and the BRD3 protein, a member of the bromodomain and extra-terminal domain family of proteins. BRD3 and H3K18ac interact and occupy promoters and enhancers of genes in embryonic stem cells. Furthermore, DIGIT is enriched in H3K18ac-modified chromatin regions, and BRD3 occupancy decreases as DIGIT is depleted, suggesting that DIGIT recruits BRD3 to the H3K18ac locus via the bromodomain. DIGIT-induced BRD3 has the properties of a phase-separated condensate that regulates definitive endoderm differentiation (*68*).

5. Interaction between miRNAs and phase separation

Genes encoding miRNAs in the nucleus are transcribed by RNA polymerase to produce primary transcripts (pri-miRNAs) of several thousand bases in length. These pri-miRNAs are mainly processed via the protein complex of the micro-processor. This complex, which is approximately 400-500 kDa in size, mainly consists of two proteins, Drosha and Pasha. Drosha is an RNase III protein, whereas Pasha is a double-stranded RNA-binding protein involved in substrate recognition by Drosha. pri-miRNAs are further processed into precursor miRNAs (pre-miRNAs) containing 60-70 nt of stem-loop structure by the action of Drosha. pre-miRNAs are transported from the nucleus to the cytoplasm via the RanGTP/Exportin-5 transport protein (*69*). Nuclear pores responsible for transporting pre-miRNAs exhibit hydrogel characteristics (*70*). In the cytoplasm, pre-miRNAs are recognized by Dicer and form miRNA: miRNA* dimers through shearing and modification of the stem-loop structure. miRNA: miRNA* dimers are destabilized by the action of decapping enzymes and eventually generate mature, functional single-stranded miRNAs, which subsequently bind to the miRNP complex, whereas miRNA* is rapidly degraded. The mature miRNA, along with other proteins, forms the RISC, which degrades or inhibits the translation of the target mRNA (*71*). Both cleavage steps are catalyzed by the cleavage complex (D-body), which contains three core components, including the RNase III family protein Dicer-like 1 (DCL1), the double-stranded RBP, Hyponastic leaf 1 (HYL1) and the zinc finger protein Serrate (SE) (*6*).

5.1. miRNA regulates stress responses through phase separation

miRNAs can simultaneously regulate multiple targets and rapidly adapt to cellular metabolism in response to reversible stress. Drosophila miR-980 is one of the stress response factors and is associated with ovarian germ line differentiation. In a study, under stress conditions, miR-980 levels were significantly reduced, whereas Rbfox1 mRNA levels were increased, and a luciferase reporter gene assay verified that miR-980 directly targeted Rbfox1 in vitro and in the ovary. miR-980 also affects the protein levels of Rbfox1. Drosophila Rbfox1 protein contains multiple LCRs and can undergo phase separation under stress to form condensates, which can be disintegrated by 1,6-hexanediol. Therefore, miR-980-mediated Rbfox1 phase separation is a novel stress-responsive signaling cascade with profound effects on the survival, growth and differentiation of cells, and on the ability of organisms to survive under stress (Table 3) (*72*).

5.2. Phase separation regulates miRNA processing

The D-bodies are 0.2-0.8 μM in diameter and are essential for miRNA processing. They are central to miRNA biogenesis and represent phase-separated condensates of SE proteins, which contain IDRs (*73*). SE can form phase-separated droplets through weak intermolecular interactions generated by its N-terminal IDR and can subsequently recruit DCL1, HYL1 and pri/pre-miRNAs into the droplets to form D-bodies (*74*). As cleavage proceeds, pri-miRNAs are gradually consumed, intermolecular collisions are reduced, the interaction between HYL1 and SE is weakened and pri-miRNAs are released outside the cleavage vesicle. The release of miRNAs from D-bodies is achieved via co-transport.

Table 3. List of other ncRNAs (except lncRNAs) interacting with phase separation

ncRNA	Localization	Structure	Interaction	Function	References
miR-980	Cytoplasm	Not known	Rbfox1	Cell survival	*72*
miR-223	Cytoplasm	Not known	YBX1	Packaging RNAs into exosomes	*79*
snRNA	Nucleus	Not known	Coilin	Forming Cajal bodies	*86-88*
snoRNA					
piRNA	Cytoplasm	Uridine at the 5' end and 2'-O-methyl modification at the 3' end	Piwi proteins	Regulating gene silencing	*90-91* *96* *99*
circVAMP3	Cytoplasm	Closed loop	CAPRIN1	Inhibiting Hepatocellular Carcinoma	
ThymoD	Nucleus	Not known	Cohesin	Repositioning of the Bcl11b enhancer	

D-bodies are involved in gene transcription, RNA variable splicing and transposon silencing in addition to contributing to the maturation and release of miRNAs through phase separation. Therefore, phase separation may also play a role in these processes (*6*). Consistently, Seung Cho Lee and Robert A Martienssen reported that D-bodies are sites of miRNA biogenesis in plants and represent phase separated condensates of SE proteins containing IDRs.

FUS is one of the RNA-binding proteins containing an IDR and is associated with neurodegeneration. Mutations in its PrLD or nuclear localization signal enhance the conversion of FUS from liquid to solid deposits. FUS promotes Drosha recruitment on nascent pri-miRNAs, leading to miRNA maturation (*75*). As a pathological marker of ALS and frontotemporal dementia, TDP-43 fuses with FUS and is involved in the biogenesis and metabolism of coding RNAs and ncRNAs (*76*).

Stress-induced interactions between stress granules and miRNA-associated proteins (AGO2 and DICER) can regulate miRNA biogenesis by attenuating DICER catalytic activity (*70*). In a study, in a rat model of acute ischemic stroke with middle cerebral artery occlusion, miR-335 promoted the formation of stress granules by downregulating ROCK2 (*77*). Therefore, membraneless compartments and biomolecular condensates can regulate miRNA biogenesis. In addition, miRNAs can regulate the homeostasis of many biological and physiological processes such as transcription and apoptosis (*78*).

5.3. Phase separation specifically sorts miRNAs into exosomes

The RNA-binding protein YBX1 not only forms liquid biomolecular condensates in cells, but also undergoes phase separation in vitro via tyrosine- and arginine-rich motifs in the IDR. Two structural domains of YBX1, namely, CSD and IDR, are sorted together into cell-secreted exosomes, leading to the recruitment of miR-223 to droplets. Overexpression of YBX1 increases miR-223 levels in HEK293T and U2OS exosomes, and YBX1 phase separation is critical for the recruitment of miR-223 to exosomes (*79*). Point mutations in YBX1 inhibit its phase separation, prevent biomolecular condensation resulting from the admixture of YBX1 protein into cells and interfere with the sorting of miR-233 into exosomes. Increasing the RNA/YBX1 ratio initially promotes droplet size until the droplet is unstable or not produced.

5.4. Phase separation contributes to miRNA-mediated translational repression

The AGO family and miRNAs assemble into miRISCs that synergize with the GW182 protein to bind to target mRNAs with sequence complementarity to miRNAs, leading to their degradation (*80*). Nup358, a mutated protein present in patients with acute necrotizing encephalopathy, interacts with the C-terminal silencing domain of GW182 in its N-terminal region, affecting the inhibition of miRNA-mediated translation (*81*). miRISCs can assemble into phase-separated condensates through the interaction between AGO2 and GW182. Recent studies have also supported the phase-separating property of Nup358. Therefore, it can be speculated that Nup358 phase separation contributes to the stable binding of miRISCs to target mRNAs in the cytoplasm and hence plays a role in miRNA-mediated translational repression (*82*). In addition, AGO proteins undergo lipid-mediated phase separation to control de novo peptide ubiquitination (*83*).

FMRP is enriched in neuronal granules and can bind to RNA to regulate translation. It contains LCRs that form droplet-like condensates with 4E-BP2 and miR-125b, which coincides with in vitro inhibition of translation. Phosphorylation of the LCRs of FMRP increases its phase separation tendency and facilitates particle assembly, whereas their methylation decreases the phase separation tendency and facilitates particle disassembly (*84*).

6. Interaction between other ncRNAs and phase separation

snRNA and snoRNA

Cajal bodies are organelles present in the nucleus of proliferating cells. They are often close to the nucleolus, which is the most prominent nucleolar structure (*85*). Cajal bodies are structures that coordinate, facilitate and investigate the nuclear phase of spliceosomal snRNP biogenesis. They undergo fusion and fission events

and are sensitive to 1,6-hexanediol treatment, which is a key feature of the droplet organelle. Cajal body-associated regions are enriched with highly expressed histone genes and U sn/snoRNA loci that form intra- and inter-chromosomal clusters (*86*). snRNAs and snoRNAs act as scaffolds for local recruitment through RNA-protein interactions at high protein concentrations. The Cajal body structural protein, coilin, is both highly phosphorylated and methylated, which is essential for nucleosome assembly (*87*). Coilin, along with another essential Cajal body protein called SMN1, can oligomerize and interact with various key effector proteins and snRNPs. It forms a shell around the periphery of Cajal bodies, whereas SMN1 forms the inner core. Notably, Logan *et al*. demonstrated that disruption of Cajal bodies is associated with altered levels of primary and mature miRNAs and the let-7a mRNA target HMGA2 and suggested that Cajal bodies and miRNA processing mechanisms functionally interact to promote the biogenesis of miRNAs and snRNPs (88).

piRNA

piRNAs are small ncRNAs (24-31 nt) that can form complexes with Piwi proteins of the AGO family (*89*). piRNAs are characterized by uridine at the 5' end and 2'-O-methyl modification at the 3' end (*90*). They are mainly found in mammalian germ cells and stem cells and form piRNA complexes by binding to Piwi proteins. They regulate gene silencing pathways by binding to piRNA complexes.

In *Drosophila*, Yb bodies are liquid-like multivalent condensates that are considered the site of piRNA biogenesis in ovarian somatic cells. Yb is the major component of Yb bodies and consists of the C-terminus of helicase, RNA helicase and the extended Tudor structural domain; among which, the structural domain of RNA helicase is required for Yb-RNA interactions, Yb body formation and piRNA biogenesis (*91*).

AGO2 and TNRC6B are core protein components of RISCs, and multivalent interactions between the glycine/tryptophan-rich structural domain of TNRC6B and the tryptophan-binding pocket in the PIWI structural domain of AGO2 facilitate the formation of phase-separated droplets (*92*). Phase separation can isolate miRNA targets and accelerate their deadenylation, thereby regulating the rate of mRNA translation and decay. In a study on *Caenorhabditis elegans*, an IP assay verified that DEPS-1 binds to the PIWI protein PRG-1 through its PIWI binding site and forms elongated cohesions *in vivo* that mediate piRNA-dependent silencing (93).

circRNA

circRNAs are a unique class of RNA molecules. Unlike traditional linear RNAs, circRNA molecules have a closed-loop structure, are not affected by RNA exonucleases and are more stably expressed and less susceptible to degradation (*94*). Recent studies have shown that circRNAs are rich in miRNA binding sites, which act as miRNA sponges in cells, thereby relieving the repressive effect of miRNAs on their target genes and elevating their expression levels. This mechanism of action is known as the competitive endogenous RNA (ceRNA) mechanism (*95*). Several studies have shown that circRNAs play an important regulatory role in diseases.

circVAMP3, formed by reverse splicing of exons 3 and 4 of the VAMP3 gene, is lowly expressed in hepatocellular carcinoma tissues and correlates negatively with patient prognosis. In a study, an immunoprecipitation assay showed that antibodies to the RNA-binding protein CAPRIN1 or G3BP1 significantly enriched circVAMP3 relative to IgG antibodies. In addition, circVAMP3 promoted CAPRIN1 and G3BP1 phase separation in a concentration-dependent manner, leading to the formation of a stress granule, inhibition of c-Myc translation to downregulate protein levels of the Myc proto-oncogene protein and impairment of HCC cell proliferation and metastasis in vitro and *in vivo* (*96*).

eRNA

The ncRNA transcribed from enhancers is defined as 'enhancer RNA' (eRNA) (*97*). Studies have shown that eRNA transcription can stabilize or enhance phase separation. Super enhancer RNA promotes phase separation of super enhancer through RNA-RNA interactions and successfully forms local liquid condensates (*98*).

T cells are the primary performers of human immune function, and their developmental progression is regulated by a combination of transcriptional regulators. During T cell development, the intergenic region of Bcl11b containing enhancers is relocated from the lamina to the nuclear interior to direct the Bcl11b enhancer to the Bcl11b promoter. Enhancer RNA ThymoD mediates the repositioning of Bcl11b enhancers. ThymoD transcription promotes the demethylation of CTCF binding sites, recruiting the cohesin complex to the transcribed region to activate the cohesin-dependent loop that induces loop formation of Bcl11b enhancers and promoters, allowing epigenetic labeling of the activated deposited loop domain to facilitate phase separation (*99*).

7. Conclusion

Protein phase separation has emerged as a potential mechanism for regulating biological function. RNA-protein condensates are essentially hydrogel-like droplets rich in RNA and RNA-associated proteins. Stress-induced phase separation via RNA-binding proteins may be an evolutionarily conserved mechanism for cellular adaptation to and survival against environmental

stress (*76*). Notably, the sequence-specific base-pairing properties of RNAs can lead to their phase separation and are responsible for many neurological disorders such as Huntington's disease, muscular dystrophy, and ALS (*100*). The genomes of higher eukaryotes are commonly transcribed to produce large amounts of ncRNAs, which are key regulators of embryonic development, DNA damage response, and human diseases such as neuronal diseases, heart dysfunction and cancer.

The extensive involvement of ncRNAs and phase separation in the regulation of physiological processes suggests that their functional significance remains to be discovered. Therefore, specific regulatory mechanisms of ncRNAs associated with phase separation should be assessed to enhance the understanding of ncRNA regulation and function.

The association between ncRNAs and phase separation offers novel insights into the biology of enigmatic ncRNAs. Although the properties that allow ncRNAs to act as a scaffold structure remain uncertain, the underlying mechanisms can be explained with further investigation. Several questions remain to be addressed: What are the physicochemical mechanisms underlying the assembly of MLOs? How can precise IDRs and drug target regions be predicted? Can specific kinetics of lncRNA-driven phase separation be used as markers of disease? Can specific MLOs be targeted by targeting the corresponding RNAs? How can phase separation be accurately controlled? More research into phase separation in diseases is required to derive medical benefits from therapeutic strategies, early and accurate diagnosis and disease prevention.

Funding: This work was supported by Six talent peaks project in Jiangsu Province, Jiangsu Provincial Medical Innovation Team (CXTDA2017025).

Conflict of Interest: The authors have no conflicts of interest to disclose.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Zhang H, Ji X, Li P, Liu C, Lou J, Wang Z, Wen W, Xiao Y, Zhang M,Zhu X. Liquid-liquid phase separation in biology: mechanisms, physiological functions and human diseases. Sci China Life Sci. 2020; 63:953-985.
2. Dolgin E. What lava lamps and vinaigrette can teach us about cell biology. Nature. 2018; 555:300-302.
3. Li P, Banjade S, Cheng H C, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth J V, King D S, Banani S F, Russo P S, Jiang Q X, Nixon B T,Rosen M K. Phase transitions in the assembly of multivalent signalling proteins. Nature. 2012; 483:336-40.
4. Nott T J, Petsalaki E, Farber P, Jervis D, Fussner E, Plochowietz A, Craggs T D, Bazett-Jones D P, Pawson T, Forman-Kay J D,Baldwin A J. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. Mol Cell. 2015; 57:936-947.
5. Onoguchi-Mizutani R,Akimitsu N. Long noncoding RNA and phase separation in cellular stress response. J Biochem. 2022; 171:269-276.
6. Xie D, Chen M, Niu J, Wang L, Li Y, Fang X, Li P,Qi Y. Phase separation of SERRATE drives dicing body assembly and promotes miRNA processing in Arabidopsis. Nat Cell Biol. 2021; 23:32-39.
7. Alberti S, Gladfelter A,Mittag T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. Cell. 2019; 176:419-434.
8. Elguindy M M,Mendell J T. NORAD-induced Pumilio phase separation is required for genome stability. Nature. 2021; 595:303-308.
9. Feng Z, Chen X, Zeng M,Zhang M. Phase separation as a mechanism for assembling dynamic postsynaptic density signalling complexes. Curr Opin Neurobiol. 2019; 57:1-8.
10. Nakagawa S, Yamazaki T,Hirose T. Molecular dissection of nuclear paraspeckles: towards understanding the emerging world of the RNP milieu. Open Biol. 2018; 8:180150
11. El Fatimy R, Boulaassafre S, Bouchmaa N, El Khayari A, Vergely C, Malka G,Rochette L. The emerging role of miRNA-132/212 cluster in neurologic and cardiovascular diseases: Neuroprotective role in cells with prolonged longevity. Mech Ageing Dev. 2021; 199:111566.
12. Ling Z, Wang X, Tao T, Zhang L, Guan H, You Z, Lu K, Zhang G, Chen S, Wu J, Qian J, Liu H, Xu B,Chen M. Involvement of aberrantly activated HOTAIR/EZH2/miR-193a feedback loop in progression of prostate cancer. J Exp Clin Cancer Res. 2017; 36:159.
13. Lin X, Zhuang S, Chen X, Du J, Zhong L, Ding J, Wang L, Yi J, Hu G, Tang G, Luo X, Liu W,Ye F. lncRNA ITGB8-AS1 functions as a ceRNA to promote colorectal cancer growth and migration through integrin-mediated focal adhesion signaling. Mol Ther. 2022; 30:688-702.
14. Pei X, Wang X,Li H. LncRNA SNHG1 regulates the differentiation of Treg cells and affects the immune escape of breast cancer via regulating miR-448/IDO. Int J Biol Macromol. 2018; 118:24-30.
15. Elbaum-Garfinkle S, Kim Y, Szczepaniak K, Chen C C, Eckmann C R, Myong S,Brangwynne C P. The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. Proc Natl Acad Sci U S A. 2015; 112:7189-94.
16. Tsang B, Pritisanac I, Scherer S W, Moses A M,Forman-Kay J D. Phase Separation as a Missing Mechanism for Interpretation of Disease Mutations. Cell. 2020; 183:1742-1756.
17. Zeng M, Shang Y, Araki Y, Guo T, Huganir R L,Zhang M. Phase Transition in Postsynaptic Densities Underlies Formation of Synaptic Complexes and Synaptic Plasticity. Cell. 2016; 166:1163-1175 e12.
18. Bergeron-Sandoval L P, Safaee N,Michnick S W. Mechanisms and Consequences of Macromolecular Phase Separation. Cell. 2016; 165: 1067-1079.
19. McSwiggen D T, Mir M, Darzacq X,Tjian R. Evaluating phase separation in live cells: diagnosis, caveats, and functional consequences. Genes Dev. 2019; 33:1619-1634.
20. Ray S, Singh N, Kumar R, *et al*. alpha-Synuclein aggregation nucleates through liquid-liquid phase

separation. Nat Chem. 2020; 12:705-716.
21. Gussmann F, Hansen-Goos H, Dietrich S,Roth R. Liquid-liquid phase separation in an inhomogeneous ternary colloid-polymer mixture. J Chem Phys. 2021; 154:224504.
22. Yu M, Peng Z, Qin M, *et al*. Interferon-gamma induces tumor resistance to anti-PD-1 immunotherapy by promoting YAP phase separation. Mol Cell. 2021; 81: 1216-1230e9.
23. Wang S, Dai T, Qin Z, Pan T, Chu F, Lou L, Zhang L, Yang B, Huang H, Lu H,Zhou F. Targeting liquid-liquid phase separation of SARS-CoV-2 nucleocapsid protein promotes innate antiviral immunity by elevating MAVS activity. Nat Cell Biol. 2021; 23:718-732.
24. Lu B, Zou C, Yang M, He Y, He J, Zhang C, Chen S, Yu J, Liu K Y, Cao Q,Zhao W. Pharmacological Inhibition of Core Regulatory Circuitry Liquid-liquid Phase Separation Suppresses Metastasis and Chemoresistance in Osteosarcoma. Adv Sci (Weinh). 2021; 8:e2101895.
25. Sun Y, Lau S Y, Lim Z W, Chang S C, Ghadessy F, Partridge A,Miserez A. Phase-separating peptides for direct cytosolic delivery and redox-activated release of macromolecular therapeutics. Nat Chem. 2022; 14:274-283.
26. Hofweber M, Hutten S, Bourgeois B, Spreitzer E, Niedner-Boblenz A, Schifferer M, Ruepp M D, Simons M, Niessing D, Madl T,Dormann D. Phase Separation of FUS Is Suppressed by Its Nuclear Import Receptor and Arginine Methylation. Cell. 2018; 173:706-719e13.
27. Ambadipudi S, Biernat J, Riedel D, Mandelkow E,Zweckstetter M. Liquid-liquid phase separation of the microtubule-binding repeats of the Alzheimer-related protein Tau. Nat Commun. 2017; 8:275.
28. Boulay G, Sandoval G J, Riggi N, Iyer S, Buisson R, Naigles B, Awad M E, Rengarajan S, Volorio A, McBride M J, Broye L C, Zou L, Stamenkovic I, Kadoch C,Rivera M N. Cancer-Specific Retargeting of BAF Complexes by a Prion-like Domain. Cell. 2017; 171:163-178e19.
29. Hnisz D, Abraham B J, Lee T I, Lau A, Saint-Andre V, Sigova A A, Hoke H A,Young R A. Super-enhancers in the control of cell identity and disease. Cell. 2013; 155:934-47.
30. Schepers A,Clevers H. Wnt signaling, stem cells, and cancer of the gastrointestinal tract. Cold Spring Harb Perspect Biol. 2012; 4:a007989.
31. Cai D, Liu Z,Lippincott-Schwartz J. Biomolecular Condensates and Their Links to Cancer Progression. Trends Biochem Sci. 2021; 46:535-549.
32. Alberti S,Hyman A A. Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. Nat Rev Mol Cell Biol. 2021; 22:196-213.
33. Peng W X, Koirala P,Mo Y Y. LncRNA-mediated regulation of cell signaling in cancer. Oncogene. 2017; 36:5661-5667.
34. Soltys K,Ozyhar A. Transcription Regulators and Membraneless Organelles Challenges to Investigate Them. Int J Mol Sci. 2021; 22:12758.
35. Caudron-Herger M, Pankert T, Seiler J, Nemeth A, Voit R, Grummt I,Rippe K. Alu element-containing RNAs maintain nucleolar structure and function. EMBO J. 2015; 34:2758-74.
36. Imamura K, Imamachi N, Akizuki G, *et al*. Long noncoding RNA NEAT1-dependent SFPQ relocation from promoter region to paraspeckle mediates IL8 expression upon immune stimuli. Mol Cell. 2014; 53:393-406.
37. McCluggage F,Fox A H. Paraspeckle nuclear condensates: Global sensors of cell stress? Bioessays. 2021; 43:e2000245.
38. Hirose T, Yamazaki T,Nakagawa S. Molecular anatomy of the architectural NEAT1 noncoding RNA: The domains, interactors, and biogenesis pathway required to build phase-separated nuclear paraspeckles. Wiley Interdiscip Rev RNA. 2019; 10:e1545.
39. Fox A H, Nakagawa S, Hirose T,Bond C S. Paraspeckles: Where Long Noncoding RNA Meets Phase Separation. Trends Biochem Sci. 2018; 43:124-135.
40. Fei J, Jadaliha M, Harmon T S, Li I T S, Hua B, Hao Q, Holehouse A S, Reyer M, Sun Q, Freier S M, Pappu R V, Prasanth K V,Ha T. Quantitative analysis of multilayer organization of proteins and RNA in nuclear speckles at super resolution. J Cell Sci. 2017; 130:4180-4192.
41. Goyal B, Yadav S R M, Awasthee N, Gupta S, Kunnumakkara A B,Gupta S C. Diagnostic, prognostic, and therapeutic significance of long non-coding RNA MALAT1 in cancer. Biochim Biophys Acta Rev Cancer. 2021; 1875:188502.
42. Sawyer I A, Sturgill D,Dundr M. Membraneless nuclear organelles and the search for phases within phases. Wiley Interdiscip Rev RNA. 2019; 10:e1514.
43. Wang X, Liu C, Zhang S, Yan H, Zhang L, Jiang A, Liu Y, Feng Y, Li D, Guo Y, Hu X, Lin Y, Bu P,Li D. N(6)-methyladenosine modification of MALAT1 promotes metastasis via reshaping nuclear speckles. Dev Cell. 2021; 56:702-715e8.
44. Wang M, Tao X, Jacob M D, Bennett C A, Ho J J D, Gonzalgo M L, Audas T E,Lee S. Stress-Induced Low Complexity RNA Activates Physiological Amyloidogenesis. Cell Rep. 2018; 24:1713-1721e4.
45. Valgardsdottir R, Chiodi I, Giordano M, Cobianchi F, Riva S,Biamonti G. Structural and functional characterization of noncoding repetitive RNAs transcribed in stressed human cells. Mol Biol Cell. 2005; 16:2597-604.
46. Ninomiya K, Adachi S, Natsume T, Iwakiri J, Terai G, Asai K,Hirose T. LncRNA-dependent nuclear stress bodies promote intron retention through SR protein phosphorylation. EMBO J. 2020; 39:e102729.
47. Prasanth K V, Rajendra T K, Lal A K,Lakhotia S C. Omega speckles - a novel class of nuclear speckles containing hnRNPs associated with noncoding hsr-omega RNA in Drosophila. J Cell Sci. 2000; 113 Pt 19:3485-97.
48. Dumbovic G, Biayna J, Banus J, Samuelsson J, Roth A, Diederichs S, Alonso S, Buschbeck M, Perucho M,Forcales S V. A novel long non-coding RNA from NBL2 pericentromeric macrosatellite forms a perinucleolar aggregate structure in colon cancer. Nucleic Acids Res. 2018; 46:5504-5524.
49. Pitchiaya S, Mourao M D A, Jalihal A P, Xiao L, Jiang X, Chinnaiyan A M, Schnell S,Walter N G. Dynamic Recruitment of Single RNAs to Processing Bodies Depends on RNA Functionality. Mol Cell. 2019; 74:521-533 e6.
50. Thakur J,Henikoff S. Architectural RNA in chromatin organization. Biochem Soc Trans. 2020; 48:1967-1978.
51. Guh C Y, Hsieh Y H,Chu H P. Functions and properties of nuclear lncRNAs-from systematically mapping the interactomes of lncRNAs. J Biomed Sci. 2020; 27:44.
52. Pessina F, Giavazzi F, Yin Y, Gioia U, Vitelli V, Galbiati A, Barozzi S, Garre M, Oldani A, Flaus A, Cerbino R, Parazzoli D, Rothenberg E,d'Adda di Fagagna F. Functional transcription promoters at DNA double-strand breaks mediate RNA-driven phase separation of damage-

response factors. Nat Cell Biol. 2019; 21:1286-1299.
53. Fijen C,Rothenberg E. The evolving complexity of DNA damage foci: RNA, condensates and chromatin in DNA double-strand break repair. DNA Repair (Amst). 2021; 105:103170.
54. Thapar R, Wang J L, Hammel M, *et al*. Mechanism of efficient double-strand break repair by a long non-coding RNA. Nucleic Acids Res. 2020; 48:10953-10972.
55. Novo C L. A Tale of Two States: Pluripotency Regulation of Telomeres. Front Cell Dev Biol. 2021; 9:703466.
56. Yamashita A, Watanabe Y, Nukina N,Yamamoto M. RNA-assisted nuclear transport of the meiotic regulator Mei2p in fission yeast. Cell. 1998; 95:115-23.
57. Ding D Q, Okamasa K, Katou Y, Oya E, Nakayama J I, Chikashige Y, Shirahige K, Haraguchi T,Hiraoka Y. Chromosome-associated RNA-protein complexes promote pairing of homologous chromosomes during meiosis in *Schizosaccharomyces pombe*. Nat Commun. 2019; 10:5598.
58. He P, Zhang C, Ji Y, Ge M K, Yu Y, Zhang N, Yang S, Yu J X, Shen S M,Chen G Q. Epithelial cells-enriched lncRNA SNHG8 regulates chromatin condensation by binding to Histone H1s. Cell Death Differ. 2022; 29:1569-1581
59. Patil D P, Chen C K, Pickering B F, Chow A, Jackson C, Guttman M,Jaffrey S R. m(6)A RNA methylation promotes XIST-mediated transcriptional repression. Nature. 2016; 537:369-373.
60. Cerase A, Armaos A, Neumayer C, Avner P, Guttman M,Tartaglia G G. Phase separation drives X-chromosome inactivation: a hypothesis. Nat Struct Mol Biol. 2019; 26:331-334.
61. Onoguchi-Mizutani R, Kirikae Y, Ogura Y, Gutschner T, Diederichs S,Akimitsu N. Identification of a heat-inducible novel nuclear body containing the long noncoding RNA MALAT1. J Cell Sci. 2021; 134:jcs253559.
62. Wang C, Duan Y, Duan G, Wang Q, Zhang K, Deng X, Qian B, Gu J, Ma Z, Zhang S, Guo L, Liu C,Fang Y. Stress Induces Dynamic, Cytotoxicity-Antagonizing TDP-43 Nuclear Bodies via Paraspeckle LncRNA NEAT1-Mediated Liquid-Liquid Phase Separation. Mol Cell. 2020; 79:443-458e7.
63. Wang R, Cao L, Thorne R F, Zhang X D, Li J, Shao F, Zhang L,Wu M. LncRNA GIRGL drives CAPRIN1-mediated phase separation to suppress glutaminase-1 translation under glutamine deprivation. Sci Adv. 2021; 7:eabe5708
64. Li R H, Tian T, Ge Q W, *et al*. A phosphatidic acid-binding lncRNA SNHG9 facilitates LATS1 liquid-liquid phase separation to promote oncogenic YAP signaling. Cell Res. 2021; 31:1088-1105.
65. Yap K, Mukhina S, Zhang G, Tan J S C, Ong H S,Makeyev E V. A Short Tandem Repeat-Enriched RNA Assembles a Nuclear Compartment to Control Alternative Splicing and Promotes Cell Survival. Mol Cell. 2018; 72:525-540e13.
66. Wu M, Xu G, Han C, Luan P F, Xing Y H, Nan F, Yang L Z, Huang Y, Yang Z H, Shan L, Yang L, Liu J,Chen L L. lncRNA SLERT controls phase separation of FC/DFCs to facilitate Pol I transcription. Science. 2021; 373:547-555.
67. Daneshvar K, Pondick J V, Kim B M, Zhou C, York S R, Macklin J A, Abualteen A, Tan B, Sigova A A, Marcho C, Tremblay K D, Mager J, Choi M Y,Mullen A C. DIGIT Is a Conserved Long Noncoding RNA that Regulates GSC Expression to Control Definitive Endoderm Differentiation of Embryonic Stem Cells. Cell Rep. 2016; 17:353-365.
68. Daneshvar K, Ardehali M B, Klein I A, *et al*. lncRNA DIGIT and BRD3 protein form phase-separated condensates to regulate endoderm differentiation. Nat Cell Biol. 2020; 22:1211-1222.
69. Correia de Sousa M, Gjorgjieva M, Dolicka D, Sobolewski C,Foti M. Deciphering miRNAs' Action through miRNA Editing. Int J Mol Sci. 2019; 20:6249.
70. Kucherenko M M,Shcherbata H R. miRNA targeting and alternative splicing in the stress response - events hosted by membrane-less compartments. J Cell Sci. 2018; 131:jcs202002.
71. Lee S C,Martienssen R A. Phase separation in plant miRNA processing. Nat Cell Biol. 2021; 23:5-6.
72. .Kucherenko M M,Shcherbata H R. Stress-dependent miR-980 regulation of Rbfox1/A2bp1 promotes ribonucleoprotein granule formation and cell survival. Nat Commun. 2018; 9:312.
73. Gao S, Wang J, Jiang N, *et al*. Hyponastic Leaves 1 protects pri-miRNAs from nuclear exosome attack. Proc Natl Acad Sci U S A. 2020; 117:17429-17437.
74. Wu X, Shi Y, Li J, Xu L, Fang Y, Li X,Qi Y. A role for the RNA-binding protein MOS2 in microRNA maturation in Arabidopsis. Cell Res. 2013; 23:645-57.
75. Pessina F, Gioia U, Brandi O, Farina S, Ceccon M, Francia S,d'Adda di Fagagna F. DNA Damage Triggers a New Phase in Neurodegeneration. Trends Genet. 2021; 37:337-354.
76. Ling S C. Synaptic Paths to Neurodegeneration: The Emerging Role of TDP-43 and FUS in Synaptic Functions. Neural Plast. 2018; 2018:8413496.
77. Si W, Ye S, Ren Z, Liu X, Wu Z, Li Y, Zhou J, Zhang S, Li Y, Deng R,Chen D. miR335 promotes stress granule formation to inhibit apoptosis by targeting ROCK2 in acute ischemic stroke. Int J Mol Med. 2019; 43:1452-1466.
78. Jie M, Feng T, Huang W, Zhang M, Feng Y, Jiang H,Wen Z. Subcellular Localization of miRNAs and Implications in Cellular Homeostasis. Genes (Basel). 2021; 12:856.
79. Liu X M, Ma L, Schekman R. Selective sorting of microRNAs into exosomes by phase-separated YBX1 condensates. Elife. 2021; 10:e71982
80. Niaz S,Hussain M U. Role of GW182 protein in the cell. Int J Biochem Cell Biol. 2018; 101:29-38.
81. Sahoo M R, Gaikwad S, Khuperkar D, *et al*. Nup358 binds to AGO proteins through its SUMO-interacting motifs and promotes the association of target mRNA with miRISC. EMBO Rep. 2017; 18:241-263.
82. Deshmukh P, Singh A, Khuperkar D,Joseph J. Acute necrotizing encephalopathy-linked mutations in Nup358 impair interaction of Nup358 with TNRC6/GW182 and miRNA function. Biochem Biophys Res Commun. 2021; 559:230-237.
83. Gao Y, Zhu Y, Wang H, Cheng Y, Zhao D, Sun Q,Chen D. Lipid-mediated phase separation of AGO proteins on the ER controls nascent-peptide ubiquitination. Mol Cell. 2022; 82:1313-1328e8.
84. Tsang B, Arsenault J, Vernon R M, Lin H, Sonenberg N, Wang L Y, Bah A,Forman-Kay J D. Phosphoregulated FMRP phase separation models activity-dependent translation through bidirectional control of mRNA granule formation. Proc Natl Acad Sci U S A. 2019; 116:4218-4227.
85. Trinkle-Mulcahy L,Sleeman J E. The Cajal body and the nucleolus: "In a relationship" or "It's complicated"? RNA Biol. 2017; 14:739-751.

86. Meier U T. RNA modification in Cajal bodies. RNA Biol. 2017; 14:693-700.
87. Ogg S C,Lamond A I. Cajal bodies and coilin--moving towards function. J Cell Biol. 2002; 159:17-21.
88. Logan M K, McLaurin D M,Hebert M D. Synergistic interactions between Cajal bodies and the miRNA processing machinery. Mol Biol Cell. 2020; 31:1561-1569.
89. Seto A G, Kingston R E,Lau N C. The coming of age for Piwi proteins. Mol Cell. 2007; 26:603-609.
90. Shigematsu M, Kawamura T, Morichika K, Izumi N, Kiuchi T, Honda S, Pliatsika V, Matsubara R, Rigoutsos I, Katsuma S, Tomari Y,Kirino Y. RNase kappa promotes robust piRNA production by generating 2',3'-cyclic phosphate-containing precursors. Nat Commun. 2021; 12:4498.
91. Hirakata S, Ishizu H, Fujita A, Tomoe Y,Siomi M C. Requirements for multivalent Yb body assembly in transposon silencing in Drosophila. EMBO Rep. 2019; 20:e47708.
92. Sheu-Gruttadauria J,MacRae I J. Phase Transitions in the Assembly and Function of Human miRISC. Cell. 2018; 173:946-957e16.
93. Suen K M, Braukmann F, Butler R, Bensaddek D, Akay A, Lin C C, Milonaityte D, Doshi N, Sapetschnig A, Lamond A, Ladbury J E,Miska E A. DEPS-1 is required for piRNA-dependent silencing and PIWI condensate organisation in Caenorhabditis elegans. Nat Commun. 2020; 11:4242.
94. Salzman J. Circular RNA Expression: Its Potential Regulation and Function. Trends Genet. 2016; 32:309-316.
95. Zang J, Lu D,Xu A. The interaction of circRNAs and RNA binding proteins: An important part of circRNA maintenance and function. J Neurosci Res. 2020; 98:87-97.
96. Chen S, Cao X, Zhang J, Wu W, Zhang B, Zhao F. circVAMP3 Drives CAPRIN1 Phase Separation and Inhibits Hepatocellular Carcinoma by Suppressing c-Myc Translation. Adv Sci (Weinh). 2022; 9:e2103817.
97. Ye R, Cao C, Xue Y. Enhancer RNA: biogenesis, function, and regulation. Essays Biochem. 2020; 64:883-894.
98. Debek S, Juszczynski P. Super enhancers as master gene regulators in the pathogenesis of hematologic malignancies. Biochim Biophys Acta Rev Cancer. 2022; 1877:188697.
99. Isoda T, Moore A J, He Z, Chandra V, Aida M, Denholtz M, Piet van Hamburg J, Fisch K M, Chang A N, Fahl S P, Wiest D L,Murre C. Non-coding Transcription Instructs Chromatin Folding and Compartmentalization to Dictate Enhancer-Promoter Communication and T Cell Fate. Cell. 2017; 171:103-119e18.
100. Fujino Y, Nagai Y. The molecular pathogenesis of repeat expansion diseases. Biochem Soc Trans. 2022; 50:119-134.

Received July 9, 2022; Revised September 7, 2022; Accepted September 29, 2022.

[§]These authors contributed equally to this work.
**Address correspondence to:*
Bin Xu and Ming Chen, Department of Urology, Affiliated Zhongda Hospital of Southeast University, Nanjing, China.
E-mail: njxbseu@seu.edu.cn; mingchenseu@126.com

Released online in J-STAGE as advance publication October 21, 2022.

Original Article

DOI: 10.5582/bst.2022.01358

Epigenetic modification of *Kiss1* gene expression in the AVPV is essential for female reproductive aging

Ruoxi Dai[1,§], Wen Xu[1,§], Wei Chen[2], Liyuan Cui[1], Lisha Li[1], Jing Zhou[1], Xueling Jin[1], Yan Wang[1], Ling Wang[1,3,4,*], Yan Sun[1,3,4,*]

[1] Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, China;
[2] Department of Urology, Zhongshan Hospital of Fudan University, Shanghai, China;
[3] The Academy of Integrative Medicine of Fudan University, Shanghai, China;
[4] Shanghai Key Laboratory of Female Reproductive Endocrine-related Disease, Shanghai, China.

SUMMARY Female reproductive senescence is heralded by hypothalamus region-specific changes in the transcription of genes such as *Kiss1* under estradiol (E2) positive feedback, associated with luteinizing hormone (LH) surge dysfunction and reproductive decline. The current study explored whether the anteroventral periventricular nucleus (AVPV) displayed epigenetic changes mediated by age-related dysregulation of gene expression and whether an epigenetic-based intervention could alleviate an aging-related neuroendocrine disorder. Chromatin immunoprecipitation sequencing (ChIP-seq) and ChIP-qPCR were used to assess the differential acetylation of histone H3 in the AVPV and the expression of genes in hormone-primed middle-aged rats. The association between acetylated histone H3 and *Kiss1* expression and the underlying mechanisms of dysregulation were determined using pharmacological inhibitors and molecular experiments *in vitro* and *in vivo*. An AVPV gene expression program failed to initiate in middle-aged females displaying typical genome-wide hypoacetylation of histone H3, and this coincided with decreased LH. Hypoacetylation of histone H3 at the 3' intergenic region of *Kiss1* in particular was associated with enhanced chromatin looping between the promoter and enhancer. Restoration of physiological histone H3 acetylation by intracerebroventricular injection of trichostatin A (TSA) restored the expression of *Kiss1* by modifying chromatin looping and led to the restoration of *Kiss1* neuronal activation and *Kiss1* synthesis as well as circulating LH. These findings have revealed novel epigenetic-associated changes in gene expression in female reproductive aging. These results also suggest that HDAC enzyme-based treatment is a potential therapeutic approach for insufficient preovulatory LH release in aging females.

Keywords aging, AVPV, estradiol, histone, acetylation, *Kiss1*

1. Introduction

Altered gene expression induced by estradiol (E2)-positive feedback in hypothalamic anteroventral periventricular nucleus (AVPV) neurons results in diminished neurotransmitter output and is well established as a key contributor to neuroendocrine changes in reproductive aging. Female reproductive aging is characterized by reduced gonadotropin-releasing hormone (GnRH) neuronal activation and dysfunction of the luteinizing hormone (LH) surge (*1-4*). Several studies have yielded findings regarding understanding of the molecular mechanisms that contribute to age-related dysregulation of genes in the AVPV. However, an unbiased view of diverse species undergoing reproductive aging has revealed that such gene alterations under E2-positive feedback were not caused by disparities among ovarian steroid exposure (*5*), altered estrogen receptor expression related to age (*6,7*), or in combination with decreased sex steroid receptors in the hypothalamus (*8*).

Epigenetic regulation, and especially histone modification, has recently been highlighted as a key mechanism to control gene expression (*9-11*) and is closely related to neuronal activation (*12*) and/or dysfunction (*13-16*). A global view of histone acetylation patterns has revealed a correlation between the level of histone acetylation at specific loci and the transcriptional activity of the gene (*17-19*). Hyperacetylation of histone in the gene promoter/enhancer region leads to chromatin organization and transcriptional activation (*20,21*).

Hypoacetylation of histone H3 in the coding region severely inhibits transcription by active genes (*20*) and may represent a critical aspect of the aging process (*22*). Moreover, histone acetylation is hypothesized to regulate binding between ERα and the estrogen response element (ERE) within the regulatory region of E2-responsive genes, mediating their transcription (*23-26*). Consistent with this hypothesis, the current authors recently reported that under E2-positive feedback conditions, acetylated histone H3 (AcH3)-immunoreactive (IR) cells in the preoptic area (POA) and AVPV increased, activating GnRH neurons for a robust LH surge (*27,28*). Of particular interest, the current authors found that females in middle age experienced differential histone H3 acetylation (H3ac) characteristics in the two nuclei as well as significant changes in HDACs (*29*), coincident with the down-regulation of *Kiss1* (anterior hypothalamus-AVPV) and a decreased LH surge (*28*). Based on these findings, more detailed and extensive information on genome-wide changes in gene expression related to acetylated histone H3 binding in the AVPV of middle-aged individuals needs to be assembled.

The *Kiss1* gene-encoded neuropeptide kisspeptin in the AVPV potently drives GnRH neuronal activation and the subsequent LH surge (*30-33*). Female rats that exhibit reproductive aging display reduced total *Kiss1* mRNA (*34*) and less *Kiss1* mRNA per cell in the AVPV as well as decreased Kiss1 cellular activation under E2-positive feedback conditions (*2*). Under E2 positive feedback, ERα recruitment and histone acetylated in the *Kiss1* promoter region consequently promote chromatin looping between the promoter and enhancer (*35,36*), which is associated with specific upregulation of AVPV *Kiss1*. However, there are few studies on the association between epigenetic changes and *Kiss1* expression in reproductive senescence. Therefore, the current hypothesis is that reduced *Kiss1* in the AVPV of middle-aged individuals may be mainly attributed to suppressed H3ac-induced chromatin loop formation, limiting the potential for interaction between a remote enhancer and a *Kiss1* gene promoter.

The current study used chromatin immunoprecipitation sequencing (ChIP-seq) analysis to characterize the contribution of altered H3ac to impaired gene expression in the AVPV of middle-aged females. The *Kiss1* and *Period 1* (*Per1*) with reduced mRNA in the AVPV were found to be epigenetically regulated *via* H3ac. Chromatin looping between the promoter and intergenic region was analyzed to determine the interaction of a *Kiss1* enhancer-promoter using chromosome conformation capture (3C) (*37,38*). Presented here is *in vivo* and *in vitro* evidence that treatment with a pharmacological histone-acetylating agent increases *Kiss1* mRNA expression and avoids age-related neuroendocrine alterations. These data provide mechanistic insight into H3ac-dependent gene expression changes in the AVPV; *Kiss1* in particular may underlie female reproductive aging characteristics, thus representing a potential therapeutic target for an LH release disorder of hypothalamic origin.

2. Materials and Methods

2.1. Animal care

Young (2-3 months) and middle-aged (9-10 months, retired breeders) female rats (Sprague Dawley, Charles River, Beijing) that were given free access to food and water were divided into groups. A 12:12 L/D cycle (lights on at 7 A.M.) was maintained. Estrous cyclicity was monitored with a daily vaginal smear for at least 2 cycles. Only rats with 2 regular estrous cycles (4-5 days) were randomly assigned to groups (*2,39,40*). All experimental procedures performed on animals were approved by the Institutional Animal Care and Use Committee of Fudan University.

2.2. Surgery and tissue harvest

2.2.1. Ovariectomy and hormone administration

After animals were anesthetized with pentobarbital sodium (30 mg/kg, ip), an ovariectomy (OVX) was performed. At 9 A.M. on day 7 after OVX, the females received a daily subcutaneous injection of 2 μg estradiol benzoate (E2; Hangxiang Inc., China) dissolved in 0.1 mL of peanut oil for two days. Forty-eight hours after the first E2 injection, the rats were treated with 500 μg of progesterone (P; Steraloids, Inc.). This hormone treatment protocol reliably stimulates neuron activation and an LH surge (*2,28,39,40*).

2.2.2. Stereotaxic surgery and cannula placement

After OVX, rats were immobilized in a RWD stereotaxic apparatus for cannula placement. A 22-gauge intracerebroventricular (icv) guide cannula (RWD Life Science, China) was positioned into the third ventricle stereotaxically (anterior/posterior + 0.2 mm; medial/lateral + 0.0 mm; dorsal/ventral - 9.8 mm relative to the bregma). A 26-gauge dummy was plugged and extended 1 mm below the guide and anchored with dental acrylic (*2,39,40*). The position of the guide cannula was confirmed by dye injection and brain sectioning to track the cannula path at the end of all experiments. Females with an accurately placed cannula were used in data analysis. A seven-day recovery was provided before animals were primed with exogenous hormone.

2.2.3. Hypothalamic dissection

The brain was quickly removed and set in a chilled stainless steel brain matrix (RWD Surgical Instrument Co.) to cut a coronal section containing the entire anterior hypothalamus, and the thickness was approximately 2

mm. The AVPV block was micro-dissected out with a microblade (Flying Eagle Razor, Gillette) and pooled in light of experimental requirements. For example, tissue from 10 young females was pooled into a single sample prior to ChIP-seq or qPCR. All tissues were frozen instantly in liquid nitrogen and transferred to a -80°C freezer.

2.3. ChIP and qPCR

2.3.1. ChIP

The ChIP-IT High Sensitivity Kit (Active motif) was used for ChIP assays. Generally, the dissected AVPV tissues were fixed with 1% formaldehyde at room temperature for 10 min, transferred into 500 μL of lysis buffer, and sonicated for 10 min. Chromatin was sheared using an Ultrasonic Processor (Sonics VCX130) and cleared by centrifugation. The solubilized chromatin was pulled down using anti-acetylated H3 (Millipore, 06-599). The beads were rinsed and the immunoprecipitated (IP) complex was eluted after incubation overnight. Libraries were constructed with IP DNA and an un-immunoprecipitated control (Input) that had been refined.

2.3.2. ChIP-seq library construction and sequencing

A NEB Ultra DNA library kit was used for library preparation, for which 50 ng of each sample was needed. A library containing the input DNA (no IP) was constructed. The quantity, size distribution, and purity of the libraries were qualified using a Qubit fluorometer and an Agilent 2100 Bioanalyzer. Sequencing was performed by an Illumina HiSeq X Ten platform (Novogene, Beijing, China). Approximately 30 million reads per sample were obtained.

2.3.3. ChIP-seq data analysis and gene ontology

Quality control was performed using the Saicheng Biology (Guangzhou, China) Sequencing Core with the ENCODE chip pipeline (version 1.8.4). The rattus norvegicus genome version (Rnor 6.0/rn6) was used for mapping with BWA (version 0.7.10). Peaks of enriched H3ac compared to the background input were identified using MACS2 (*41*). BedTools (version 2.27.0) was used to manipulate basic genomic regions, encompassing intersections, and windows (*42*). The enriched peaks were annotated using ChIPpeakAnno (*43*) and ChIPseeker (*44*) based on the rat genome. The promoter regions were determined to be – 3 kb to + 3 kb from the position of the transcription start site (TSS). The differential H3ac modified regions were determined as described previously (*45,46*). In brief, after H3ac regions were confirmed by BedTools (version 2.27.0), the number of reads in those regions was normalized with peaks within 3 kb. The signal was calculated using the IP sample reads per kilobase per million mapped reads (RPKM) minus the input RPKM. As a result, peaks indicating an increase of over 2-fold or a decrease of more than 1/2 between the rats of different ages were defined as differential H3ac regions. The regions enriched with H3ac were identified using the Integrated Genome Viewer (IGV) in alignment with the mapped reads.

2.3.4. qPCR detection of chromatin immunoprecipitated DNA

ChIP-seq data were validated with ChIP-qPCR. The input DNA sample was used for normalization. Primers were selected within the H3Ac-enriched regions detected using ChIP-seq (Table S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=122*). Primers from non-H3Ac region were used as the negative control. Real-time qPCR was performed in triplicate using SYBR Premix Ex Taq (Takara Biomedical Technology, Japan). The relative amount of each amplified fragment was evaluated according to the amplification acquired from input DNA.

2.3.5. RNA extraction and RT-qPCR

The Neasy lipid minikit (Qiagen) was used to purify DNA-free total RNA. The high-capacity cDNA RT kit with ribonuclease inhibitor (Applied Biosystems) was used to perform RT-qPCR. Gene expression was detected with quantitative PCR using the Lightcycle Roche 480 SYBR green master mix (Roche) and the Applied Biosystem 7700 Real-time PCR cycler. With GAPDH as a normalizer, the quantity of the amplified transcripts was calculated using the comparative threshold cycle method. The mean CT values and the ΔΔCT were calculated for duplicate samples. The primer sequences are shown in Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=122*).

2.4. Cell culture

The GT1-7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 10% (vol/vol) FBS (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37°C in 5% (vol/vol) CO_2. Cells were cultured in media replenished with 10% dextran and charcoal-stripped serum 24 h before treatment. Cells were exposed to E2 (10 mol) or not exposed for 48 h and then exposed to trichostatin A (TSA) (Sigma-Aldrich) at 200 nM for 24 h. Control wells were processed in parallel with the vehicle.

2.5. Drug administration

For LH measurements, TSA (Sigma-Aldrich) was dissolved in 5% dimethyl sulfoxide (DMSO) in saline

to a concentration of 50 ng/mL, 200 ng/mL, or 500 ng/mL. On the day of infusion, middle-aged rats received an icv infusion into the third ventricle *via* microliter syringe (RWD Life Science, China) attached with a plastic connector. The effect of TSA (500 ng/mL) on H3ac was observed in AVPV cells. This concentration was chosen based on its efficacy on LH release. E2 was purchased from Hangxiang Inc. (Wuhan, China) and prepared as previously described (*5*).

2.6. Combined fluorescence in situ hybridization (FISH) and immunohistochemistry

OVX animals were treated with E2 and P and sacrificed during the LH surge as previously described (*39,40*). Blood was collected, and animals were decapitated. The brain was instantly removed, cryopreserved on dry ice, and stored at -80°C until cryogenic sectioning. Brains were coronally cut into five sets of 20-μm sections, thaw-mounted onto Superfrost-plus slides, and stored at -80°C. One set was used for a combined FISH and immunohistochemistry assay.

After fixation in 4% paraformaldehyde, elution in 0.1 M phosphate buffer (pH 7.0), and immersion in proteinase K (20 μg/mL) for 5 min, slide-mounted sections were then placed in prehybridization buffer for 1 h and then with Fam-labeled sense and antisense RNA probes specific to the *Kiss1* sequence (sequences of probes are listed in Table S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=122*). Subsequently, the hybridized sections were rinsed with 2 × SSC, 1 × SSC and 0.5 × SSC, blocked with 1% bovine serum albumin in PBS with 0.25% TritonX-100 for 30 min, incubated overnight with rabbit anti-acetylated histone H3 (AcH3, 1:1200, Millipore) and rabbit anti-c-Fos (1:100, Servicebio) antibodies in blocking solution, and washed in PBS. After incubation with FITC anti-rabbit secondary antibody and counter staining with DAPI, the sections were mounted with anti-fluorescence quenching sealing tablets.

2.7. 3C assay

A 3C assay was performed as in previous studies (37) with some modifications. After 600 U of Kpnl (Roche) was digested, crosslinked chromatin was incubated overnight at 37°C. It was then ligated in 6 mL of 1 × ligation buffer. The *Kiss1* locus encompasses four Kpnl sites; the primers flanking the Kpnl sites were designated K1F: CATGCCAGGTTATACCC CAAC, K1R: CAGAAGTCTGGATCACCAAC, K2F: CTGTG TCATGAGGACGTG, K2R: GGTTCCCGTGATGAAG TAG, K3F: GTGAGAAGAAGACA CTCGTG, K3R: C TGGTGTTATAGCACGTTG, and K-anchor-F: CTGGTGTTATAGCACG TTG. The chromatin loop of the *Kiss1* gene was detected using the primer K-anchor F along with one of the other primers. A loading control region was amplified with CAGGACTGAGGGACGGAAG and GCATCCCTGCC CTGCAAAC. The PCR workflow was as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min; and final extension at 72°C for 10 min. The PCR products were detected using agarose gel electrophoresis.

2.8. LH assay

Samples of trunk blood were obtained between 3 P.M. and 5:30 P.M. on the expected day of an LH surge. Serum LH was measured using enzyme-linked immunosorbent assay (ELISA) while LH reagents were provided by the Beijing Sino-UK Institute of Biological Technology (Chaoyang, Beijing).

2.9. Statistical processing

Statistical analyses were performed using the software GraphPad Prism 9.0. Data are expressed as the mean ± SEM. The *t-test* was used to detect differences in the $H3ac^+$ and c-Fos^+ Kiss1 cells in the AVPV and gene expression between groups. Analysis of variance (ANOVA) was used to evaluate the effects of TSA on the *Kiss1* mRNA expression in GT1-7 cells and LH release. Bonferroni or Tukey's post-hoc test was used to identify individual group differences following ANOVA.

3. Results

3.1. Genome-wide ChIP-seq analyses revealed age-associated epigenomic changes in the rat AVPV

Cells containing acetylated histone H3 decreased in the AVPV of middle-aged females as previously reported (*28*), but the total histone H3 did not change (Figure S1 and S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=122*). ChIP-seq was performed to generate a genome-wide landscape of H3ac in the AVPV for OVX middle-aged and young rats that were exposed to E2-positive feedback (Figure 1A). Peak calling analysis was performed using a model-based analysis for ChIP-seq with a false discovery rate (FDR) < 0.05. In total, 91,708 and 143,186 H3ac peaks were identified in middle-aged and young rats, respectively. As shown in Figure 1B, H3ac signals peaked within ± 3 kb from the TSS in both groups.

The feature sets spanning sub-types were defined by genomic locations (Figure 1C) according to the description in the ChIPseeker (*44*): promoter (within ± 3 kb region from the TSS), exon, intron, downstream (3 kb downstream of the transcription termination site, TTS), untranslated region (UTR), and distal intergenic region. Most H3ac modification regions were distributed in the proximal promoter region (≤ 3 kb) and distal intergenic regions. Redistribution to both of the TSSes (≤ 3 kb)

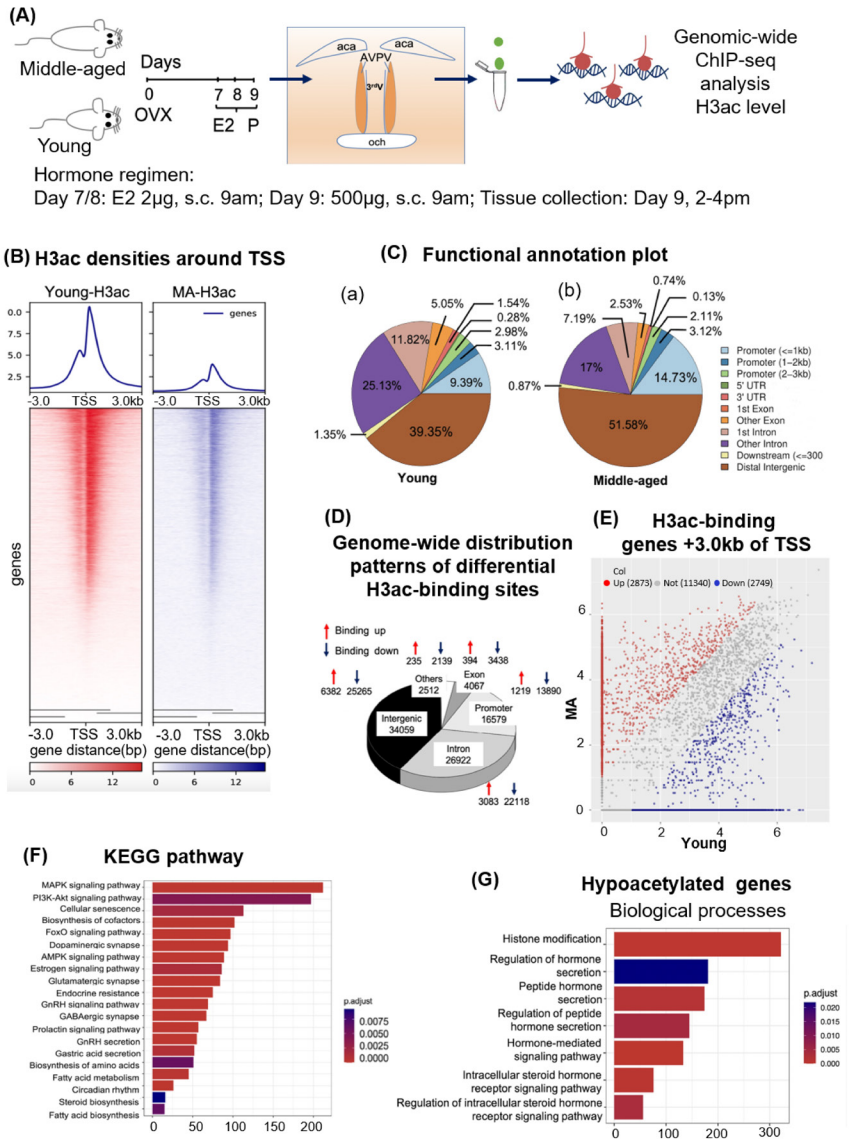

Figure 1. Preponderance of hypoacetylations in the AVPV of middle-aged females compared to young controls and the biological process of hypoacetylated genes under E2 positive feedback. (A) Schematic illustration of the experimental design. Chromatin immunoprecipitation was performed in the AVPV of E2-primed middle-aged and young rats, followed by next-generation sequencing (ChIP-seq). **(B)** Profiles of the active genes (H3ac enrichment) around the TSS. Heatmap showing the H3ac signal from 3 kb upstream to 3 kb downstream of H3ac peaks in middle-aged and young females after normalization. **(C)** Functional annotation plot showing the age-related differential proportion of H3ac regions with several genomic features. **(D)** Pie chart showing the genome-wide distribution patterns of differential H3ac-binding sites in the AVPV in the middle-aged and young groups. Arrows represent sites displaying a significant E2-induced increase (red) or decrease (blue) in H3ac binding. **(E)** The H3ac-binding gene expression pattern in the AVPV of middle-aged and young rats around + 3.0 kb of the TSS. The difference in counts between groups was evaluated using the scatter plot of log2 (RPKM). Red and blue dots represent 3.0 kb upstream of the TSS with different hypoacetylated or hyperacetylated signals in the AVPV of middle-aged animals compared to the controls, while grey dots correspond to unchanged H3ac modification in the AVPV. **(F and G)** Functional enrichment analysis performed with DAVID on the genes associated with hypoacetylated regions were compared in middle-aged and young rats. GO biological processes and KEGG pathways related to histone modification and hormone secretion are shown. The significance is indicated as the log10 *P* value.

and remote intergenic regions was noted in middle-aged females (Figure 1C). Peaks with an increase of over 2-fold or a decrease of more than 1/2 between middle-aged and young females were subsequently defined as differential H3ac regions. As shown in Figure 1D, the H3ac-binding signals markedly decreased in middle-aged rats, and most of the peaks found in the promoter, intergenic, and intron regions were 13,890, 25,265 and 22,118, respectively. The H3ac-binding up regions located in those regions were 1219, 6382 and 3083, respectively.

Most of the H3ac-binding down regions were enriched within ± 3 kb from the TSS (Figure 1B), so the differentially H3ac-associated genes between the two groups were assessed (Figure 1E), and a scatter plot of log2 (RPKM) was prepared. Two thousand eight hundred and seventy-three genes were identified as down-

regulated (16.9%) in the AVPV of middle-aged rats, 2,749 genes were up-regulated (16.2%), and the expression of 11,340 genes did not change (66.9%) (Figure 1E).

Gene ontology (GO) term enrichment analysis was subsequently performed and revealed distinct functional categories for the H3ac-associated gene lists. Within the category of biological processes of the hypoacetylated genes, histone modification and the regulation of hormone secretion were listed as related functions (Figure 1G). Regarding the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, the hypoacetylated genes were participants in the mitogen-activated protein kinase (MAPK), neurotransmitter, and hormone signaling pathways (Figure 1F). The top 20 significantly downregulated genes and pathways are shown in Figures S3 and S4 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=122*).

3.2. Histone H3 hypoacetylation in the loci of *Kiss1* and *Per1* genes in middle-aged rats

A genome-wide view shows that impaired H3ac in middle-aged females occurred quite homogenously across all chromosomes (Figure 2A). Moreover, hypoacetylated H3 was evaluated in the loci of the pivotal genes involved in the GnRH-LH surge such as *Kiss1* and circadian clock genes with functional histone modification including *Per1*, *Per2*, *Bmal1*, and *Clock* in the AVPV of middle-aged rats (for a representative Integrative Genome Viewer view, see Figure 2B). This finding was confirmed using ChIP followed by qPCR to show that hypoacetylated histone H3 is associated with a significant reduction in *Kiss1*, *Per1*, and *Per2* mRNA (Figure 2C) in middle-aged rats. Consistent with these findings, a 2-fold decrease in *Kiss1* and a 1.5-fold decrease in *Per1* was revealed by qPCR validation analysis versus young controls (Figure 2D, $P < 0.05$, respectively). ChIP-qPCR using primers from non-H3Ac regions revealed no significant differences (data not shown). Overall, these experiments identified several genes associated with the hormone-mediated LH surge and reproductive regulation and with the altered H3ac profile in the AVPV of middle-aged females undergoing a reproductive decline.

3.3. Suppressed H3ac in AVPV Kiss1 cells is associated with attenuated *Kiss1* transcriptional activation in middle-aged rats

FISH and immunofluorescence revealed that the cells expressing *Kiss1* mRNA were abundant in the AVPV (Figure 3A), but few of them were H3ac^{+} in young and middle-aged OVX rats (Figure 3A-a and g). In line with a previous study (*28*), the AVPV from young females

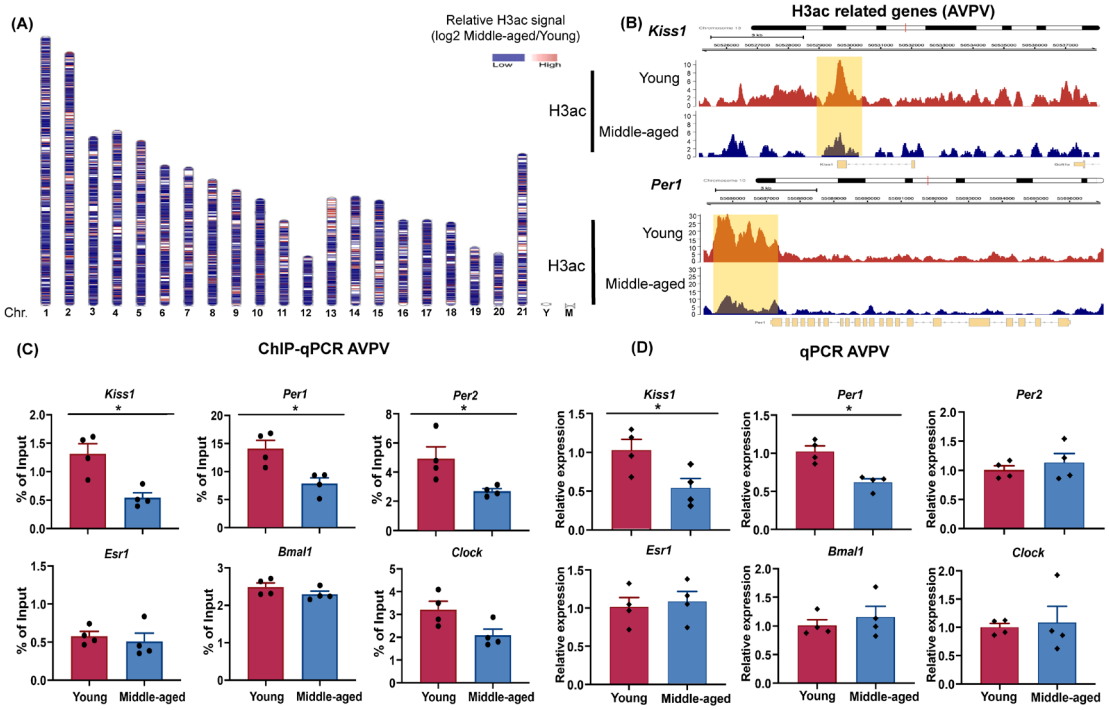

Figure 2. Altered chromosomal distribution of H3ac reads and acetylation signatures in the AVPV of middle-aged rats. (A) Chromosome ideogram showing the level of H3ac peaks across the chromosomes of middle-aged female rats compared to young female rats. The color key from blue to red indicates low to high relative levels of H3ac, respectively. **(B)** Representative tracks of *Kiss1* and *Per1* ChIP-seq data showing pronounced loss of the H3ac signal in the AVPV of middle-aged rats. **(C)** Chromatin was isolated from the AVPV removed from middle-aged ($n = 4$) and young ($n = 4$) females. ChIP-qPCR experiments performed in the two groups of animals. Unpaired Mann-Whitney *t-test*. **(D)** qRT-PCR analyses using primers against the genes listed were performed in AVPV tissues of young ($n = 4$) and middle-aged offspring ($n = 4$). Unpaired Mann-Whitney *t-test*. Data in **(C)** and **(D)** are expressed as the mean ± SEM. $^{*}P < 0.05$.

primed with E2 exhibited a marked increase in H3ac immunoreactivity and H3ac^{+} was strikingly identified within Kiss1 cells. Detailed quantification subsequently revealed that approximately 42% of *Kiss1*-expressing cells located in the AVPV were H3ac^{+} ($P < 0.001$, Figure 3A-f and l). However, H3ac^{+} induced in AVPV Kiss1 cells by E2 was disrupted to approximately 24% in middle-aged females (Figure 3A-l and o, $P < 0.001$). The percentage of H3ac^{+} Kiss1 cells decreased markedly by approximately 60% compared to that in young controls treated with E2.

Given the close association between histone acetylation and chromatin accessibility and the transcriptional activity of cells (*20,36,47*), whether the suppressed H3ac in AVPV Kiss1 cells observed in middle-aged females (Figure 3A-l and o) was associated with a parallel decline in *Kiss1* transcriptional activation was determined next. C-Fos immunoreactivity was used as a marker for Kiss1 neuronal activation in the AVPV (Figure 3B) based on previous studies by the current authors (*39,40*). Approximately 32% of AVPV Kiss1 cells from E2-primed young females had c-Fos^{+} nuclei (Figure 3B-a and c). FISH and IF profiles revealed marked impairment in c-Fos immunoreactivity in middle-aged rats (Figure 3B-d, $P < 0.05$); that is, the percentage of c-Fos^{+} Kiss1 cells decreased to approximately 20% (Figure 3B-b and c, $P < 0.05$).

3.4. TSA promotes *Kiss1* transcription and counteracts neuroendocrine dysfunction in middle-aged females by enhancing H3ac

ChIP-seq analyses pointed to a preponderance of hypoacetylation in the AVPV of middle-aged rats, so the therapeutic potential of TSA was examined next (Figure 4). The effects of TSA on *Kiss1* expression in GT1-7 cells were first tested using a mouse-derived immortalized GnRH-secreting neuronal cell line (Figure 4A-a). E2 exposure (10 mol) induced a significant 20-fold increase in *Kiss1* expression in the cell line. *Kiss1* mRNA increased significantly in GT1-7 cells as a result of TSA (200 nmol). Moreover, the level was strikingly upregulated by the simultaneous presence of both TSA and E2 (Figure 4A-b). Thereafter, cycling middle-aged

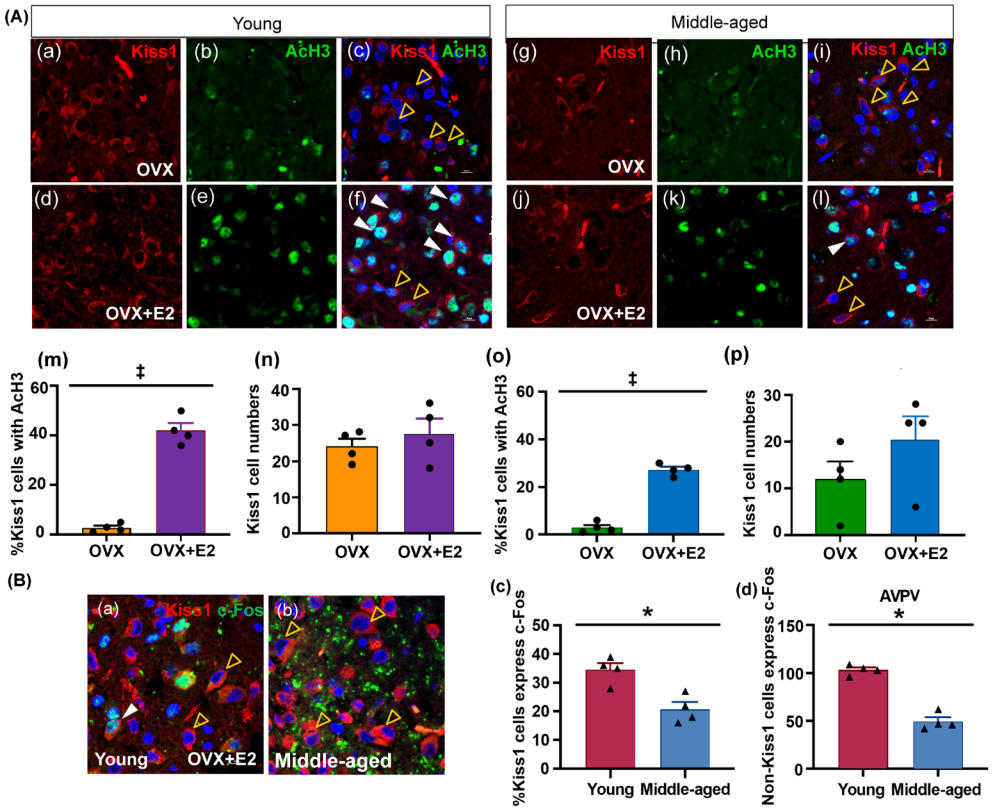

Figure 3. Middle-aged female rats exhibit suppressed histone H3ac in Kiss1^{AVPV} associated with reduced c-Fos expression. (A) Confocal representative photomicrographs showing *Kiss1* and H3ac immunoreactivity in the AVPV. 3V, third ventricle. Bars, 10 μm. **(a–f)** and **(g-l)** Kiss1^{AVPV} cells with (white solid triangles) or without H3ac (yellow open triangles) in young and middle-aged rats primed with E2 or oil, as determined by FISH combined with IF (*Kiss1* mRNA, red; H3ac, green). **(m-n)** and **(o-p)** Quantitative analysis of the percentage of H3ac^{+} Kiss1^{AVPV} cells in young ($n = 4$) and middle-aged females ($n = 4$) after E2 or oil treatment. **(B)** C-Fos^{+} Kiss1 neurons decreased in the AVPV of middle-aged females treated with E2. **(a)** Representative AVPV section showing Kiss1 cells expressing c-Fos in young rats. **(b)** Kiss1 cells lack activation to express c-Fos in middle-aged rats. **(c)** and **(d)** Statistical analysis of c-Fos expression in Kiss1 and non-Kiss1 cells in the AVPV of young and middle-aged rats. Bars, 100 μm. The statistical significance for all analyses was $^{*}P < 0.05$; $^{‡}P < 0.001$.

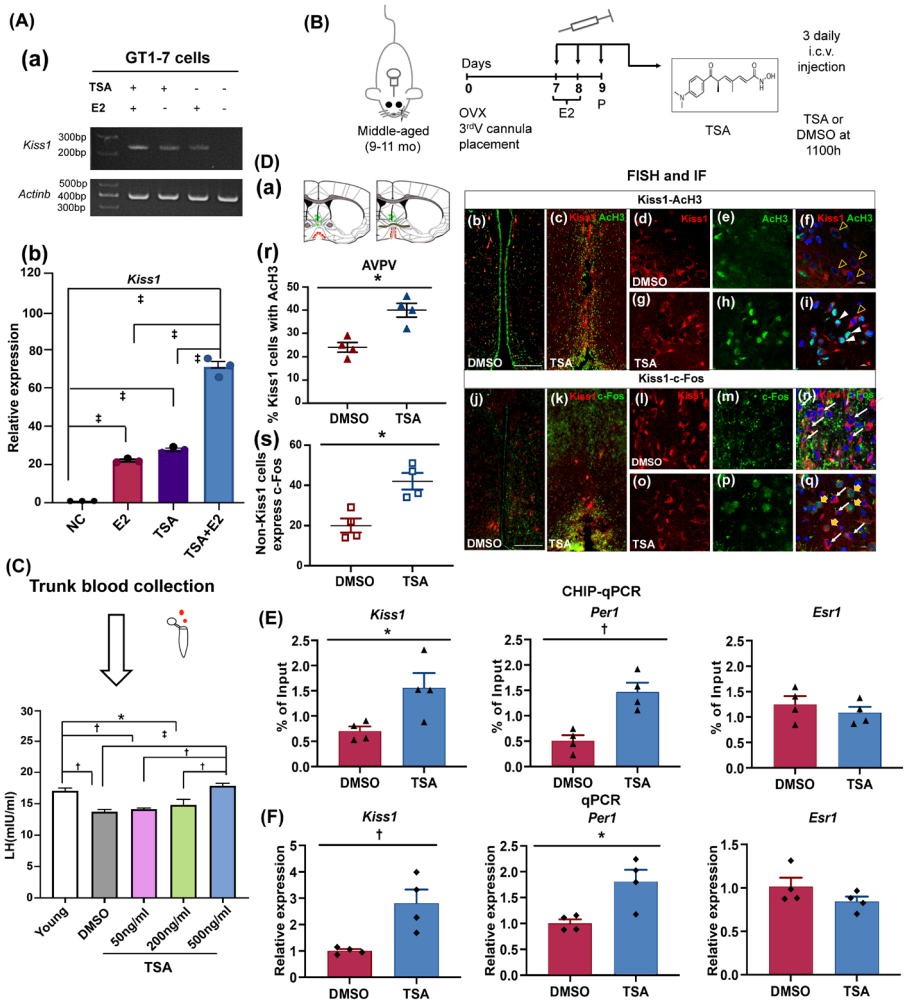

Figure 4. Histone deacetylase inhibitor TSA rescues neuroendocrine dysfunction in middle-aged females by modulating hypoacetylated genes in the AVPV. (A) Effects of inhibitors of histone deacetylation on *Kiss1* expression in the immortalized GT1-7 cell line. RT-PCR analysis of *Kiss1* gene expression in GT1-7 cells treated with 200 nM of TSA. **(B)** Schematic illustration of the experimental design. TSA or DMSO was injected into the third cerebral ventricle of middle-aged rats treated with E2. **(C)** Mean LH levels were measured using trunk blood collected from middle-aged (3-4 h after TSA or DMSO injection) and young rats treated with E2. **(D)** Icv injection of TSA enhances H3ac and improves c-Fos expression in Kiss1^{AVPV}. **(a)** Brain sections of the hypothalamic AVPV were collected. **(b-i)** Representative FISH combined with IF images showing H3ac or non-H3ac in Kiss1^{AVPV} after TSA or DMSO treatment. **(j-q)** Representative FISH combined with IF pictures showing c-Fos or non-c-Fos expressed Kiss1^{AVPV} cells in the groups treated with TSA or DMSO. **(r)** Quantitative analysis of the percentage of H3ac^{+} in Kiss1^{AVPV} cells in the groups treated with TSA (n = 4) or DMSO (n = 4). **(s)** Quantitative analysis of the percentage of Kiss1^{AVPV} cells expressing c-Fos in the groups treated with TSA (n = 4) or DMSO (n = 4). **(E)** and **(F)** Statistical analysis of ChIP-qPCR and qPCR data on young and middle-aged rats treated with TSA or DMSO (n = 4, respectively). The statistical significance for all analyses was $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$.

rats were selected and treated with daily icv injections of DMSO or different doses of TSA (Figure 4B). Trunk blood samples were collected upon sacrifice of the rats for LH measurement on the predicted day of an LH surge. Control animals had a high level of LH release, while 500 ng/mL of TSA restored LH release compared to that in females treated with DMSO ($P < 0.001$, Figure 4C).

Next, whether TSA had a directly effect on Kiss1 neurons was examined. In line with previous data (see Figure 3A), there were few H3ac^{+} Kiss1 neurons in middle-aged rats treated with DMSO (Figure 4D-b and f). The TSA treatment significantly induced a 1.5-fold increase in H3ac^{+} Kiss1 cells in the AVPV ($P < 0.05$, Figure 4D-c, i and r). Similarly, rats treated with TSA had a marked increase in the c-Fos^{+} nuclei of the AVPV Kiss1 cells by approximately 50% compared to that in rats treated with DMSO ($P < 0.05$, Figure 4D-k, q and s). Whether LH restored by TSA (Figure 4C) was associated with an epigenetic effect of H3ac was also determined. ChIP-qPCR assays were performed to target the different gene loci of *Kiss1*, *Per1*, and *Esr1*. A marked increase in H3ac was observed in the enhancer region of *Kiss1* ($P < 0.05$) and in the promoter region of *Per1* as a result of TSA ($P < 0.01$). Changes in H3ac were not observed at *Esr1* (Figure 4E).

To further evaluate the effect of TSA on the levels of gene expression, qRT-PCR of *Kiss1*, *Per1*, and *Esr1* was performed (Figure 4F). Levels of *Esr1* transcription were comparable among the different groups, but *Kiss1*

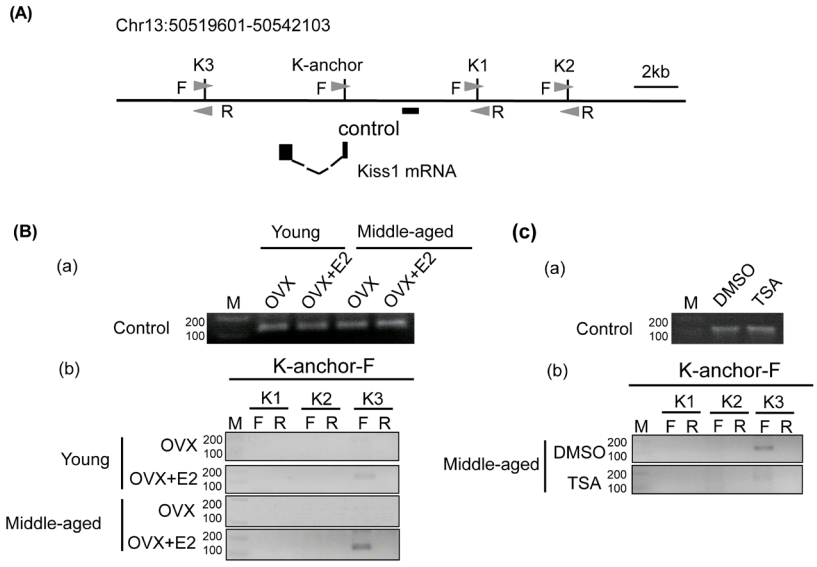

Figure 5. Icv administration of TSA modifies chromatin loop formation at the *Kiss1* gene locus in the AVPV of middle-aged rats. (A) Diagram of the *Kiss1* locus; filled boxes indicate exons, and thin dotted lines indicate introns. Kpnl restriction endonuclease sites are indicated by vertical lines (labeled as K1–K3). Arrows show the positions of primers used in 3C assays. The thick horizontal bar indicates the region used in loading control PCR. **(B)** Chromatin loop formation at the *Kiss1* gene locus in the AVPV of middle-aged rats. **(a)** PCR for loading control. Tissue from the AVPV was sampled in young and middle-aged OVX rats with (OVX+E2) or without E2 treatment (OVX). **(b)** 3C analysis of the *Kiss1* locus obtained from AVPV tissue from young and middle-aged females exposed or not exposed to E2. 3C assays were performed, and the PCR products were analyzed using agarose gel electrophoresis. The PCR products were generated using the primer K-anchor forward (K-anchor-F) along with one of the other Kpnl primers (K1–K3) as indicated. **(C)** TSA induces chromatin conformational loop changes at the *Kiss1* gene locus in the AVPV of middle-aged rats. **(a)** PCR for loading control. Tissue from the AVPV was sampled from OVX+E2-primed middle-aged rats injected with TSA or DMSO. **(b)** PCR products of a 3C assay were analyzed using agarose gel electrophoresis. Primers and PCR products were generated as described above.

and *Per1* were significantly upregulated in the group treated with TSA (Figure 4F; $P < 0.01$ and $P < 0.05$, respectively).

3.5. The mechanism of reduced *Kiss1* gene expression in middle-age includes changes in chromatin looping associated with histone hypoacetylation

Chromatin access (opening) to transactivating factors driven by histone acetylation is the major epigenetic mechanism for gene transcription (*47*). Whether the chromatin conformational loop changed at the AVPV *Kiss1* gene loci after E2 treatment was assessed using 3C primers as shown in Figure 5A. The intensity of PCR products for the controls did not differ between the groups (Figure 5B-a). No PCR products were observed with the K2F-K1R, K3F-K1R, and K1F-K2R primers in the OVX young or middle-aged groups ($n = 4$, Figure 5B-b), indicating that there were no looping interactions without E2. E2 treatment increased the 3′ PCR products with K-anchor-K3F primers (Figure 5B-b) in the AVPV of young rats. These results indicate that chromatin loop formation between the promoter and the 3' intergenic region of *Kiss1* is enhanced by E2. The patterns of chromatin loop formation within the *Kiss1* loci differ between young and middle-aged rats. The PCR product obtained using primers between the promoter and 3′ intergenic region markedly increased in the rats, suggesting that an intense loop was formed in the 3′ region under E2-positive feedback conditions (Figure 5B-b). Whether H3ac drove the differential chromatin looping conformation of the AVPV *Kiss1* gene was further examined in middle-aged females using TSA. PCR products of the 3C assay with the K-anchor-K3F primer sets displayed less intensity. The intensity of the PCR product decreased to a level similar to that in young females treated with E2 (Figure 5C-b), indicating that H3ac enhancement by TSA normalized loop formation in the 3′ intergenic region, which in turn permitted *Kiss1* transcription. There were no differences in the amounts of PCR products for the controls among the groups (Figure 5C-a).

4. Discussion

The current study has provided the first high-throughput sequencing profile of epigenome changes in the AVPV. This key E2-positive feedback region is causally involved in neuroendocrine impairment in female reproductive aging. Given the importance of the hyperacetylation of histones in the promoter region to gene expression induced by steroid hormones (*24,35,48,49*), advanced reproductive age heavily contributed to differential H3ac peaks enriched by the TSS in the AVPV; a preponderance of hypoacetylation in particular contributed to the altered gene expression in middle-aged females under E2-

positive feedback conditions. This conclusion is backed up by AVPV ChIP-seq data showing the hypoacetylation of histone H3 coupled with changes in the expression of 33.1% of genes. Despite the number of sequences and regions involved in histone acetylation and the complex chromatin alterations that exist alongside gene expression (*10*), the current data expand our understanding that age-dependent epigenomic changes ultimately defining the AVPV cellular transcriptional response to E2 may cause the dysregulation of transcriptional and chromatin networks in advanced age. Interestingly, GO and KEGG analyses revealed that age-related differences in histone acetylation occurring in specific genes and pathways were associated with reproduction and hormone secretion as well as neurotransmitter synapses. The selective analysis of epigenomic changes in several neuronal and non-neuronal cells using genetic strategies such as the fluorescently tagged technique combined with fluorescence activated nuclear sorting (FANS) to isolate specific cell types from the AVPV would further increase our understanding of aging GnRH neuronal networks.

Tomikawa *et al.* found that increased E2-promoted H3ac and ERα binding in the promoter region of *Kiss1*^{AVPV} positively enhanced *Kiss1* gene expression (*35*), suggesting that the promoter region is critical to E2-activated *Kiss1* gene expression. Bioinformatic analysis predicted that a reduction in *Kiss1* in the AVPV of middle-aged rats was the result of the hypoacetylation of histone H3 at the 3′ intergenic region of the *Kiss1* loci. Removal of the 3' region, which is known to be a functional estrogen-responsive enhancer for *Kiss1*, in Tg-2 mice reduced GFP expression induced by estrogen in *Kiss1* (*35*). The acetylation of histones changes the chromatin flexibility in the nucleus and the interaction between the coding gene and its remote enhancer, but a low acetylation threshold destabilizes higher-order folding, which has a similar effect to N-terminal removal (*21,50*). Therefore, suppressed H3ac in the 3′ region of *Kiss1* may weaken its facilitating role in chromatin-looping conformation for *Kiss1* transcription. As indicated by the 3C assay, the interaction between the promoter and 3′ region of *Kiss1 via* chromatin looping is likely to be associated with E2-dependent *Kiss1* expression in the AVPV of young females. H3ac decreased markedly in middle-aged rats, but an intensified chromatin loop between the promoter and 3′ region of *Kiss1* was notably detected and was associated with reduced *Kiss1* expression. These discordant findings can be explained by the 3′ enhancer region of *Kiss1*, which may be a specific locus that loses sensitivity to E2 when females transition to middle age, or by the hypoacetylation of histone H3, which is continuously responsible for looping conformation but which finally changes chromatin to an "off" state. Nevertheless, age-associated changes in H3ac may induce a stable repressive higher-order chromatin structure *via* a CCCTC binding factor (CTCF) in the AVPV nuclei of middled-aged animals to suppress gene expression despite E2 positive feedback.

The current study also found that expression of a circadian gene, *Per1*, was affected by the hypoacetylation of histone H3. Previous studies have reported the circadian regulation of higher-order chromatin formation (*37*), long-range interchromosomal interplay (*51*), and short-range looping at specific loci (*52,53*). AVPV *Kiss1* cells express *Per1* with an E2-sensitive circadian rhythm (*54,55*); however, whether the H3ac-mediated suppression of *Per1* ultimately limited *Kiss1* expression remains unknown. Evaluating the complex dynamics between *Per1*, *Kiss1*, and other molecular players is an important topic of future research.

Even more strikingly, the current results revealed that the administration of TSA to E2-primed middle-aged rats corrected neuroendocrine alterations and rescued GnRH/LH release. Consistent with previous studies which found that TSA intensified the transcription of the *c-fos* and *c-jun* genes in neurons after kainite stimulation (*56*), TSA significantly increased c-Fos activation in the Kiss1 cells of middle-aged females, suggesting an appropriate level of histone acetylation is required to activate *Kiss1* transcription. The current findings are also consistent with those of a recent study which found that histone hyperacetylation induced by TSA can induce *Kiss1* expression in the mouse hypothalamic cell line N6 (*35*), suggesting that reduced H3ac may result in negative down-stream effects on GnRH neuron activation and LH release. An HDAC inhibitor is clinically used for cancer treatment (*57-59*). The current study cannot rule out the possibility that TSA promoted some other gene expression or protein acetylation in the GnRH neuronal network, but the current results highlight the therapeutic potential of acetylation agents as promising epigenetic therapies to treat women with LH surge dysfunction.

Under E2 positive feedback, *Esr1* is required for E2-regulated gene expression in the hypothalamus (*6,60*). Acetylation of the *Esr1* hinge region manipulates transcriptional transactivation and hormone sensitivity (*61*), but the level of H3ac at the *Esr1* promoter and the *Esr1* mRNA in middle-aged females is similar to that in young females. These data do not support the contention that decreased H3ac at the regulatory sequences of *Esr1* reduces *Kiss1* expression in the AVPV of middle-aged females.

In summary, the current study provides evidence showing that altered H3ac plays a pivotal role in age-related neuroendocrine changes, including LH disorders. That said, specific histone H3 lysine acetylation remains to be determined. Other epigenetic modifications cannot be excluded as contributing factors, but recent studies have confirmed the unique role of H3ac in orchestrating E2-induced gene expression under positive feedback. Therefore, H3ac seems to be of particular importance to transcriptional activation as characterized by hyperacetylation along gene bodies. In addition, the

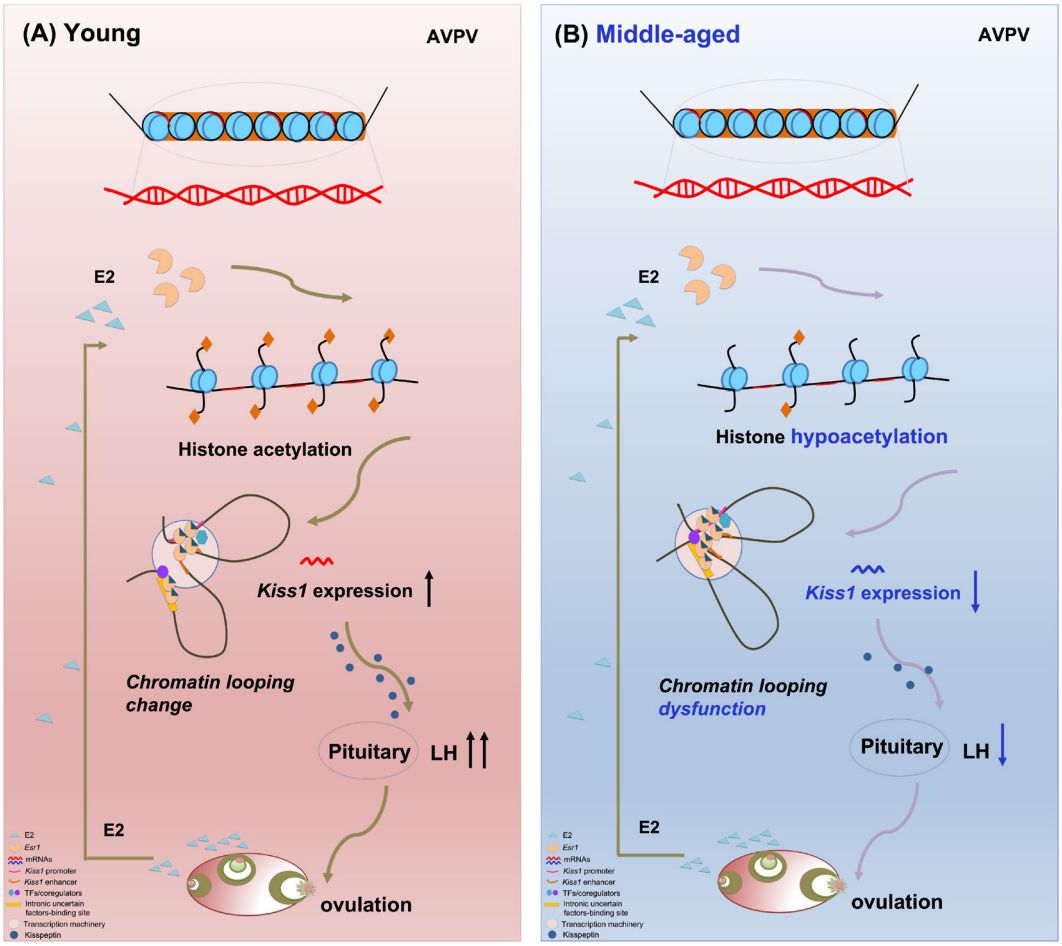

Figure 6 Schematic representation of the proposed mechanism of epigenomic alterations modifying the expression of genes such as *Kiss1* in the rat AVPV to control female reproductive aging. This diagram shows possible epigenomic regulation of gene expression in the AVPV under E2-positive feedback conditions. **(A)** In young females, the E2-ESR1 complex induces histone H3ac and opens the chromatin structure, consequently enhancing the formation of a chromatin loop between the *Kiss1* promoter and the 3′ enhancer region, promoting transcription. **(B)** In middle-aged females, decreased histone H3 acetylation associated with an altered chromatin state may cause transcriptional inaction by formatting the inhibitory chromatin loop between the *Kiss1* promoter and the 3′ enhancer region.

dysregulation of H3ac in middle age is mainly found along with reduced gene expression. Altered H3ac may manipulate *Kiss1* expression in particular by remodeling chromatin (Figure 6). This work presents important insights into the molecular mechanisms underlying reduced responsiveness to E2-positive feedback in specific regions of the hypothalamus when females transition into perimenopause.

Acknowledgements

The authors wish to sincerely thank Peng Li and Suna Tian for their assistance in preparing the figures in this manuscript.

Funding: This research was supported by the Shanghai Natural Science Foundation (grant no.17ZR1403300 to Y Sun), the National Natural Science Foundation of China for Young Investigators (grant no. 31800987 to Y Sun) and the National Natural Science Foundation of China (grant no. 82074531 to Y Sun), a project under the Scientific and Technological Innovation Action Plan of the Shanghai Natural Science Fund (grant no. 20ZR1409100 to L Wang), a project of the Chinese Association of Integration of Traditional and Western Medicine special foundation for Obstetrics and Gynecology-PuZheng Pharmaceutical Foundation (grant no. FCK-PZ-08 to L Wang), a project for hospital management of the Shanghai Hospital Association (grant no. X2021046 to L Wang), and a clinical trial project of the Special Foundation for Healthcare Research of the Shanghai Municipal Health Commission (grant No. 202150042 to L Wang).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Le WW, Wise PM, Murphy AZ, Coolen LM, Hoffman GE. Parallel declines in Fos activation of the medial anteroventral periventricular nucleus and LHRH neurons

in middle-aged rats. Endocrinology. 2001; 142:4976-4982.
2. Kauffman AS, Sun Y, Kim J, Khan AR, Shu J, Neal-Perry G. Vasoactive intestinal peptide modulation of the steroid-induced LH surge involves kisspeptin signaling in young but not in middle-aged female rats. Endocrinology. 2014; 155:2222-2232.
3. Xie Q, Kang Y, Zhang C, Xie Y, Wang C, Liu J, Yu C, Zhao H, Huang D. The role of kisspeptin in the control of the hypothalamic-pituitary-gonadal axis and reproduction. Front Endocrinol (Lausanne). 2022; 13:925206.
4. Austad SN. Sex differences in health and aging: A dialog between the brain and gonad? Geroscience. 2019; 41:267-273.
5. Neal-Perry GS, Zeevalk GD, Santoro NF, Etgen AM. Attenuation of preoptic area glutamate release correlates with reduced luteinizing hormone secretion in middle-aged female rats. Endocrinology. 2005; 146:4331-4339.
6. Chakraborty TR, Hof PR, Ng L, Gore AC. Stereologic analysis of estrogen receptor alpha (ER alpha) expression in rat hypothalamus and its regulation by aging and estrogen. J Comp Neurol. 2003; 466:409-421.
7. Chakraborty TR, Ng L, Gore AC. Age-related changes in estrogen receptor beta in rat hypothalamus: A quantitative analysis. Endocrinology. 2003; 144:4164-4171.
8. Brown TJ, MacLusky NJ, Shanabrough M, Naftolin F. Comparison of age- and sex-related changes in cell nuclear estrogen-binding capacity and progestin receptor induction in the rat brain. Endocrinology. 1990; 126:2965-2972.
9. Engmann O, Labonté B, Mitchell A*, et al.* Cocaine-induced chromatin modifications associate with increased expression and three-dimensional looping of Auts2. Biol Psychiatry. 2017; 82:794-805.
10. Ferrari R, de Llobet Cucalon LI, Di Vona C, Le Dilly F, Vidal E, Lioutas A, Oliete JQ, Jochem L, Cutts E, Dieci G, Vannini A, Teichmann M, de la Luna S, Beato M. TFIIIC binding to Alu elements controls gene expression *via* chromatin looping and histone acetylation. Mol Cell. 2020; 77:475-487.e411.
11. Anderson EM, Taniguchi M. Epigenetic effects of addictive drugs in the nucleus accumbens. Front Mol Neurosci. 2022; 15:828055.
12. Fernandez-Albert J, Lipinski M, Lopez-Cascales MT, Rowley MJ, Martin-Gonzalez AM, Del Blanco B, Corces VG, Barco A. Immediate and deferred epigenomic signatures of *in vivo* neuronal activation in mouse hippocampus. Nat Neurosci. 2019; 22:1718-1730.
13. Bonnaud EM, Suberbielle E, Malnou CE. Histone acetylation in neuronal (dys)function. Biomol Concepts. 2016; 7:103-116.
14. Girdhar K, Hoffman GE, Jiang Y, *et al.* Cell-specific histone modification maps in the human frontal lobe link schizophrenia risk to the neuronal epigenome. Nat Neurosci. 2018; 21:1126-1136.
15. Kubben N, Misteli T. Shared molecular and cellular mechanisms of premature ageing and ageing-associated diseases. Nat Rev Mol Cell Biol. 2017; 18:595-609.
16. Fallah MS, Szarics D, Robson CM, Eubanks JH. Impaired regulation of histone methylation and acetylation underlies specific neurodevelopmental disorders. Front Genet. 2020; 11:613098.
17. Kurdistani SK, Tavazoie S, Grunstein M. Mapping global histone acetylation patterns to gene expression. Cell. 2004; 117:721-733.
18. Shvedunova M, Akhtar A. Modulation of cellular processes by histone and non-histone protein acetylation. Nat Rev Mol Cell Biol. 2022; 23:329-349.
19. Ozawa H. Masterful papers that broke open understanding of reproductive neuroendocrinology. Nat Rev Endocrinol. 2022.
20. Kristjuhan A, Walker J, Suka N, Grunstein M, Roberts D, Cairns BR, Svejstrup JQ. Transcriptional inhibition of genes with severe histone H3 hypoacetylation in the coding region. Mol Cell. 2002; 10:925-933.
21. Li B, Carey M, Workman JL. The role of chromatin during transcription. Cell. 2007; 128:707-719.
22. Yi SJ, Kim K. New insights into the role of histone changes in aging. Int J Mol Sci. 2020; 21.
23. Fan L, Zhao Z, Orr PT, Chambers CH, Lewis MC, Frick KM. Estradiol-induced object memory consolidation in middle-aged female mice requires dorsal hippocampal extracellular signal-regulated kinase and phosphatidylinositol 3-kinase activation. J Neurosci. 2010; 30:4390-4400.
24. Gagnidze K, Weil ZM, Faustino LC, Schaafsma SM, Pfaff DW. Early histone modifications in the ventromedial hypothalamus and preoptic area following oestradiol administration. J Neuroendocrinol. 2013; 25:939-955.
25. Frick KM, Tuscher JJ, Koss WA, Kim J, Taxier LR. Estrogenic regulation of memory consolidation: A look beyond the hippocampus, ovaries, and females. Physiol Behav. 2018; 187:57-66.
26. Kovács T, Szabó-Meleg E, Ábrahám IM. Estradiol-induced epigenetically mediated mechanisms and regulation of gene expression. Int J Mol Sci. 2020; 21.
27. Xu W, An X, Zhang N, Li L, Zhang X, Wang Y, Wang L, Sun Y. Middle-aged female rats lack changes in histone H3 acetylation in the anterior hypothalamus observed in young females on the day of a luteinizing hormone surge. Biosci Trends. 2019; 13:334-341.
28. Xu W, Huang J, Li L, Zhang X, Wang Y, Tong G, Sun Y. Alterations of estradiol-induced histone H3 acetylation in the preoptic area and anteroventral periventricular nucleus of middle-aged female rats. Biochem Biophys Res Commun. 2019; 516:894-899.
29. Xu W, Zhang N, Li LS, Wang Y, Wang L, Du MR, Li DJ, Sun Y. Gene expression pattern of histone acetylation enzymes changed in the hypothalamus of middle-aged female rats: A putative mechanism for female reproductive aging. Reprod Dev Med. 2018; 2:65-73.
30. Uenoyama Y, Inoue N, Nakamura S, Tsukamura H. Kisspeptin neurons and estrogen-estrogen receptor alpha signaling: Unraveling the mystery of steroid feedback system regulating mammalian reproduction. Int J Mol Sci. 2021; 22.
31. Adams C, Stroberg W, DeFazio RA, Schnell S, Moenter SM. Gonadotropin-releasing hormone (GnRH) neuron excitability is regulated by estradiol feedback and kisspeptin. J Neurosci. 2018; 38:1249-1263.
32. Sobrino V, Avendaño MS, Perdices-López C, Jimenez-Puyer M, Tena-Sempere M. Kisspeptins and the neuroendocrine control of reproduction: Recent progress and new frontiers in kisspeptin research. Front Neuroendocrinol. 2022; 65:100977.
33. Kimura M, Ishii MN, Seki N, Sakai Y, Yamashita T, Awatsuji H, Kanda K, Matsumoto K, Matsui H. Reduction of Kiss1 expression in the anteroventral periventricular nucleus is associated with atrazine-induced attenuation of the luteinizing hormone surge in female rats. Biol Reprod. 2019; 100:41-48.
34. Lederman MA, Lebesgue D, Gonzalez VV, Shu J, Merhi

ZO, Etgen AM, Neal-Perry G. Age-related LH surge dysfunction correlates with reduced responsiveness of hypothalamic anteroventral periventricular nucleus kisspeptin neurons to estradiol positive feedback in middle-aged rats. Neuropharmacology. 2010; 58:314-320.
35. Tomikawa J, Uenoyama Y, Ozawa M, *et al.* Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. Proc Natl Acad Sci U S A. 2012; 109:E1294-1301.
36. Semaan SJ, Kauffman AS. Emerging concepts on the epigenetic and transcriptional regulation of the *Kiss1* gene. Int J Dev Neurosci. 2013; 31:452-462.
37. Zhao H, Sifakis EG, Sumida N, *et al.* PARP1- and CTCF-Mediated interactions between active and repressed chromatin at the lamina promote oscillating transcription. Mol Cell. 2015; 59:984-997.
38. Akgol Oksuz B, Yang L, Abraham S, *et al.* Systematic evaluation of chromosome conformation capture assays. Nat Methods. 2021; 18:1046-1055.
39. Sun Y, Todd BJ, Thornton K, Etgen AM, Neal-Perry G. Differential effects of hypothalamic IGF-I on gonadotropin releasing hormone neuronal activation during steroid-induced LH surges in young and middle-aged female rats. Endocrinology. 2011; 152:4276-4287.
40. Sun Y, Shu J, Kyei K, Neal-Perry GS. Intracerebroventricular infusion of vasoactive intestinal peptide rescues the luteinizing hormone surge in middle-aged female rats. Front Endocrinol (Lausanne). 2012; 3:24.
41. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W, Liu XS. Model-based analysis of ChIP-seq (MACS). Genome Biol. 2008; 9:R137.
42. Quinlan AR, Hall IM. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics. 2010; 26:841-842.
43. Zhu LJ, Gazin C, Lawson ND, Pagès H, Lin SM, Lapointe DS, Green MR. ChIPpeakAnno: A bioconductor package to annotate ChIP-seq and ChIP-chip data. BMC Bioinformatics. 2010; 11:237.
44. Yu G, Wang LG, He QY. ChIPseeker: An R/Bioconductor package for ChIP peak annotation, comparison and visualization. Bioinformatics. 2015; 31:2382-2383.
45. Tamura I, Ohkawa Y, Sato T, Suyama M, Jozaki K, Okada M, Lee L, Maekawa R, Asada H, Sato S, Yamagata Y, Tamura H, Sugino N. Genome-wide analysis of histone modifications in human endometrial stromal cells. Mol Endocrinol. 2014; 28:1656-1669.
46. Troutman TD, Kofman E, Glass CK. Exploiting dynamic enhancer landscapes to decode macrophage and microglia phenotypes in health and disease. Mol Cell. 2021; 81:3888-3903.
47. Clayton AL, Hazzalin CA, Mahadevan LC. Enhanced histone acetylation and transcription: A dynamic perspective. Mol Cell. 2006; 23:289-296.
48. Chen H, Lin RJ, Xie W, Wilpitz D, Evans RM. Regulation of hormone-induced histone hyperacetylation and gene activation *via* acetylation of an acetylase. Cell. 1999; 98:675-686.
49. Frick KM, Kim J. Mechanisms underlying the rapid effects of estradiol and progesterone on hippocampal memory consolidation in female rodents. Horm Behav. 2018; 104:100-110.
50. Tse C, Sera T, Wolffe AP, Hansen JC. Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase iii. Mol Cell Biol. 1998; 18:4629-4638.
51. Aguilar-Arnal L, Hakim O, Patel VR, Baldi P, Hager GL, Sassone-Corsi P. Cycles in spatial and temporal chromosomal organization driven by the circadian clock. Nat Struct Mol Biol. 2013; 20:1206-1213.
52. Xu Y, Guo W, Li P, Zhang Y, Zhao M, Fan Z, Zhao Z, Yan J. Long-range chromosome interactions mediated by cohesin shape circadian gene expression. PLoS Genet. 2016; 12:e1005992.
53. Furlan-Magaril M, Ando-Kuri M, Arzate-Mejía RG, Morf J, Cairns J, Román-Figueroa A, Tenorio-Hernández L, Poot-Hernández AC, Andrews S, Várnai C, Virk B, Wingett SW, Fraser P. The global and promoter-centric 3D genome organization temporally resolved during a circadian cycle. Genome Biol. 2021; 22:162.
54. Chassard D, Bur I, Poirel VJ, Mendoza J, Simonneaux V. Evidence for a putative circadian Kiss-Clock in the hypothalamic AVPV in female mice. Endocrinology. 2015; 156:2999-3011.
55. Shao S, Zhao H, Lu Z, Lei X, Zhang Y. Circadian rhythms within the female HPG axis: From physiology to etiology. Endocrinology. 2021; 162.
56. Sng JC, Taniura H, Yoneda Y. Inhibition of histone deacetylation by trichostatin a intensifies the transcriptions of neuronal c-fos and c-jun genes after kainate stimulation. Neurosci Lett. 2005; 386:150-155.
57. Damaskos C, Garmpis N, Valsami S, *et al.* Histone deacetylase inhibitors: An attractive therapeutic strategy against breast cancer. Anticancer Res. 2017; 37:35-46.
58. Li X, Xu W. HDAC1/3 dual selective inhibitors - New therapeutic agents for the potential treatment of cancer. Drug Discov Ther. 2014; 8:225-228.
59. Zhou N, Xu W, Zhang Y. Histone deacetylase inhibitors merged with protein tyrosine kinase inhibitors. Drug Discov Ther. 2015; 9:147-155.
60. Hewitt SC, Korach KS. Oestrogen receptor knockout mice: Roles for oestrogen receptors alpha and beta in reproductive tissues. Reproduction. 2003; 125:143-149.
61. Wang C, Fu M, Angeletti RH, Siconolfi-Baez L, Reutens AT, Albanese C, Lisanti MP, Katzenellenbogen BS, Kato S, Hopp T, Fuqua SA, Lopez GN, Kushner PJ, Pestell RG. Direct acetylation of the estrogen receptor alpha hinge region by p300 regulates transactivation and hormone sensitivity. J Biol Chem. 2001; 276:18375-18383.

Received August 15, 2022; Revised October 14, 2022; Accepted October 20, 2022.

[§]These authors contributed equally to this work.
*Address correspondence to:
Yan Sun, Hospital & Institute of Obstetrics and Gynecology, Fudan University, 128 Shenyang Road, Yangpu, Shanghai 200081, China.
E-mail: ysunsh@126.com

Ling Wang, Laboratory for Reproductive Immunology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, China.
E-mail: Dr.wangling@fudan.edu.cn

Released online in J-STAGE as advance publication October 23, 2022.

Correspondence

DOI: 10.5582/bst.2022.01453

Liquid-liquid phase separation: A new perspective to understanding aging and pathogenesis

Jufeng Xia*

Arai Japan Medical Institute, Tokyo, Japan.

SUMMARY Mounting evidence has suggested that phase separation, and especially liquid-liquid phase separation (LLPS), underlies the formation of membraneless organelles, which are supramolecular assemblies of proteins and RNA molecules in cells. These membraneless organelles are also called biomolecular condensates. Evidence is now growing that condensates, such as stress granules, P bodies, Cajal bodies, and nucleoli, play vital roles in biological processes, like RNA storage and processing, signaling regulation, transcription regulation, gene regulation, and transport. Conversely, condensates may cause diseases, such as neurodegenerative diseases and tumors, when they go wrong. Condensates initially have liquid-like properties, but accumulating biological and chemical mutations with age render them into a more solid-like state, like amyloids in Alzheimer's disease, Huntington's disease, and Parkinson's disease. Research into phase separation is still in its infancy, but this field is a promising avenue for treatment of aging-related diseases.

Keywords phase separation, condensate, aging, cancer, amyloid

Liquid-liquid phase separation (LLPS) describes a phenomenon whereby proteins and/or nucleic acids in a solution separate into a dense phase that resembles liquid droplets when their concentration rises above a certain level, just like oil drops forming in water. The dense phase will form biomolecular condensates such as membraneless organelles (MOs). MOs such as nucleoli were described as early as the 1830s (*1*). Recently, Keizer *et al*. found that, outside of cell division phases, chromosomes are actually almost liquid and that this structure may be formed through LLPS (*2*). Condensates have been reported to have several functions including the enhancement or inhibition of cellular reactions, sensing changes in the niche (the environment surrounding cells in aging tissue), and buffering biomolecule concentration (*3*). In a lot of biological processes, condensates play an important role and thus condensate assembly, a phase change, the quality control system (QCS), and the relationship between condensates and aging-related diseases need to be understood.

Condensate assembly

When a liquid phase is formed by LLPS, the liquid state is maintained by continuous interactions between biomolecules in the liquid phase. A common way that proteins form a liquid phase is through multivalence of phase-separating molecules (*4*). Multivalence is derived from folded interaction domains or intrinsically disordered regions (*5*). Besides proteins, RNAs have also been found to be key factors in the formation and composition of condensates (*6*). Cooperation between folded protein domains and nucleic acids may be necessary for most phase separation processes.

When condensates begin to assemble, two types of molecules are needed, scaffold molecules and client proteins. Scaffold molecules have numerous valences and play a role in promoting LLPS, and client proteins have lower valences and specifically bind to elements in the scaffolds (*7*). Cells employ various mechanisms to regulate the formation of condensates. Phosphorylation and/or methylation of proteins was reported to change the saturation concentration of proteins *via* post-translational modification (*8,9*). That said, LLPS is very sensitive to RNA concentrations and cells can control transcription to regulate condensates. In addition, other factors including temperature, the concentration of metabolites, pH, and ions also regulate the formation of condensates and cooperate to change them.

Phase change and aging

Although condensates are initially in a liquid phase, they will age and change to a more solid phase under certain conditions (*10*). The liquid phase can age into

a gel or glass state. In gelation, the physical crosslinks between condensate components reach a percolation threshold and the condensate changes to a gel. However, condensates are soft glasses themselves and can harden into a solid-like state as a result of changes in temperature or density. Both a gel and glass state can slow down protein dynamics and promote aggregation. There are various types of aggregated proteins, such as oligomers, amyloid fibrils, and disordered or amorphous protein aggregates. A leading villain, amyloid fibrils lead to various neurodegenerative diseases; once formed, they can become seeds and change other soluble proteins into an amyloid state (*11*). Aging is a two-edged sword: it can preserve the biological structure and suppress redundant biochemical reactions during stress adaption, but it is also linked to various diseases (*12*).

A few factors have been reported to promote condensate aging, such as high protein concentrations, loss of binding partners, and insufficient water. Conversely, increasing the heterogeneity of the protein composition in condensates could be a way to suppress aging; as an example, adding a few binding partners such as RNA and RNA-binding proteins (RBPs) will delay or prevent the aging of condensates (*13*).

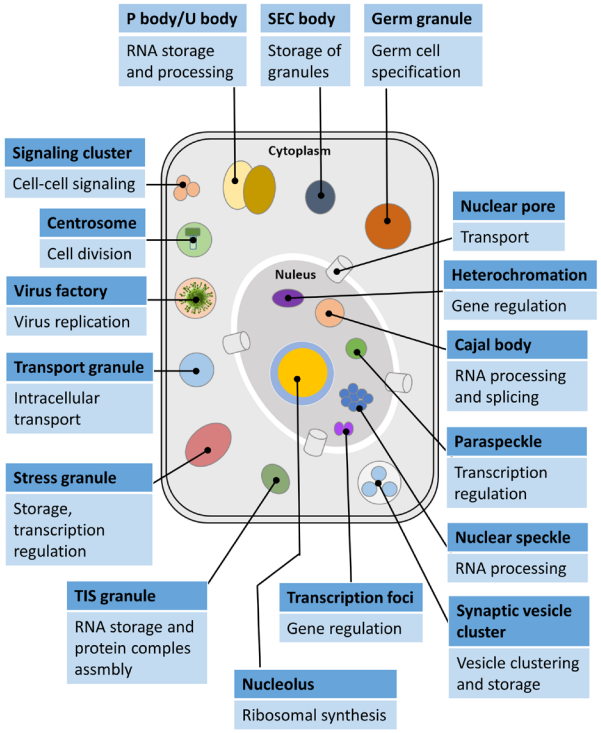

Figure 1. Functions of condensates. This graph provides an overview of the different condensates that have been studied in cells to date.

QCS for condensates

The QCS has gradually been recognized as an important regulator of condensate assembly, disassembly, and dynamic equilibrium. Recently, a study suggested that misfolding proteins accumulate in condensates and lead to aging (*14*). For example, stress granules combine with RNA and operate under stress, and they dissolve and release RNA once stress resolves. However, when misfolded proteins bind to stress granules, they are unable to release RNA (*15*). The same study found that the molecular chaperone 70-kDa heat shock protein (HSP70) can prevent the accumulation of misfolded protein in stress granules and even disassemble stress granules containing misfolded protein; those granules change from a liquid state to a solid-like state. In addition to chaperones, the ubiquitin-proteasome system has also been put forward as a component of the QCS. The ubiquitin-proteasome system is reported to play a key role in stress granule clearance, but the functions of most of the components in this system are still unclear (*16*). Another way to remove misfolded proteins is autophagy, which can selectively degrade condensates. For example, P62 was reported to be able to disassemble ubiquitin-positive stress granules. There are a few potential mechanisms that operate in the QCS, but in most instances they have not been studied in detail and more studies should be conducted to elucidate those mechanisms (Figure 1).

Mechanisms by which condensates are involved in aging-related diseases

Mounting evidence has indicated that abnormal condensate assembly or disassembly will lead to various diseases. Numerous diseases, such as tumors and neurodegenerative diseases, are driven by genetic or epigenetic mutations. A mutation in a LLPS protein will not only affect the protein itself but the interaction between it and other surrounding condensate proteins. Mutations in condensate-forming proteins could inhibit the formation of a condensate or affect condensate stability by changing the saturation concentration of scaffold proteins.

Abnormal condensate formation leads to various diseases. This happens in the following ways. Firstly, aberrant condensates disrupt proteostasis. Many neurological diseases are caused by mutations in QCS factors. For instance, a mutation in an autophagy protein led to an early onset neurodegenerative phenotype in an experiment with mice (*17*). Enhancing the QCS may be a viable option for preventing aging-related diseases. Secondly, aberrant condensates can disturb epigenetic gene regulation and heterochromatin formation. Abnormal LLPS could affect many epigenetic and chromatin regulatory factors and also promote oncogenic transformation-related enhancers and gene promoters (*18*). An aging-related abnormality in gene expression could be driven by aberrant condensates in the nucleus. Keeping condensates in good condition is crucial to maintaining tissues and organs, and especially in stem cells. Thirdly, aberrant condensates are involved in genome instability. Cancer cells frequently carry

point mutations, deletions, insertions, and fusions in their genome. To prevent these changes, cells employ a complicated system to repair the damaged DNA, and condensates are reported to be involved in the repair process. The aging process seems to weaken the ability of condensates to repair DNA (*19*). Fourthly, condensates lose their assembly sites on telomeres. As telomere attrition occurs with aging, condensate assembly is affected, and this may lead to cellular senescence and stem cell exhaustion. Moreover, tumor cells always form telomere clusters, which may be a backdoor for tumor cells to escape apoptosis (*20*). Fifthly, aberrant condensates disturb signaling in cells. There is increasing evidence that abnormal signaling events are frequently seen in cancers, and many of these may be linked to aberrant LLPS and condensate formation (*21*).

More than eight hundred RBPs exhibit LLPS, and a few of them tend to accumulate as protein aggregates in diseases (*22*). The relationship between RBPs and neurodegenerative diseases was first unveiled with the discovery of TDP-43 as the key factor that accumulates in the spinal cords of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (*23,24*). A nuclear protein, TDP-43 can phase separate; its fibrils are able to induce further TDP-43 aggregation, and the protein irreversibly accumulates in neurons over time (*25*). RBPs also contribute to the pathophysiology of tauopathies, including Alzheimer's disease (AD). Many proteins can assemble into highly ordered structures called amyloids, which are characterized by fibrillary arrangement. Amyloid fibrils are often very stable assemblies that exclude water and contain a large proportion of cross-ß-sheets. A study has suggested that cross-ß-sheets underlie the formation of condensates (*26*). However, the role that cross-ß-sheets playing in neurodegeneration is still unclear.

Cancer cells can activate oncogenes and inactivate tumor suppressor genes through genomic alterations. Both oncogene activation and suppressor inhibition involve phase separation. Dysregulated condensates have been found in various cancers (*27*). Stress granules, a type of condensate, promote cell survival under stress. There are usually microenvironment characteristics such as hypoxia and a high level of reactive oxygen species in cancer cells that induce stress granule assembly. For example, *KRAS* is one of the most frequently mutated oncogenes in human cancers, and it is linked to stress granule assembly in pancreatic ductal adenocarcinoma, colorectal adenocarcinoma, and lung adenocarcinoma (*28*). Paraspeckles are nuclear bodies that are able to regulate gene expression. Abnormal assembly of paraspeckles has often been observed in various tumors. In hepatocellular carcinoma, paraspeckle assembly increases as a result of inflammation, which induces *STAT3* activation. Over-activation of *STAT3* promotes cell survival, inflammation, epithelial to mesenchymal transition, and cancer cell maintenance (*29*) (Figure 2).

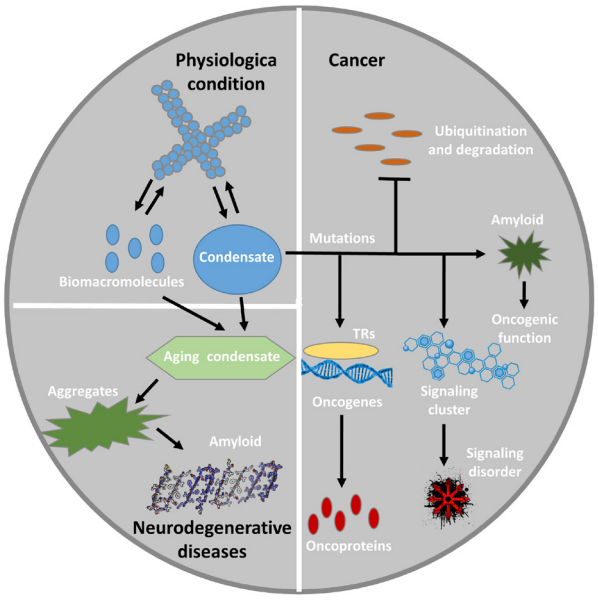

Figure 2. LLPS in cells and its relationship to neurodegenerative diseases and cancers.

Conclusions and perspectives for the future

Our understanding of phase separation and biomolecular condensates has increased greatly over the past decade, but it is still in its beginning stages. The role played by many factors and domains in condensate assembly and maturation is unclear. In the QCS, a few well-known proteins such as chaperones are also key components in regulating condensates. Further elucidation of the components of the QCS offers promise since it will reveal how cells cooperate with condensates and also uncover the link between out of control condensates and diseases. Numerous studies on aging-related diseases have indicated that condensates can promote neurodegenerative diseases and cancers by forming amyloids, affecting genetic and epigenetic regulation, losing the ability to repair DNA, and disturbing signaling in cells. Phase separation and condensates have given us new insights into aging-related diseases and unveiled new molecular mechanisms of those diseases. This could lead to the development of new diagnostic methods and therapies. Moreover, condensates and related factors could be new targets for drug development. This offers fresh hope for the treatment of intractable diseases such as cancers and neurodegenerative diseases.

Funding: None.

Conflict of Interest: The author has no conflicts of interest to disclose.

References

1. Alberti S, Gladfelter A, Mittag T. Considerations and challenges in studying liquid-liquid phase separation and

biomolecular condensates. Cell. 2019; 176:419-434.
2. Keizer VIP, Grosse-Holz S, Woringer M, Zambon L, Aizel K, Bongaerts M, Delille F, Kolar-Znika L, Scolari VF, Hoffmann S, Banigan EJ, Mirny LA, Dahan M, Fachinetti D, Coulon A. Live-cell micromanipulation of a genomic locus reveals interphase chromatin mechanics. Science. 2022; 377:489-495.
3. Shin Y, Chang YC, Lee DSW, Berry J, Sanders DW, Ronceray P, Wingreen NS, Haataja M, Brangwynne CP. Liquid nuclear condensates mechanically sense and restructure the genome. Cell. 2018; 175:1481-1491.
4. Li P, Banjade S, Cheng HC, Kim S, Chen B, Guo L, Llaguno M, Hollingsworth JV, King DS, Banani SF, Russo PS, Jiang QX, Nixon BT, Rosen MK. Phase transitions in the assembly of multivalent signalling proteins. Nature. 2012; 483:336-340.
5. Molliex A, Temirov J, Lee J, Coughlin M, Kanagaraj AP, Kim HJ, Mittag T, Taylor JP. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. Cell. 2015; 163:123-133.
6. Roden C, Gladfelter AS. RNA contributions to the form and function of biomolecular condensates. Nat Rev Mol Cell Biol. 2021; 22:183-195.
7. Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. Compositional control of phase-separated cellular bodies. Cell. 2016; 166:651-663.
8. Monahan Z, Ryan VH, Janke AM, Burke KA, Rhoads SN, Zerze GH, O'Meally R, Dignon GL, Conicella AE, Zheng W, Best RB, Cole RN, Mittal J, Shewmaker F, Fawzi NL. Phosphorylation of the FUS low-complexity domain disrupts phase separation, aggregation, and toxicity. EMBO J. 2017; 36:2951-2967.
9. Hofweber M, Hutten S, Bourgeois B, Spreitzer E, Niedner-Boblenz A, Schifferer M, Ruepp MD, Simons M, Niessing D, Madl T, Dormann D. Phase separation of FUS is suppressed by its nuclear import receptor and arginine methylation. Cell. 2018; 173:706-719.
10. Alberti S, Hyman AA. Are aberrant phase transitions a driver of cellular aging? Bioessays. 2016; 38:959-968.
11. Serio TR, Cashikar AG, Kowal AS, Sawicki GJ, Moslehi JJ, Serpell L, Arnsdorf MF, Lindquist SL. Nucleated conformational conversion and the replication of conformational information by a prion determinant. Science. 2000; 289:1317-1321.
12. Alberti S, Dormann D. Liquid-liquid phase separation in disease. Annu Rev Genet. 2019; 53:171-194.
13. Marrone L, Drexler HCA, Wang J, *et al*. FUS pathology in ALS is linked to alterations in multiple ALS-associated proteins and rescued by drugs stimulating autophagy. Acta Neuropathol. 2019; 138:67-84.
14. White MR, Mitrea DM, Zhang P, Stanley CB, Cassidy DE, Nourse A, Phillips AH, Tolbert M, Taylor JP, Kriwacki RW. *C9orf72* poly(PR) dipeptide repeats disturb biomolecular phase separation and disrupt nucleolar function. Mol Cell. 2019; 74:713-728.
15. Mateju D, Franzmann TM, Patel A, Kopach A, Boczek EE, Maharana S, Lee HO, Carra S, Hyman AA, Alberti S. An aberrant phase transition of stress granules triggered by misfolded protein and prevented by chaperone function. EMBO J. 2017; 36:1669-1687.
16. Turakhiya A, Meyer SR, Marincola G, Böhm S, Vanselow JT, Schlosser A, Hofmann K, Buchberger A. ZFAND1 recruits p97 and the 26S proteasome to promote the clearance of arsenite-induced stress granules. Mol Cell. 2018; 70:906-919.
17. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature. 2006; 441:885-889.
18. Stegeman R, Weake VM. Transcriptional signatures of aging. J Mol Biol. 2017; 429:2427-2437.
19. Mediani L, Guillén-Boixet J, Vinet J, Franzmann TM, Bigi I, Mateju D, Carrà AD, Morelli FF, Tiago T, Poser I, Alberti S, Carra S. Defective ribosomal products challenge nuclear function by impairing nuclear condensate dynamics and immobilizing ubiquitin. EMBO J. 2019; 38:e101341.
20. Min J, Wright WE, Shay JW. Clustered telomeres in phase-separated nuclear condensates engage mitotic DNA synthesis through BLM and RAD52. Genes Dev. 2019; 33:814-827.
21. Su X, Ditlev JA, Hui E, Xing W, Banjade S, Okrut J, King DS, Taunton J, Rosen MK, Vale RD. Phase separation of signaling molecules promotes T cell receptor signal transduction. Science. 2016; 352:595-599.
22. King OD, Gitler AD, Shorter J. The tip of the iceberg: RNA-binding proteins with prion-like domains in neurodegenerative disease. Brain Res. 2012; 1462:61-80.
23. Neumann M, Sampathu DM, Kwong LK, *et al*. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science. 2006; 314:130-133.
24. Cairns NJ, Bigio EH, Mackenzie IRA, *et al*. Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: Consensus of the Consortium for Frontotemporal Lobar Degeneration. Acta Neuropathol. 2007; 114:5-22.
25. Brettschneider J, Arai K, Tredici KD, *et al*. TDP-43 pathology and neuronal loss in amyotrophic lateral sclerosis spinal cord. Acta Neuropathol. 2014; 128:423-437.
26. Hughes MP, Sawaya MR, Boyer DR, Goldschmidt L, Rodriguez JA, Cascio D, Chong L, Gonen T, Eisenberg DS. Atomic structures of low-complexity protein segments reveal kinked β sheets that assemble networks. Science. 2018; 359:698-701.
27. Kastenhuber ER, Lowe SW. Putting p53 in context. Cell. 2017; 170:1062-1078.
28. Cox AD, Fesik SW, Kimmelman AC, Luo J, Der CJ. Drugging the undruggable RAS: Mission possible? Nat Rev Drug Discov. 2014; 13:828-851.
29. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: New and unexpected biological functions. Nat Rev Cancer. 2014; 14:736-746.

Received September 20, 2022; Revised October 17, 2022; Accepted October 21, 2022.

**Address correspondence to:*
Jufeng Xia (Shunichi Arai), Arai Japan Medical Institute, 14-21 Nihonbashi-ōdenmachō, Chuo-ku, Tokyo 103-0011, Japan.
E-mail: xia-tky@umin.ac.jp

Released online in J-STAGE as advance publication October 25, 2022.

Correspondence

DOI: 10.5582/bst.2022.01394

Notch signaling pathway plays a critical role in chemotherapeutic drug-induced vestibular injury

Wen Jiang[1], Jie Yu[2], Maorong Cao[3], Bin Xiao[1], Feifan Li[3], Chengfang Chen[4], Huiming Yang[4], Ziwen Wang[3], Fenye Liu[5,*], Shudong Yu[4,*]

[1] The First Faculty of Clinical Medicine, Shandong University of Traditional Chinese Medicine, Ji'nan, Shandong, China;
[2] Department of Otorhinolaryngology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China;
[3] The Clinical Medical College, Shandong First Medical University (Shandong Academy of Medicine), Ji'nan, Shandong, China;
[4] Department of Otolaryngology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Ji'nan, Shandong, China;
[5] Department of Traditional Chinese Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Ji'nan, Shandong, China.

SUMMARY The vestibule of the inner ear is susceptible to certain chemotherapeutic agents in clinical practice. Therefore, it is of great significance to discover molecular pathways and targets that can protect the vestibule from chemotherapeutic drugs. The Notch signaling pathway is closely related to hair cell regeneration in the inner ear. However, the role of Notch signaling in chemotherapeutic drug-induced vestibular injury still remains unclear. The aim of this study was first to evaluate the role of Notch signaling in chemotherapy-induced vestibular injury. Cisplatin-induced vestibular injury of mice was evaluated by the swimming test. Changes of vestibular hair cells and the expression levels of Notch1, Jagged1, and Hes1 in Notch signaling were observed by immunofluorescence. The results showed that Notch signaling was found activated in cisplatin-induced injured vestibular cells, while, DAPT (Notch signaling inhibitor) could reversed this effect. In conclusion, the Notch signaling pathway may play a critical role in chemotherapeutic drug-induced vestibular injury and, therefore, serves as a promising therapeutic target for vestibular injury.

Keywords Vestibular injury, chemotherapeutic drug, Notch signaling pathway

Vestibular injury can be caused by a variety of factors, including ototoxic drugs, excessive noise, and aging (*1*). Indeed, ototoxicity is a major toxic side effect of several beneficial pharmaceutical drugs, including chemotherapy agents such as cisplatin. Cisplatin-induced ototoxicity has a mean rate of 62% (*2*). Chemotherapeutic drugs can cause irreversible damage to the vestibule of the inner ear, leading to oscillopsia, blurry vision, distorted perception of self-orientation, imbalance, gait ataxia, and abnormal posture (*3*). In contrast to the non-regenerative cochlear hair cells of many mammals, vestibular hair cells exhibit spontaneous regeneration. However, the regeneration of vestibular hair cells is very limited, and vestibular function cannot be restored by their spontaneous regeneration (*4*). Therefore, it is very important to protect the vestibular hair cells from damage.

Hair cell production in the adult utricle is regulated by the Notch signaling pathway (*5*). In mammals, the Notch signaling pathway consists of four receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Jagged1, Jagged2, DLL1, DLL3, and DLL4). Ligands attach to receptors, and the activated form of Notch protein is cleaved by γ-secretase and released into the cytoplasm (*6*). Subsequently, it may activate the downstream target gene Hes1 (*7*). DAPT, a γ-secretase inhibitor, can block Notch signaling. Therefore, DAPT has been used as a tool drug to inhibit the Notch signaling pathway in a large number of studies. Involvement of the Notch signaling pathway in development of the inner ear has garnered increasing attention in recent years. Notch signaling induces hair cell formation through lateral inhibition and is involved in hair cell proliferation and apoptosis (*8*). Moreover, the activation of Notch signaling has been linked to cochlea hair cells loss (*9*). However, whether the Notch signaling pathway plays a role in chemotherapeutic drug-induced vestibular injury has not been investigated yet.

Therefore, we investigated the role of the Notch signaling in chemotherapeutic drug-induced vestibular injury. To our knowledge, this is the first time this

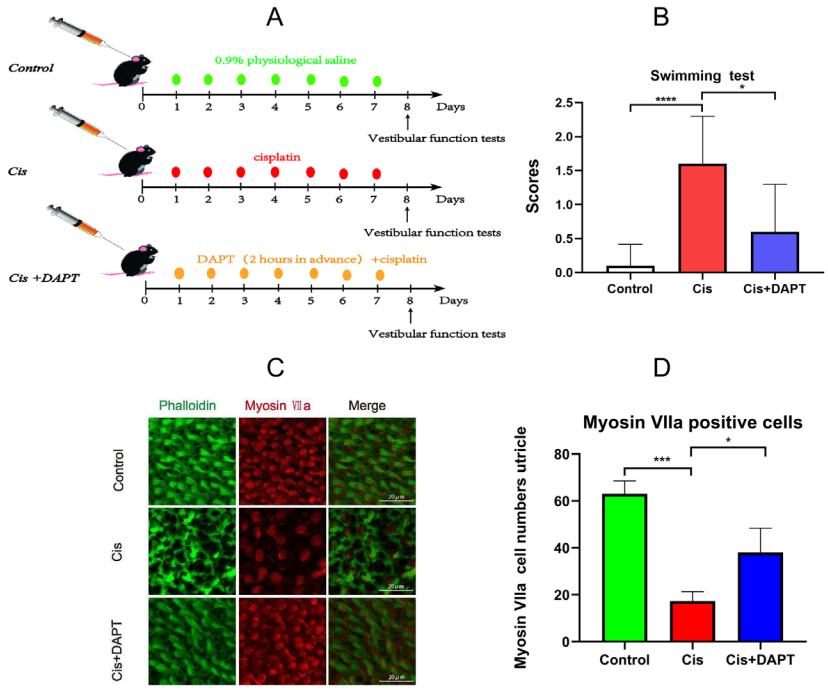

Figure 1. Inhibition of Notch signaling protected against cisplatin-induced vestibular injury. (A) Three groups of C57BL/6 mice received intraperitoneal medication injections as illustrated. **(B)** The scoring criteria swimming test was as follows. Zero points were scored if the mouse could keep its head out of the water and swim straight to the side of the container. One point was scored if the mouse could keep its head out of the water and maintain a slightly circular swimming posture. Two points were scored if the mouse could not keep its head above water and swim slightly in a circular manner. Three points were scored if the mouse could not keep its head above water and swim severely in a circular manner. **(C)** Cuticular plate and hair cell stereociliary bundles are marked with phalloidin (green), while hair cells are labeled with myosin VIIa (red). **(D)** Quantitative analysis of myosin VIIa-positive cell numbers in the utricles.

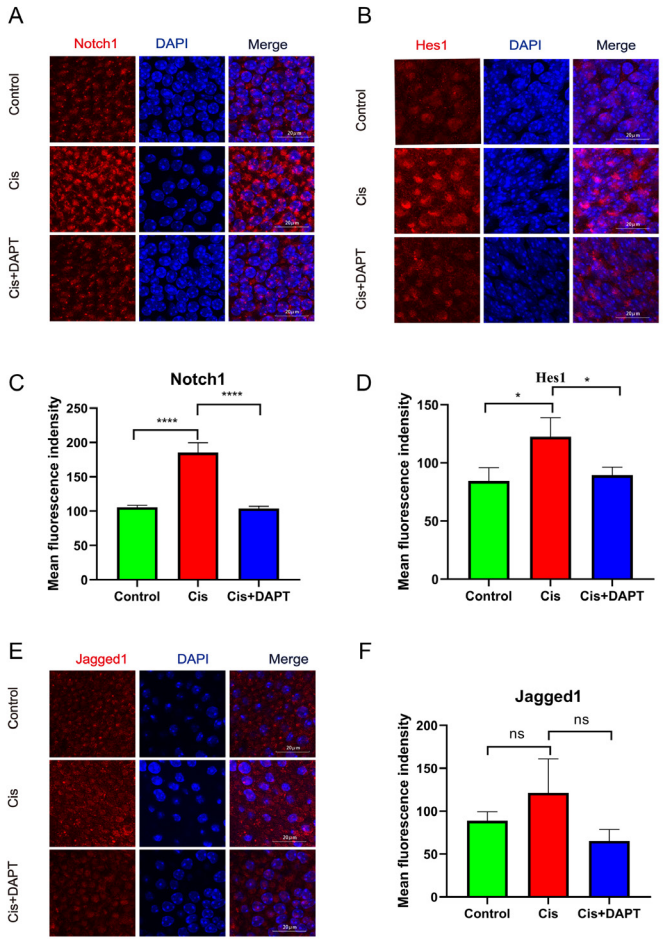

Figure 2. Effects of Notch signaling inhibition on cisplatin-induced vestibular injury in mice. (A) and **(B)** Immunofluorescence with Notch1 and Hes1 (red) antibodies and DAPI (blue) in utricles from control, cisplatin, and cisplatin + DAPT groups. **(C)** and **(D)** Quantitative analysis of Notch1 and Hes1 fluorescence intensity in the utricles. **(E)** Immunofluorescence with Jagged1 (red) antibodies and DAPI (blue) in utricles from control, cisplatin, and cisplatin + DAPT groups. **(F)** Quantitative analysis of Jagged1 fluorescence intensity in the utricles. Control: 0.9% physiological saline, Cis: cisplatin, Cis+DAPT: cisplatin + DAPT. Scale bars = 20 μm. Data were showed as mean ± SD. (Statistical analysis was performed using one-way analysis of variance (ANOVA), ns means no significant difference, $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}P < 0.0001$).

has been investigated, the results have important implications for the prophylaxis and treatment of vestibular injury.

Inhibition of Notch signaling protected against cisplatin-induced vestibular injury

In this study, thirty C57BL/6 mice (aged 7 weeks) were randomly divided into control, cisplatin, and cisplatin + DAPT groups, with 10 mice in each group. Mice in the control group were intraperitoneally injected with 0.9% physiological saline (0.6 mL/100g). Mice in the cisplatin group were intraperitoneally injected with cisplatin (3 mg/kg; Sigma, St. Louis, MO, USA). Mice in the cisplatin + DAPT group were first intraperitoneally injected with DAPT (10 mg/kg; Sigma), and 2 h later were injected intraperitoneally with cisplatin (3 mg/kg). All the above operations were performed continuously for 7 days (Figure 1A).

Vestibular function was assessed by observing the swimming posture of mice. It was showed that the vestibular injury was less severe in the cisplatin + DAPT group than in the cisplatin group. Mice in the cisplatin group displayed a severe circular swimming posture and were unable to keep their heads above water. In addition, mice in the cisplatin group showed abnormal vestibular function compared with mice in the control group. Mice in the cisplatin + DAPT group had a steady swimming posture and kept their heads above water, indicating that their vestibular function was not significantly impaired. Indeed, swimming test scores were significantly improved in the cisplatin + DAPT group compared with the cisplatin group (Figure 1B).

Changes of vestibular hair cells were observed by immunofluorescence. In the cisplatin group, the number of hair bundles and hair cells in the utricles was significantly lower, and were disorganised and spread out. In comparison, hair bundles and hair cells were effectively preserved, morphologically normal, and tightly arranged in the cisplatin + DAPT group (Figure 1C). The results of quantitative hair cell analysis were consistent with the swimming test (Figure 1D). It is therefore likely that inhibition of Notch signaling protected against cisplatin-induced vestibular injury.

Notch-related expression in the mouse utricle after Notch signaling pathway inhibition

Immunofluorescence were used to observe protein expression levels of Notch signaling pathway molecules in the utricles. As shown in Figures 2A and 2B, Notch1 and Hes1 protein levels were elevated in the cisplatin group compared with the control group. In addition, the cisplatin + DAPT group showed decreased protein levels of Notch1 and Hes1 in the utricles compared with the cisplatin group (Figures 2C and 2D). However, there was no significant difference in Jagged1 protein expression levels between cisplatin and cisplatin + DAPT groups (Figures 2E and 2F).

The relationship between Jagged1, Notch1, and Hes1 expression remains unclear in the inner ear. After cochlear hair cell injury, Jagged1 expression levels in cochlea hair cells did not change (*10*). Other studies have shown that Jagged1 is mainly expressed in support cells (*11*). However, Notch1 can interact with Jagged2, DLL1, DLL3, and DLL4 to activate the Notch signaling pathway. In addition, other signaling pathways (such as Fgf, Bmp, or Wnt) can regulate the Notch signaling pathway (*12,13*). In our study, we found that the Notch signaling pathway was activated after vestibular injury. Ligands or signaling pathways that interact with Notch1 in the vestibular system require further study.

In conclusion, our study revealed that the Notch signaling pathway may play a critical role in chemotherapeutic drug-induced vestibular injury. These results lay the foundation for preventing the side effects of chemotherapy. It should be pointed out that this study is a preliminary exploration of the role of the Notch signaling pathway in the vestibule. Accordingly, future research is still needed to explore the molecular mechanism by which Notch signaling reduces chemotherapeutic drug-induced vestibular injury. In addition, DAPT is a γ-secretase blocker, and it is necessary to explore whether other Notch signaling blockers or approaches have the above-mentioned effects.

Funding: This study was supported by the Shandong Foundation Project of Natural Science (grant number ZR2020MH376).

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Yilmaz N, Ila K. Effect of vibration on the vestibular system in noisy and noise-free environments in heavy industry. Acta Otolaryngol. 2019; 139:1014-1018.
2. Marshak T, Steiner M, Kaminer M, Levy L, Shupak A. Prevention of Cisplatin-Induced Hearing Loss by Intratympanic Dexamethasone: A Randomized Controlled Study. Otolaryngol Head Neck Surg. 2014; 150:983-990.
3. Lanvers-Kaminsky C, Zehnhoff-Dinnesen AA, Parfitt R, Ciarimboli G. Drug-induced ototoxicity: Mechanisms, Pharmacogenetics, and protective strategies. Clin Pharmacol Ther. 2017; 101:491-500.
4. Kawamoto K, Izumikawa M, Beyer LA, Atkin GM, Raphael Y. Spontaneous hair cell regeneration in the mouse utricle following gentamicin ototoxicity. Hear Res. 2009; 247:17-26.
5. Bucks SA, Cox BC, Vlosich BA, Manning JP, Nguyen TB, Stone JS. Supporting cells remove and replace sensory receptor hair cells in a balance organ of adult mice. Elife. 2017; 6.
6. Siebel C, Lendahl U. Notch Signaling in Development,

Tissue Homeostasis, and Disease. Physiol Rev. 2017; 97:1235-1294.
7. Luo X, Zhang L, Han GD, Lu P, Zhang Y. MDM2 inhibition improves cisplatin-induced renal injury in mice *via* inactivation of Notch/hes1 signaling pathway. Hum Exp Toxicol. 2021; 40:369-379.
8. Roccio M, Edge ASB. Inner ear organoids: new tools to understand neurosensory cell development, degeneration and regeneration. Development. 2019; 146.
9. Li Y, Wu Y, Liu H, Lou X. Influence of 24-diamino-5-phenylthiazole on neomycin ototoxicity in cultured organ of Corti explants. Neuroreport. 2018; 29:1011-1016.
10. Oesterle EC, Campbell S, Taylor RR, Forge A, Hume CR. Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear. J Assoc Res Otolaryngol. 2008; 9:65-89.
11. Morrison A, Hodgetts C, Gossler A, Hrabé de Angelis M, Lewis J. Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. Mechanisms of development. 1999; 84:169-172.
12. Bai H, Yang S, Xi C, Wang X, Xu J, Weng M, Zhao R, Jiang L, Gao X, Bing J, Zhang M, Zhang X, Han Z, Zeng S. Signaling pathways (Notch, Wnt, Bmp and Fgf) have additive effects on hair cell regeneration in the chick basilar papilla after streptomycin injury *in vitro*: Additive effects of signaling pathways on hair cell regeneration. Hear Res. 2021; 401:108161.
13. Lush ME, Diaz DC, Koenecke N, Baek S, Boldt H, St Peter MK, Gaitan-Escudero T, Romero-Carvajal A, Busch-Nentwich EM, Perera AG, Hall KE, Peak A, Haug JS, Piotrowski T. scRNA-Seq reveals distinct stem cell populations that drive hair cell regeneration after loss of Fgf and Notch signaling. Elife. 2019; 8.

Received August 2, 2022; Revised September 26, 2022; Accepted October 4, 2022.

**Address correspondence to:*
Fenye Liu, Department of Traditional Chinese Medicine, Shandong Provincial Hospital Affiliated to Shandong First Medical University, 324 Jingwuweiqi Road, Ji'nan, Shandong 250021, China.
E-mail: drzhang1978@163.com

Shudong Yu, Department of Otolaryngology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, 324 Jingwuweiqi Road, Ji'nan, Shandong 250021, China.
E-mail: yushudong@126.com

Released online in J-STAGE as advance publication October 6, 2022.

Correspondence

DOI: 10.5582/bst.2022.01331

A brief summary regarding the roles of interleukin-11 in neurological diseases

Marat Airapetov[1,2,*], Sergei Eresko[1,3], Polina Ignatova[1], Andrei Lebedev[1], Evgenii Bychkov[1], Petr Shabanov[1,4]

[1] Department of Neuropharmacology, Institute of Experimental Medicine, St. Petersburg, Russia;
[2] Department of Pharmacology, St. Petersburg State Pediatric Medical University, St. Petersburg, Russia;
[3] Research and Education Center for Molecular and Cellular Technologies, St. Petersburg State Chemical Pharmaceutical University, St. Petersburg, Russia;
[4] Department of Pharmacology, Kirov Military Medical Academy, St. Petersburg, Russia.

SUMMARY Interleukin 11 (IL-11) was discovered in 1990 in fibrocyte-like stromal cells of the bone marrow, but there has recently been an increased interest in the cytokine. Understanding the physiological roles of cytokines will allow their use as pharmacological agents in clinical practice. Studies have indicated that IL-11 affects the mechanism for the development of a number of pathologies of the nervous system. IL-11 plays a significant role in the central nervous system. The local expression of this cytokine by nerve cells has been observed. The current work summarizes the results of studies which found that the cytokine affects the mechanism of development of pathologies of the central nervous system. In the near future, this cytokine may be used clinically to fix the mechanisms that are involved in the development of pathological conditions of the nervous system.

Keywords IL-11, IL-6 family, gp130, brain, neuroinflammation, neurodegeneration

In 1990, a new cytokine named interleukin 11 (IL-11) was discovered in fibrocyte-like stromal cells of the bone marrow (*1*). More than 30 years have passed since the discovery of IL-11. Initially, a study indicated that IL-11 is important to the process of hematopoiesis, and especially for the maturation of megakaryocytes (*1*). A study by Mehler *et al*. posited that there are similarities in the regulation of the processes of hemalymphopoiesis and neurogenesis by means of cytokines (*2*). The study's results confirmed that there are indeed similarities between the mechanisms that regulate these processes.

Further studies have shown that IL-11 and its components of the receptor complex are widely localized among various parts of the central nervous system (CNS), which may indicate the involvement of IL-11 in cascades of reactions that regulate various physiological processes in the CNS (*3-11*). IL-11 is expressed in hippocampal neurons, stimulating the proliferation of progenitor cells, so the cytokine is involved in the process of neurogenesis (*2*). These initial studies were probably the reason for further examination of this cytokine's involvement.

The current work has summarized information about the roles that IL-11 can play in the development of pathological conditions of the nervous system. The results of such studies can help to discover new ways to treat these pathological conditions where this cytokine and signaling cascades of IL-11 are involved.

1. IL-11 and its intracellular signaling cascade of reactions

IL-11 is a protein with an approximate molecular weight of 19 kDa. The IL-11 precursor (pre-IL-11) consists of 199 amino acid residues. The pre-IL-11 gene, consisting of 5 exons and 4 introns, is located on the 19th human chromosome (*12-15*). There are data on expression of the IL-11 protein in many tissues in the body (*16*).

IL-11 and components of its receptor complex are expressed in the brain (*3-7,9-11*). The expression of IL-11 was noted in the olfactory bulb, amygdala, basal ganglia, thalamus, midbrain, bridge, medulla oblongata, hippocampus, cerebral cortex, and cerebellum (*8-11*). Localization of the key receptor component of the gp130 protein was also found in the brain of rats (immunoreactivity was observed in both glial and neuronal cells) (*9*). Electron microscopy revealed that both types of gp130 immunoreactivity are mainly associated with the cytoplasmic membrane and are not precisely localized in synaptic sites (*9*). The results of RNA-Seq analysis also revealed the presence of IL-

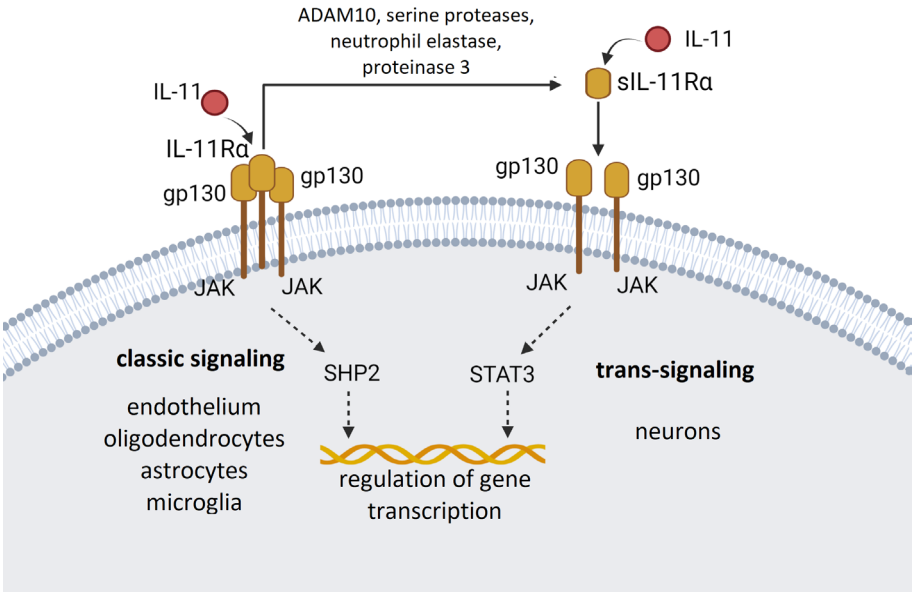

Figure 1. The interaction of IL-11 with the receptor complex serves as a signal to activate the JAK/SHP2–dependent pathway of intracellular signal transmission.

11, IL-11Ra, and gp130 mRNA among various brain structures. The level of IL-11 mRNA is insignificant in all types of cells of the nervous system, but IL-11Ra and gp130 mRNA is found in the greatest amount in astrocytes, microglia, and the endothelium (*10-11*).

Currently, there is no high-resolution structural data on the IL-11 signaling complex, although there is evidence that IL-11Ra and IL-6Ra are structurally similar (*17*). The interaction of IL-11 with the receptor complex serves as a signal to activate the JAK/SHP2 – dependent pathway of intracellular signal transmission (Figure 1). Another signaling pathway begins with phosphorylation of the STAT1 and STAT3 proteins (*12-15*). Unlike many cells that can serve as a source of cytokines such as IL-11, the expression of receptor subunits is more limited, which determines the more specific effect of cytokines on certain populations of cells in the body that can be directly activated by the IL-11 cytokine (*18*). In addition, part of the IL-11Ra receptor complex may not only be localized on the cell membrane but may exist in soluble form (*18*). Metalloprotease ADAM10, as well as serine proteases, neutrophil elastase, and proteinase 3 can cleave the ectodomain from IL-11Ra, and the ectodomain formed during cleavage serves as a soluble form of the receptor - sIL-11R (soluble IL-11R).

2. IL-11 affects the mechanism of development of neurological diseases

2.1. Alzheimer's disease

The ability of IL-11 to regulate the mechanism of pathologies in the nervous system was indicated in a neuroblastoma cell culture model of Alzheimer's disease (AD) (*16*). AD is characterized by the deposition of β-amyloid in the brain, and the increased production of β-amyloid peptide is considered to be one of the early events in the pathogenesis of AD. β-amyloid was found to activate L-phosphoserine phosphatase in neuronal cells (B104 rat neuroblastoma cells); that activation was inhibited by IL-11 while IL-11 inhibited neurotoxicity. The aforementioned study suggested that L-phosphoserine phosphatase may play a role in changing cellular metabolism in AD by increasing neurotoxicity and that IL-11 through its receptor system can act as a neuroprotector.

2.2. Multiple sclerosis

In 2006, researchers noted an increase in IL-11 in astrocytes and expression of IL-11R by oligodendrocytes in multiple sclerosis (*19*). Studies of astrocyte and oligodendrocyte cultures concluded that the IL-11/IL-11R pathway can play an important role in protecting oligodendrocytes in multiple sclerosis. When recombinant IL-11 was added to a culture of human fetal spinal cord cells, an increase in the number of oligodendrocytes was noted, an increase in the process of oligodendroglia branching was noted, apoptotic cell death decreased and IL-11 potentiated the formation of myelin. A 2009 study of knockout mice without the IL-11Ra gene (IL-11Ra$^{-/-}$ and IL-11Ra$^{+/-}$) revealed a pronounced neuroinflammatory process in spinal cord tissues, characterized by infiltration by macrophages, followed by demyelination and a decrease in the number of nerve cells (*5*). That study evaluated the effect of exogenous IL-11 on a culture of progenitor

oligodendrocytes of rats. The use of IL-11 increased cell survival by reducing the number of cells dying due to apoptosis and potentiating the process of cell division. In addition, exogenous IL-11 was able to reduce the activation of CD4 lymphocytes by inhibiting $CD11c^{++}$ cells, and IL-11 was able to regulate the production of effector cytokines by acting on $CD11c^{++}$ cells. A study of a cuprizone model of multiple sclerosis in mice found that overexpression of IL-11 caused by local delivery of a viral vector with the IL-11 gene to the lesion site reduced the degree of demyelination with simultaneous acceleration of the remyelination process (*6*). Microglia are known to participate in myelin phagocytosis, so that study's results may indicate that IL-11 is able to make adjustments to this process, but the mechanism by which microglia are involved in the effects caused by IL-11 remains to be determined. An in vitro experiment on the BV2 microglia cell line indicated that the addition of recombinant IL-11 in a dose-dependent manner reduced the degree of myelin phagocytosis (*6*). In addition, the experiment indicated that the use of IL-11 led to an increase in the thickness of the myelin layer; this indicates the ability of IL-11 to enhance the remyelination process, but the mechanism for this has yet to be studied. Studies have described the ability of IL-11 to inhibit the synthesis of TNFα, IL-1β, IL-12, and IL-6 and the production of NO by activated macrophages (*20,21*), but those studies did not report the level of pro-inflammatory mediators.

2.3. Autoimmune encephalomyelitis

In a model of autoimmune encephalomyelitis, mice were injected with 25 or 50 mcg/kg/day of recombinant IL-11 after the appearance of signs of encephalomyelitis (*5*). A dose of 50 mcg/kg/day led to a reduction in the severity of the disease: a decrease in the degree of demyelination, a decrease in the loss of oligodendrocytes, a decrease in inflammation, and a reduction in the number of CD3 lymphocytes. The use of lower doses was not reported to have a significant effect.

2.4. Ischemic stroke

In one study, the medial cerebral artery was occluded in mice in order to simulate ischemic brain damage (*7*). Results revealed a decrease in the expression of IL-11 protein and mRNA in the first 24 hours of ischemia. The administration of recombinant IL-11 showed the anti-inflammatory effects of the cytokine, which were characterized by a decrease in the markers of activation of astrocytes and microglia and a decrease in the mRNA of pro-inflammatory cytokines (IL1ß, IL-6, and TNFβ), but the level of mRNA of the anti-inflammatory cytokine TGFß1 increased, there was an increase in the level of superoxide dismutase, and a decrease in the level of malondialdehyde. This indicates a decrease in the level of oxidative stress in brain tissues.

2.5. Other research

Another finding warrants attention. A recent study found that cytokines of the IL-6 family can mediate an increase in the chemoresistance of medulloblastoma tumor cells. Conditioned in vitro with IL-6, OSM, LIF, or IL-11 cytokines, cultured medulloblastoma cells exhibit increased activity of JAK1/STAT3 signaling, while chemoresistance to a number of drugs begins to be noted (*22*). In addition, the use of gp130 inhibitors or JAK-canis inhibitors effectively overcame the resistance of medulloblastoma to vincristine in gp130-expressing cells. These findings may indicate the existence of restrictions on the use of recombinant IL-11 in a number of individual cases, but in vivo studies need to be conducted to clarify the observed phenomenon and to examine the indirect effect of IL-6 cytokines on other types of tumor cell lines.

Compliance with ethical standards: This article does not contain any research involving human or animal subjects.

Funding: The study was funded by the Institute of Experimental Medicine (state assignment: Pharmacological analysis of the action of neurotropic agents, the study of intracellular targets and the creation of targeted delivery systems, no. 0557-2019-0004) and Saint Petersburg State Pediatric Medical University.

Conflict of Interest: The authors declare that they have no conflicts of interest.

References

1. Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM, Leary AC, Sibley B, Clark SC, Williams DA, Yang YC. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell derived lymphopoietic and hematopoietic cytokine. Proc Natl Acad Sci USA. 1990; 87:7512-7516.
2. Mehler MF, Rozental R, Dougherty M, Spray DC, Kessler JA. Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Nature. 1993; 362:62-65.
3. Wilde MI, Faulds D. Oprelvekin: A review of its pharmacology and therapeutic potential in chemotherapy-induced thrombocytopenia. BioDrugs. 1998; 10:159-171.
4. Leng SX, Elias JA. Interleukin-11. Int J Biochem Cell Biol. 1997; 29:1059-1062.
5. Gurfein BT, Zhang Y, López CB. IL-11 regulates autoimmune demyelination. J Immunol. 2009; 183:4229-4240.
6. Maheshwari A, Janssens K, Bogie J, Van Den Haute C, Struys T, Lambrichts I, Baekelandt V, Stinissen P, Hendriks JJ, Slaets H, Hellings N. Local overexpression of interleukin-11 in the central nervous system limits demyelination and enhances remyelination. Mediators Inflamm. 2013; 5:1-11.

7. Zhang B, Zhang HX, Shi ST, Bai YL, Zhe X, Zhang SJ, Li YJ. Interleukin-11 treatment protected against cerebral ischemia/reperfusion injury. Biomed Pharmacother. 2019; 115:1-11.
8. Uhlén M. Proteomics. Tissue-based map of the human proteome. Science. 2015; 347:1260419.
9. Watanabe D, Yoshimura R, Khalil M, Yoshida K, Kishimoto T, Taga T, Kiyama H. Characteristic localization of gp130 (the signal-transducing receptor component used in common for IL-6/IL-11/CNTF/LIF/OSM) in the rat brain. Eur J Neurosci. 1996; 8:1630-1640.
10. Zhang Y, Chen K, Sloan SA, *et al*. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci. 2014; 34: 11929-11947.
11. Zhang Y, Sloan SA, Clarke LE, *et al*. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. Neuron. 2016; 89:37-53.
12. Gorshkova EA, Nedospasov SA, Shilov ES. Evolutionary plasticity of IL-6 family cytokines. Mol Biol (Mosk.) 2016; 50:1039-1048. (in Russian).
13. McKinley D, Wu Q, Yang-Feng T, Yang YC. Genomic sequence and chromosomal location of human interleukin-11 gene (IL11). Genomics. 1992; 13:814-819.
14. Yang YC, Yin T. Interleukin-11 and its receptor. Biofactors. 1992; 4:15-21.
15. Stephen FA, Thomas LM, Alejandro AS, Jinghui Z, Zheng Z, Webb M, David JL. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs, Nucleic Acids Res. 1997; 25:3389-3402.
16. Heese K, Nagai Y, Sawada T. Induction of rat L-phosphoserine phosphatase by amyloid-beta (1-42) is inhibited by interleukin-11. Neurosci Lett. 2000; 288:37-40.
17. Barton V, Hall M, Hudson KR, Heath JK. Interleukin-11 signals through the formation of a hexameric receptor complex. J Biol Chem. 2000; 275:36197-36203.
18. Lokau J, Agthe M, Flynn CM, Garbers C. Proteolytic control of Interleukin-11 and Interleukin-6 biology. Biochim Biophys Acta Mol Cell Res. 2017; 1864:2105-2117.
19. Zhang Y, Taveggia C, Melendez-Vasquez C, Einheber S, Raine CS, Salzer JL, Brosnan CF, John GR. Interleukin-11 potentiates oligodendrocyte survival and maturation, and myelin formation. J Neurosci. 2006; 26:12174-12185.
20. Trepicchio WL, Wang L, Bozza M, Dorner AJ. IL-11 regulates macrophage effector function through the inhibition of nuclear factor-kappaB. J Immunol. 1997; 159:5661-5670.
21. Leng SX, Elias JA. Interleukin-11 inhibits macrophage interleukin-12 production. J Immunol. 1997; 159:2161-2168.
22. Sreenivasan L, Li LV, Leclair P, Lim CJ. Targeting the gp130/STAT3 axis attenuates tumor microenvironment mediated chemoresistance in Group 3 medulloblastoma cells. Cells. 2022; 11:381.

Received July 25, 2022; Revised October 5, 2022; Accepted October 16, 2022.

**Address correspondence to:*
Marat Airapetov, Department of Neuropharmacology, Institute of Experimental Medicine, 12 Academician Pavlova Street, St. Petersburg, 197376, Russia.
E-mail: interleukin1b@gmail.com

Released online in J-STAGE as advance publication October 19, 2022.

Comment

DOI: 10.5582/bst.2022.01390

An average of nearly 200,000 new infections per day over a six-week period: What is the impact of such a severe COVID-19 pandemic on the healthcare system in Japan?

Kenji Karako[1], Peipei Song[2,*], Yu Chen[1], Takashi Karako[3]

[1] Department of Human and Engineered Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, Chiba, Japan;
[2] Center for Clinical Sciences, National Center for Global Health and Medicine, Tokyo, Japan;
[3] International Health Care Center, National Center for Global Health and Medicine, Tokyo, Japan.

SUMMARY During a six-week period from July 20 to August 31, 2022, Japan experienced its highest level of COVID-19 infection ever, with an average of nearly 200,000 new infections per day nationwide. Cases requiring inpatient care peaked at 1,993,062. Twenty-seven prefectures (out of 47 prefectures) had an average hospital bed occupancy of 50% or higher, and bed occupancy in Kanagawa in particular reached 98% in mid-August. In Tokyo, bed occupancy by patients with severe COVID-19 reached 57% and peaked at 64% in mid-August. Although the number of new infections per day has decreased since September, hospital bed occupancy, the number of severe cases, and deaths remain high nationwide. Efforts including vaccination campaigns, domestic surveillance, and routine infection control measures based on the varied knowledge that the Japanese public already has should be thoroughly implemented to reduce the number of the infected in order to avoid an increase the number of serious cases and deaths.

Keywords COVID-19, Omicron variant, new infections per day, bed occupancy

Globally, the number of infections with the Omicron variant of SARS-CoV-2 has increased unlike ever before due to its higher transmissibility (*1,2*). Japan in particular has experienced its highest level of COVID-19 infections ever, with an average of nearly 200,000 new infections per day nationwide during a six-week period from July 20 to August 31, 2022 (Figure 1A); a cumulative total of 18,917,782 infected and 39,872 deaths were reported as of August 31, 2022 (*3*).

On July 20, 2022, the number of new infections per day in Japan exceeded 200,000 for the first time, the peak to date was reached on August 2, 2022 with 267,470 new infections (accounting for over 30% of new cases globally), and the number was 169,771 on August 31, 2022 (*3,4*). In addition, the infection status may have been underestimated in light of severe pressure on the testing system, changing healthcare-seeking behavior, and a delay in the conducting of tests and examinations and the publication of reports during the summer vacation and Obon holidays (*5*).

With an average of nearly 200,000 new infections per day over a six-week period, the number of cases requiring inpatient care, severe cases, and deaths have tended to increase (Figure 1B), causing an unprecedented shock to the healthcare system. The number of cases requiring inpatient care peaked at 1,993,062 on August 11, and 637 of those cases were severe (*3*).

Hospital bed occupancy has increased nationwide. Over a six-week period, 27 prefectures (out of 47 prefectures) had an average hospital bed occupancy of 50% or higher. Bed occupancy in Kanagawa in particular reached 98% in mid-August (Figure 2A). In addition, bed occupancy by patients with severe COVID-19 (defined based on the characteristics of the Omicron variant) has also increased. The situation was most critical in Tokyo, where average bed occupancy reached 57%, peaking at 64% in mid-August (Figure 2B).

There are unique characteristics with respect to the progression of the COVID-19 pandemic in Japan and the response of the Japanese healthcare system. This is particularly evident in declaring states of emergency during peaks with voluntary self-isolation, the low mortality rate, the sharp decline in the number of cases after the impact of the Tokyo 2020 Olympic and Paralympic Games on the healthcare system (*6*), and the unprecedented rise in the number of infections due to the current spread of the Omicron variant, with an average of nearly 200,000 new infections per day nationwide

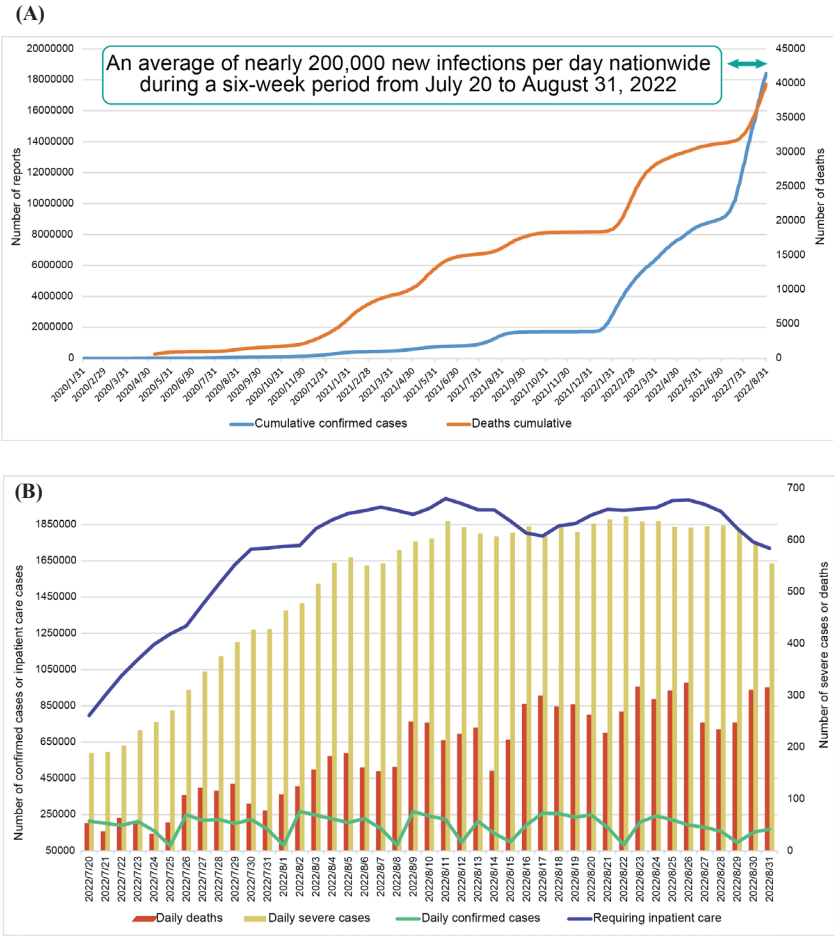

Figure 1. Number of COVID-19 cases reported in Japan. (A) Number of cases reported from 2020-2022; **(B)** Number of cases requiring inpatient care, severe cases, and deaths from July 20-August 31, 2022. Data source: *https://www.mhlw.go.jp/stf/covid-19/open-data.html* (Calculations are based on data made public by the Ministry of Health, Labor, and Welfare on September 6, 2022).

reported during a six-week period.

Japan's basic policy on COVID-19 is to curb the outbreak of infection, maintain the medical system, and focus on dealing with the severely ill (*7*). Based on this policy, Japan declared a state of emergency during peaks accompanied by a call for the public to change their behavior patterns to minimize the risk of being infected; , it allocated hospital beds and at-home care depending on the symptoms of the infected, and it preferentially supplied vaccines to the elderly. As a result of these combined efforts, the past six waves of the pandemic have been effectively contained nationwide, but the unprecedented increase in the number of infections due to the current spread of the Omicron variant has placed a significant burden on the healthcare system.

Although the number of new infections per day has decreased since September, with 112,175 newly confirmed cases reported on September 6, 2022 (*3*), hospital bed occupancy, the number of severe cases, and deaths remain high nationwide. In addition, the number of close contacts and patients receiving at-home care are still increasing in many regions. Coupled with increased instances of difficulty obtaining an emergency hospital admission and shortages of healthcare workers (*8*), the current pandemic continues to impose a significant burden in terms of both COVID-19 treatment and on the medical system in general.

In the face of the current COVID-19 pandemic, efforts including vaccination campaigns, domestic surveillance, and routine infection control measures based on the varied knowledge that the Japanese public already has should be thoroughly implemented to reduce the number of the infected in order to avoid an increase the number of serious cases and deaths. Further efforts such as use of telemedicine should be made to reinforce the medical system and reduce the burden on medical facilities and public health centers.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Takashita E, Yamayoshi S, Simon V, van Bakel H, Sordillo EM, Pekosz A, Fukushi S, Suzuki T, Maeda K, Halfmann P, Sakai-Tagawa Y, Ito M, Watanabe S, Imai M, Hasegawa H, Kawaoka Y. Efficacy of antibodies and antiviral drugs against Omicron BA.2.12.1, BA.4, and BA.5 subvariants. N Engl J Med. 2022; 387:468-470.
2. Tallei TE, Alhumaid S, AlMusa Z, Fatimawali, Kusumawaty D, Alynbiawi A, Alshukairi AN, Rabaan AA. Update on the omicron sub-variants BA.4 and BA.5. Rev Med Virol. 2022; e2391.
3. Ministry of Health, Labor, and Welfare. Changes in the number of new positive cases (by day). *https://covid19.*

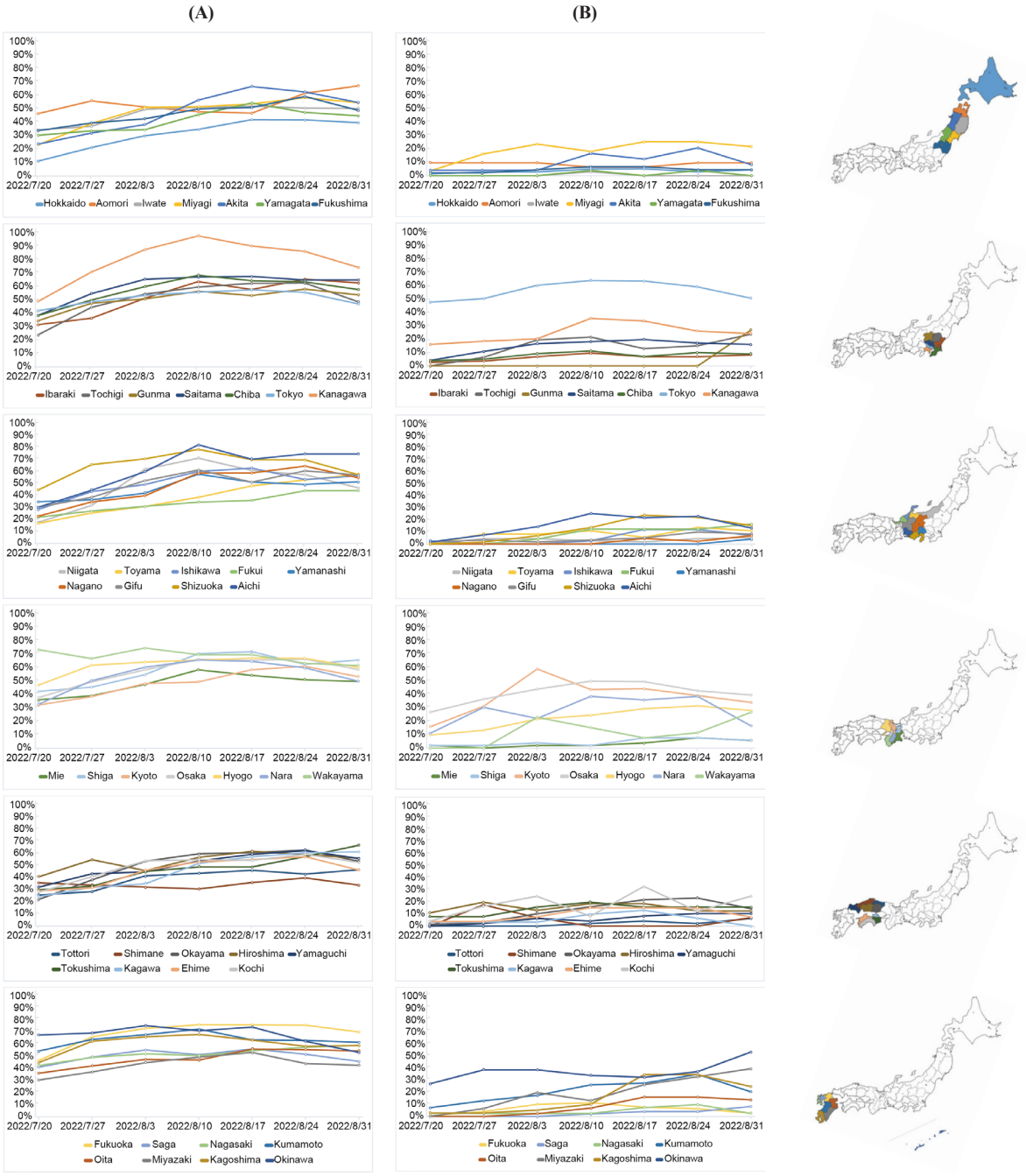

Figure 2. (A) Hospital bed occupancy and (B) bed occupancy by patients with severe COVID-19 in Japan from July 20-August 31, 2022.
Data source: *https://www.mhlw.go.jp/stf/seisakunitsuite/newpage_00023.html*

mhlw.go.jp/en (accessed September 6, 2022)
4. The World Health Organization. WHO Coronavirus (COVID-19) Dashboard (as of August 2, 2022). *https://covid19.who.int* (accessed September 2, 2022)
5. National Institute of Infectious Diseases. COVID-19 trends: Current situation (as of August 18, 2022. https://*www.niid.go.jp/niid/en/2019-ncov-e/11450-covid19-ab95th-en.html* (accessed September 2, 2022)
6. Song P, Karako T. The strategy behind Japan's response to COVID-19 from 2020-2021 and future challenges posed by the uncertainty of the Omicron variant in 2022. Biosci Trends. 2022; 15:350-352.
7. Inoue H. Japanese strategy to COVID-19: How does it work? Glob Health Med. 2020; 2:131-132.
8. National Institute of Infectious Diseases. COVID-19 trends: Current situation (as of August 31, 2022). *https://www.niid.go.jp/niid/ja/2019-ncov/11451-covid19-ab97th.html* (accessed September 6, 2022). (in Japanese)

Received September 2, 2022; Revised September 7, 2022; Accepted September 9, 2022.

*Address correspondence to:
Peipei Song, Center for Clinical Sciences National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.
E-mail: psong@it.ncgm.go.jp

Released online in J-STAGE as advance publication September 12, 2022.

Comment

DOI: 10.5582/bst.2022.01297

The role of influenza in the era of COVID-19: Can we forget it?

Ting Li[1,2,§], Tetsuya Asakawa[3,§], Hui Liu[4], Cordia Chu[2,*], Hongzhou Lu[1,*]

[1] Department of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, Shenzhen, China;
[2] Centre of Environment and Population Health, School of Medicine and Dentistry, Griffith University, Brisbane, Australia;
[3] Institute of Neurology, the Third People's Hospital of Shenzhen, Shenzhen, China;
[4] Medical Administration Department, Shenzhen Municipal Health Commission, Shenzhen, China.

SUMMARY COVID-19 has been a topic of interest since a pandemic struck in 2019. The morbidity of influenza tended to decrease due to the measures to prevent COVID-19. Indeed, influenza seems to be "ignored" in this era of COVID-19. However, influenza has not disappeared from the scene. Presented here are two examples of recent influenza epidemics in China and Australia. Possible interactions between COVID-19 and influenza are discussed. Measures against COVID-19 may reduce contact with influenza, subsequently reducing adaptive immunity against influenza in the general population. Influenza might not be center stage right now, but insufficient adaptive immunity in the population may potentially trigger a future influenza pandemic. Coinfection with COVID-19 and influenza might potentially be a thorny problem. Hence, influenza cannot be ignored. Governments around the world should take measures to prepare for a possible influenza pandemic in the future.

Keywords COVID-19, influenza, coinfection, pandemic

Influenza has been the greatest public health challenge. The terms "Spanish flu" or "the Great Influenza epidemic" were used to describe the 1918 influenza pandemic, a deadly global pandemic of the H1N1 influenza A virus that infected approximately 500 million people and caused 24.7-39.3 million deaths (*1*). The subsequent Asian flu in 1957, Hong Kong flu in 1968, and swine flu in 2009 were major public health incidents with appreciable morbidity and mortality. The World Health Organization (WHO) estimated that global there are approximately 1 billion new influenza cases per year, including 3-5 million severe cases and 290,000-650,000 deaths (*2*). However, since the COVID-19 pandemic struck in 2019, the higher morbidity and mortality of COVID-19 seem to have garnered more public attention, and influenza seems to have been forgotten. Unfortunately, influenza has never actually disappeared from the scene, even in this era of COVID-19. Data from the WHO Global Influenza Surveillance and Response System (GISRS) indicated a resurgence of influenza in 2021-2022 (Figure 1A). GISRS laboratories tested 161,959 samples reported by 107 countries from May 30 to June 12, 2022 and found influenza A in 97.5% and influenza B in 2.5% (*3*). A point worth noting is that despite the lower global total morbidity during the second wave of 2022, data from two countries, namely China (Figure 1B) and Australia (Figure 1C), revealed markedly higher numbers of influenza cases, indicating a resurgence of influenza. Clustered cases were reported in several provinces in the south of China and mainly involved primary and secondary schools. The rate of infection is known to be significantly higher in children than in adults during this wave. Data from the Chinese National Influenza Center (CNIC) revealed that cases of influenza-like-illness (ILI) reported in the past two weeks were markedly higher than during the same period in previous years and that the predominant subtype of the influenza A virus is H3N2 (*4*). CNIC also reported that Guangdong Province has the highest morbidity (*vs.* other provinces). There were 126,857 new ILI cases reported in Guangdong, which is 10.38 times the normal figure from the previous year (*5*). However, actual data on influenza morbidity might be underestimated due to the strict prophylactic measures against COVID-19 in China, which have prompted patients to refrain from seeking medical advice. The situation in Australia is also daunting. Australia is now suffering its worst epidemic of influenza. Australia's National Notifiable Communicable Disease Surveillance System (NNDSS) has reported 147,155 cases during this wave, and 55,101 of those cases were reported from June 6 to 19, 2022. Six-point-one percent of patients with severe influenza had to be admitted to the ICU. Influenza's morbidity is approximately 300 times the normal rate (Figure 1C)

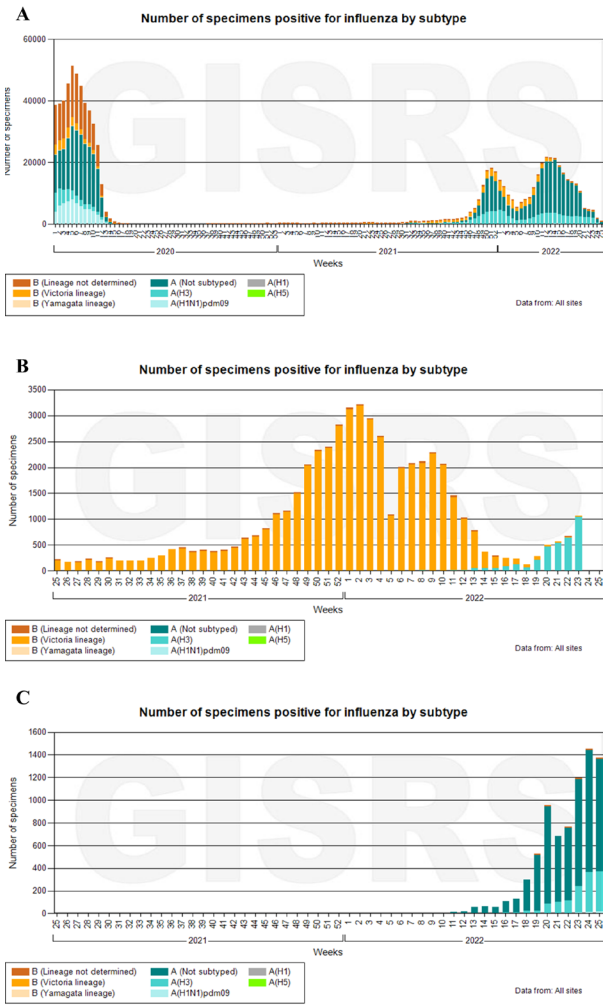

Figure 1. Status of current influenza infections from 2020 to 2022. **(A)**, Global data on influenza infections from 2020 to 2022. **(B)**, Data on influenza infections in China from 2021 to 2022. **(C)**, Data on influenza infections in Australia from 2021 to 2022. All of the data are publicly available from the WHO Global Influenza Surveillance and Response System. *http://www.who.int/flunet*.

(*6*). A "twindemic" of COVID-19 and influenza is a knotty problem now facing the Australian Government. The examples of China and Australia indicate that the influenza problem is far from being resolved despite this being an era of COVID-19.

One crucial problem, namely coinfection with COVID-19 and influenza, should be seriously considered during this "twindemic" scenario. Several recent studies reported coinfection with COVID-19 and influenza in Japan (*7*), the US, Australia, Chile, South Africa (*8*), Turkey (*9*), and the UK (*10*). Kawai *et al*. conducted a retrospective study of 193 patients with COVID-19 using a rapid diagnostic approach, and they found that no patients with COVID-19 were coinfected with influenza (*7*). They therefore concluded that coinfection with COVID-19 and influenza was rare during the winter of 2020 in Japan, but they urged that attention be paid to the coinfection problem during the next influenza season. Ozaras *et al*. reported six patients coinfected with COVID-19 and influenza among 1,103 patients with COVID-19 (0.54%) in Turkey. All of these patients with a coinfection had a mild to moderate infection (*9*). Swets *et al*. examined 6,965 patients with COVID-19 in the UK and found that 227 were coinfected with influenza (3.26%) (*10*). All of these studies reported that cases of coinfection with COVID-19 and influenza are rare at the present time but that attention needs to be paid to this situation because the clinical outcomes of coinfection with COVID-19 and influenza remain unknown. Swets *et al*. found that coinfection with COVID and influenza was significantly associated with an increased probability of death. Moreover, coinfection with COVID-19 and other respiratory viruses significantly increased the likelihood being placed on invasive mechanical ventilation (*10*). That study predicted a worse outcome due to coinfection, but the actual situation requires further investigation.

The interactions between COVID-19 and influenza are complicated and not fully understood. Several issues need to be kept in mind: 1) As respiratory viruses, both viruses have analogous transmission characteristics and common clinical manifestations. Hence, measures to prevent COVID-19 are also effective in preventing influenza (*11*). This suggests that the morbidity of influenza decreased since many measures had been taken against COVID-19 (such as mask wear, city lockdowns, and social distancing) (*12,13*). 2) Prioritizing COVID-19 while neglecting influenza might lead to diminished adaptive immunity against influenza in the general population, increased the risk of infecting with influenza particularly among specific populations of the elderly and individuals with certain underlying medical conditions. 3) A previous study indicated that infection with COVID-19 tends to involve coinfection with other respiratory viruses (*10*). However, distinguishing between COVID-19 and influenza in the early stages based solely on symptoms is extremely difficult. More effective differential diagnosis tools are needed. Other than the PCR assays, thoracic radiology findings might be useful in screening for patients coinfected with COVID-19 and influenza (*9*).

The morbidity of influenza has recently tended to decline, possibly due to the measures against COVID-19 (*13*). That said, influenza has definitely not disappeared. Measures against COVID-19 may conversely reduce contact with influenza, subsequently reducing adaptive immunity against influenza in the general population. Hence, these measures against COVID-19 are a double-edged sword with regard to influenza. Influenza might not be center stage right now, but insufficient adaptive immunity in the population may potentially trigger a future influenza pandemic. Coinfection with COVID-19 and influenza might potentially be a thorny problem in the near future. Public health authorities around the world should be aware of this situation and make preparations ahead of time, particularly with regard to the coming influenza season. More effective vaccines and antivirals should be developed. In addition, correct knowledge

regarding the prevention of COVID-19 and influenza needs to be updated and conveyed to the public. Put simply, this may be an era of COVID-19, but it may act as a Trojan horse for influenza. In any event, influenza should never be forgotten.

Funding: This work is supported by the Shenzhen Science and Technological Foundation (No. JSGG20220301090005007), the Third People's Hospital of Shenzhen Foundation (No. G2021027) and the Third People's Hospital of Shenzhen Foundation (No. G2022062)

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. Bull Hist Med. 2002; 76:105-115.
2. WHO. World Health Organization. Global influenza strategy 2019-2030. *https://apps.who.int/iris/handle/10665/311184* (accessed Jun 27, 2022).
3. CNIC. FluNet Summary. *https://www.who.int/tools/flunet/flunet-summary* (accessed June 30 2022).
4. Summary of influenza Epidemic situation in China. *https://ivdc.chinacdc.cn/cnic/zyzx/lgzb/* (accessed July, 2 2022). (in Chinese)
5. Influenza Surveillance Weekly Report (No.705). *https://ivdc.chinacdc.cn/cnic/zyzx/lgzb/202206/t20220624_259815.htm* (accessed July 2, 2022). (in Chinese)
6. NNDSS. Health AGD of Australian Influenza Surveillance Report and Activity Updates. *https://www1.health.gov.au/internet/main/publishing.nsf/Content/cda-surveil-ozflu-flucurr.htm* (accessed July 2, 2022).
7. Kawai S, Fukushima K, Yomota M, Fukuda A, Fujiwara S, Tanaka M, Kobayashi T, Yajima K, Hosomi Y, Imamura A. Number of Patients with Influenza and COVID-19 Coinfection in a Single Japanese Hospital during the First Wave. Jpn J Infect Dis. 2021; 74:570-572.
8. Olsen SJ, Azziz-Baumgartner E, Budd AP, Brammer L, Sullivan S, Pineda RF, Cohen C, Fry AM. Decreased influenza activity during the COVID-19 pandemic-United States, Australia, Chile, and South Africa, 2020. Am J Transplant. 2020; 20:3681-3685.
9. Ozaras R, Cirpin R, Duran A, Duman H, Arslan O, Bakcan Y, Kaya M, Mutlu H, Isayeva L, Kebanli F, Deger BA, Bekeshev E, Kaya F, Bilir S. Influenza and COVID-19 coinfection: Report of six cases and review of the literature. J Med Virol. 2020; 92:2657-2665.
10. Swets MC, Russell CD, Harrison EM, Docherty AB, Lone N, Girvan M, Hardwick HE, Investigators IC, Visser LG, Openshaw PJM, Groeneveld GH, Semple MG, Baillie JK. SARS-CoV-2 co-infection with influenza viruses, respiratory syncytial virus, or adenoviruses. Lancet. 2022; 399:1463-1464.
11. Sawakami T, Karako K, Song P, Sugiura W, Kokudo N. Infectious disease activity during the COVID-19 epidemic in Japan: Lessons learned from prevention and control measures. Biosci Trends. 2021; 15:257-261.
12. Luo M, Sun J, Gong Z, Wang Z. What is always necessary throughout efforts to prevent and control COVID-19 and other infectious diseases? A physical containment strategy and public mobilization and management. Biosci Trends. 2021; 15:188-191.
13. Li Q, Wang J, Lv H, Lu H. Impact of China's COVID-19 prevention and control efforts on outbreaks of influenza. Biosci Trends. 2021; 15:192-195.

Received July 5, 2022; Revised July 14, 2022; Accepted July15, 2022.

[§]These authors contributed equally to this work.
**Address correspondence to:*
Hongzhou Lu, Department of Infectious Diseases, National Clinical Research Center for Infectious Diseases, the Third People's Hospital of Shenzhen, 29 Buji Bulan Road, Shenzhen 518112, Guangdong Province, China.
E-mail: luhongzhou@fudan.edu.cn

Cordia Chu, Centre of Environment and Population Health, School of Medicine and Dentistry, Griffith University, Brisbane, Queensland 4111, Australia.
E-mail: c.chu@griffith.edu.au

Released online in J-STAGE as advance publication July17, 2022., 2022.

Perspective

DOI: 10.5582/bst.2022.01373

A well-matched marriage of immunotherapy and radiofrequency ablation to reduce the relapse and progression of hepatocellular carcinoma

Rui Liao[1,2,*], Peipei Song[2], Yuxin Duan[1], Wentao Ye[1], Kunli Yin[1], Meiqing Kang[1], Yanxi Yu[1], Jian Yang[1], Wei Tang[2,3]

[1] Department of Hepatobiliary Surgery, The First Hospital Affiliated with Chongqing Medical University, Chongqing, China;
[2] National Center for Global Health and Medicine, Tokyo, Japan;
[3] Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, The University of Tokyo Hospital, Tokyo, Japan.

SUMMARY Hepatocellular carcinoma (HCC) remains a health challenge with increasing incidence worldwide. Radiofrequency ablation (RFA) is a potentially curative option for patients with early-stage HCC. However, the high rate of tumor recurrence limits long-term survival when the tumors are larger than 2 cm and undergoing insufficient RFA (iRFA). Notably, in situ tumor necrosis due to thermal ablation is assumed to be a source of antigens that induce antitumor immunity. Therefore, mounting studies and trials have attempted to provide a rational and effective therapeutic strategy combining RFA and immunotherapy to treat HCC. Nowadays, many controversies and challenges with this combined therapeutic strategy remain to be resolved, such as the indications for adjuvant immunotherapy along with RFA in early HCC, the sequence of the two treatments in advanced HCC, and the optimal timing of immunotherapy before or after RFA. In addition, individualized treatment strategies need to be perfected for patients with HCC.

Keywords immunotherapy, radiofrequency ablation, hepatocellular carcinoma, recurrence, metastasis

Hepatocellular carcinoma (HCC) is one of the most common malignancies with typical immunogenic tumor characteristics, and it develops most frequently in the context of chronic hepatitis virus infection and cirrhosis (*1*). Although surgical resection and liver transplantation are potentially curative options for patients with early-stage HCC, the high rate of tumor recurrence limits long-term survival (*2*). A point worth noting is that treatment with radiofrequency ablation (RFA) is widely accepted as a first-line therapeutic approach for early HCC, and its advantages are a high level of efficacy, a low incidence of complications, and low cost. Compared to surgery, RFA alone has a discouraging 5-year survival rate, with a high rate of HCC recurrence as the main problem post-ablation, and particularly for tumors more than 2-3 cm in size (*3*). To resolve this dilemma, recent efforts have focused on a combination of RFA and immunotherapy as a multimodal treatment strategy. So far, there is a lack of reliable comparative clinical data as to whether immunotherapy could really reduce additional sessions of RFA to treat HCC and halt cancer progression. Therefore, the current recommendations for combined strategies involving RFA and distinct immunotherapies need to be devised for this deadly disease, and the long-term effects of this promising approach need to be validated further.

1. Use of RFA to treat HCC

RFA is a common minimally invasive therapeutic technique to destroy HCC through a Joule effect induced by the generation of a high-frequency alternating current and local heat from an electrode tip inserted into neoplastic tissues. RFA can produce tissue hyperthermia to achieve tumor necrosis at 375-480 kHz. Currently, heat-ablated lesions are surmised to consist of three zones: a central zone (> 60°C), a transitional zone (43-50°C), and the surrounding tissue unaffected by ablation (*4*). In the transitional zone, a tumor could undergo sublethal injury and result in tumor dissemination after insufficient RFA (iRFA). International clinical practice guidelines on HCC do not recommend the use of RFA for tumors larger than 5 cm due to the high risk of residual tumors (*2*). Aside from tumor size, multiple studies (*5,6*) have confirmed several clinical characteristics associated with a risk

of local recurrence, such as multiple tumor nodules, poorly defined HCC margins, and the location of the tumor near major intrahepatic blood vessels. As well as local recurrence, iRFA was also found to be significantly related to distant recurrence (*7*). Based on HCC guidelines, RFA is not a curative treatment option for patients with cancer in the intermediate (BCLC B) or advanced (BCLC C) stage. That said, if treating HCC with transarterial chemoembolization (TACE) and systemic therapies yields no better efficacy, then this task would be an insurmountable mountain. Fortunately, thanks to the advent of the genomic era as well as an in-depth understanding of the immune response generated directly by RFA in the tumor microenvironment, the combination of RFA and immunotherapy seems to be a potential therapeutic strategy for decreasing the recurrence and metastasis of HCC.

2. Immune response induced by RFA when treating HCC

Research has firmly established that HCC is not a local disease even in the early stages. In fact, ablation treatments physically eliminate local tumors but also play a considerable role in distant lesions through an immune response, named the "abscopal effect". One possible rationale is that thermally induced in situ tumor necrosis can constantly give rise to antigens that induce antitumor immunity (*8*). In 2003, Wissniowski *et al*. (*9*) reported the first study to identify an RFA-mediated antitumor response and they found a potential immunological effect on tumor growth due to a tumor-specific T-cell response elicited 2 weeks after RFA treatment. A recent study involving a mouse model revealed that iRFA enhanced the immuno-suppressive environment, increasing $CD11b^{+}CD15^{+}$ polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs) and decreasing $CD8^{+}$ T cells, and it subsequently promoted tumor growth and metastasis (*10*). However, mounting evidence has revealed that dendritic cell (DC), natural killer (NK) cell, and $CD4^{+}$ and $CD8^{+}$ T-cell responses to tumors and systemic immune variations increased significantly following RFA (*11,12*). The prevailing view posits that tumor-specific immune responses facilitate an increased innate immune response and reduced immunosuppression after RFA. However, inconsistent findings are constantly emerging. For example, $CD4^{+}$ and $CD8^{+}$ T cells played a temporary antitumor role in RFA-treated mice that was quickly tampered by active immune suppression responses and then followed by a higher regulatory T-cell to $CD8^{+}$ cell ratio and increased PD-L1/PD-1 expression (*13*). A clinical observation of patients with HCC receiving RFA revealed that the memory phenotype and survival time of enhanced tumor-associated antigen-specific T-cells following RFA were not sufficient to impede HCC recurrence (*14*). Hence, these results are thought provoking because anti-tumor immune responses initiated by RFA are not strong enough to eliminate all tumor cells, potentially leading to the recurrence of HCC.

3. Current status of and challenges with this combination therapy

In clinical practice, there is contradictory evidence regarding which population is eligible for RFA and immunotherapy. On one hand, RFA is recommended for patients with very early and early stage HCC who could not be suitable for immunotherapy. On the other hand, RFA is considered to be a curative treatment in early HCC instead of an adjuvant approach like immunotherapy for patients with HCC in the intermediate or advanced stage. The rationale for this novel combined therapeutic strategy is based on the positive results of basic studies and pilot trials. The promising results of immunotherapy in patients with advanced HCC imply that adjuvant treatment with RFA is a possible option in early to intermediate stage HCC. Notably, the STORM trial noted no benefit in terms of recurrence-free survival (RFS) in patients with HCC receiving the adjuvant sorafenib, an oral multityrosine kinase inhibitor, post-resection or ablation (*15*). Undoubtedly because of this, the combined therapy strategy is also attracting the attention of clinicians who want to know whether enhanced anti-tumor immunity may optimize RFA to treat HCC.

In a multicenter, randomized, open-label, phase 3 trial conducted in South Korea, Lee *et al*. (*16*) reported that adjuvant immunotherapy with activated cytokine-induced killer (CIK) cells increased recurrence-free and overall survival (OS) in patients with HCC who underwent curative treatment with surgical resection, RFA, or percutaneous ethanol injection. A combined treatment involving RFA and cellular immunotherapy (CIT) yielded similar results in patients with HCC (*17*). In this trial, autologous mononuclear cells inducing NK cells, γδT cells, and CIK cells were infused intravenously to patients within 8-11 days of RFA. The preliminary results implied that a combination of sequential CIT and RFA may prevent the recurrence of HCC in patients after RFA. In another clinical trial, Kitahara *et al*. also demonstrated that RFA + administration of OK432-stimulated DCs to a necrotic tumor improved RFS in patients with HCC (*18*). Based on the results of CheckMate-040 and KEYNOTE-224, nivolumab and pembrolizumab, two immune checkpoint inhibitors (ICIs) causing a PD-1 blockade, were approved as second-line therapy for patients with HCC after sorafenib failure. Interestingly, a propensity score matching analysis of recurrent HCC revealed that combination therapy with anti-PD-1 inhibitors (initially administered within 72 hours of RFA) and RFA resulted in longer RFA and OS than RFA alone (*19*). Greten *et*

al. reported a survival analyses and immune monitoring data via anti-CTLA4 (tremelimumab) treatment prior to subtotal RFA or chemoablation in patients with advanced HCC (*20*). In contrast to previous studies, the median OS rate of patients treated with RFA was 9.2 months (95% CI: 6.6–11.2 months). They also demonstrated the safety and feasibility of anti-CTLA4 treatment plus RFA and they observed the clear activation of T cell responses. Table 1 reviews clinical trials involving combinations of RFA and immunotherapy to treat HCC.

4. Conclusions and perspectives

Nowadays, many controversies and challenges remain to be resolved as various proof-of-concept clinical trials are underway. First, would adjuvant immunotherapy for early HCC beyond the Milan criterion have a further survival benefit for completely ablated HCC according to imaging? Second, which treatment should be used first in advanced HCC since both are adjuvant therapies? The frequency of ablation and immunotherapy still needs to be specified. Third, current clinical trials have no satisfactory answer regarding the optimal timing of immunotherapy to produce a synergistic effect with RFA. Most importantly, ablative immunotherapy has yielded better outcomes in some studies but the objective response rate has yet to improve, so the combination therapy could be administered to all patients with HCC. Consequently, individualized treatment strategies need to be taken into consideration in combined therapy for HCC. Nonetheless, immunotherapy is an option to compensate for the deficiencies of RFA in treating HCC. Rational and standardized clinical use of this therapy is still a long way away.

Funding: This work was supported by a grant from the Sino-Japanese Sasagawa Medical Scholarship Program, the Joint Science and Health Research Project of the City of Chongqing Municipality (2020GDRC013), and the Chongqing Medical University Program for Young Innovators in Future Medicine (W0087).

Table 1. Clinical trials registered with the NIH investigating combinations of RFA and immunotherapy to treat HCC

Study Registrant	Trial ID (Phase)	Number of Patients	Clinical Title	Treatment	Study Period
Greten TF, *et al*.	NCT01853618 (Phase I/ II)	61	A Pilot Study of Tremelimumab - A Monoclonal Antibody Against CTLA-4 in Combination with Trans-Arterial Catheter Chemoembolization (TACE), Radiofrequency Ablation (RFA), or Cryoablation in Subjects with Hepatocellular Carcinoma (HCC) or Biliary Tract Carcinomas (BTC)	RFA/TACE/Cryoablation + Tremelimumab	5/2013-6/2017
Lai K, *et al*.	NCT03124498 (Phase I/ II)	55	A Study to Evaluate Autologous CIK Cells in Patients with Hepatocellular Carcinoma After TACE, PEIT or RFA	RFA/TACE/PEIT + CIK	11/2017-6/2019
Zhao M, *et al*.	NCT03939975 (Phase II)	50	A Prospective Study of Anti-PD-1 Inhibitor Therapy in Combination with Incomplete Thermal Ablation in Patients with Advanced Hepatocellular Carcinoma	Thermal Ablation + PD-1	6/2019-7/2019
Peng ZW, *et al*.	NCT02678013 (Phase III)	210	Radiofrequency Ablation Combined with Highly-purified CTL *vs*. Radiofrequency Ablation Alone for Recurrent HCC	RFA + CTL	1/2016-1/2022
Kuansheng Ma, *et al*.	NCT05277675 (NA)	160	A Prospective Study of Radiofrequency Ablation Combined with Systematic Neoadjuvant Therapy in the Treatment of Recurrent Hepatocellular Carcinoma	RFA + Tislelizumab/Sintilimab + Lenvatinib/Bevacizumab	1/12/2021-30/10/2023
Zhou JX, *et al*.	NCT05162898 (Phase II)	90	Radiofrequency Ablation Combined with Toripalimab and Lenvatinib in the Treatment of Short-term Recurrent Hepatocellular Carcinoma	RFA + Toripalimab + Lenvatinib	1/2022-12/2025
Renier W, *et al*.	NCT04727307 (Phase II)	202	Neoadjuvant Atezo, Adjuvant Atezo + Beva Combined with RF Ablation of Small HCC: A Multicenter Randomized Phase II Trial	RFA + the Neoadjuvant Atezolizumab, and the Adjuvants Atezolizumab + Bevacizumab	1/2022-7/2027
Chen MS, *et al*.	NCT04652440 (Phase II)	30	Phase II Study of Ablation Combined With PD-1 Antibody in Patients with Hepatocellular Carcinoma	RFA/MWA + PD-1	12/2020-12/2023
Kuang M, *et al*.	NCT03067493 (Phase II)	98	RFA or Surgical Resection Combined with Neo-MASCT for Primary HCC: A Phase II Trial	RFA/Surgical Resection + Neo-MASCT	1/2022-

Abbreviations: NIH: National Institutes of Health; RFA: radiofrequency ablation; HCC: hepatocellular carcinoma; CTLA-4: cytotoxic T lymphocyte-associated protein-4; TACE: trans-arterial catheter chemoembolization; PEIT: percutaneous ethanol injection treatment; CIK: cytokine-induced killer; PD-1: programmed cell death protein-1; CTL: cytotoxic T lymphocyte; NA: not applicable; MWA: microwave ablation; MASCT: multiple antigen-stimulating cellular therapy.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, Lencioni R, Koike K, Zucman-Rossi J, Finn RS. Hepatocellular carcinoma. Nat Rev Dis Primers. 2021; 7:6.
2. Wen N, Cai Y, Li F, Ye H, Tang W, Song P, Cheng N. The clinical management of hepatocellular carcinoma worldwide: A concise review and comparison of current guidelines: 2022 update. Biosci Trends. 2022; 16:20-30.
3. Reig M, Forner A, Rimola J, *et al*. BCLC strategy for prognosis prediction and treatment recommendation: The 2022 update. J Hepatol. 2022; 76:681-693.
4. Ahmed M, Brace CL, Lee FT, Jr., Goldberg SN. Principles of and advances in percutaneous ablation. Radiology. 2011; 258:351-369.
5. Ng KK, Poon RT, Lam CM, Yuen J, Tso WK, Fan ST. Efficacy and safety of radiofrequency ablation for perivascular hepatocellular carcinoma without hepatic inflow occlusion. Br J Surg. 2006; 93:440-447.
6. Bai XM, Cui M, Yang W, Wang H, Wang S, Zhang ZY, Wu W, Chen MH, Yan K, Goldberg SN. The 10-year survival analysis of radiofrequency ablation for solitary hepatocellular carcinoma 5 cm or smaller: Primary versus recurrent HCC. Radiology. 2021; 300:458-469.
7. Sparchez Z, Mocan T, Radu P, Mocan LP, Sparchez M, Leucuta DC, Al Hajjar N. Prognostic factors after percutaneous radiofrequency ablation in the treatment of hepatocellular carcinoma. Impact of incomplete ablation on recurrence and overall survival rates. J Gastrointestin Liver Dis. 2018; 27:399-407.
8. Llovet JM, De Baere T, Kulik L, Haber PK, Greten TF, Meyer T, Lencioni R. Locoregional therapies in the era of molecular and immune treatments for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol. 2021; 18:293-313.
9. Wissniowski TT, Hansler J, Neureiter D, Frieser M, Schaber S, Esslinger B, Voll R, Strobel D, Hahn EG, Schuppan D. Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. Cancer Res. 2003; 63:6496-6500.
10. Zeng X, Liao G, Li S, *et al*. Eliminating METTL1-mediated accumulation of PMN-MDSCs prevents hepatocellular carcinoma recurrence after radiofrequency ablation. Hepatology. 2022; doi: 10.1002/hep.32585.
11. Dromi SA, Walsh MP, Herby S, Traughber B, Xie J, Sharma KV, Sekhar KP, Luk A, Liewehr DJ, Dreher MR, Fry TJ, Wood BJ. Radiofrequency ablation induces antigen-presenting cell infiltration and amplification of weak tumor-induced immunity. Radiology. 2009; 251:58-66.
12. Rochigneux P, Nault JC, Mallet F, *et al*. Dynamic of systemic immunity and its impact on tumor recurrence after radiofrequency ablation of hepatocellular carcinoma. Oncoimmunology. 2019; 8:1615818.
13. Shi L, Chen L, Wu C, Zhu Y, Xu B, Zheng X, Sun M, Wen W, Dai X, Yang M, Lv Q, Lu B, Jiang J. PD-1 blockade boosts radiofrequency ablation-elicited adaptive immune responses against tumor. Clin Cancer Res. 2016; 22:1173-1184.
14. Mizukoshi E, Yamashita T, Arai K, Sunagozaka H, Ueda T, Arihara F, Kagaya T, Yamashita T, Fushimi K, Kaneko S. Enhancement of tumor-associated antigen-specific T cell responses by radiofrequency ablation of hepatocellular carcinoma. Hepatology. 2013; 57:1448-1457.
15. Bruix J, Takayama T, Mazzaferro V, *et al*. Adjuvant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): A phase 3, randomised, double-blind, placebo-controlled trial. Lancet Oncol. 2015; 16:1344-1354.
16. Lee JH, Lee JH, Lim YS, Yeon JE, Song TJ, Yu SJ, Gwak GY, Kim KM, Kim YJ, Lee JW, Yoon JH. Adjuvant immunotherapy with autologous cytokine-induced killer cells for hepatocellular carcinoma. Gastroenterology. 2015; 148:1383-1391 e1386.
17. Cui J, Wang N, Zhao H, Jin H, Wang G, Niu C, Terunuma H, He H, Li W. Combination of radiofrequency ablation and sequential cellular immunotherapy improves progression-free survival for patients with hepatocellular carcinoma. Int J Cancer. 2014; 134:342-351.
18. Kitahara M, Mizukoshi E, Terashima T, Nakagawa H, Horii R, Iida N, Arai K, Yamashita T, Sakai Y, Yamashita T, Honda M, Nakamoto Y, Kaneko S. Safety and long-term outcome of intratumoral injection of OK432-stimulated dendritic cells for hepatocellular carcinomas after radiofrequency ablation. Transl Oncol. 2020; 13:100777.
19. Wang X, Liu G, Chen S, Bi H, Xia F, Feng K, Ma K, Ni B. Combination therapy with PD-1 blockade and radiofrequency ablation for recurrent hepatocellular carcinoma: A propensity score matching analysis. Int J Hyperthermia. 2021; 38:1519-1528.
20. Agdashian D, ElGindi M, Xie C, *et al*. The effect of anti-CTLA4 treatment on peripheral and intra-tumoral T cells in patients with hepatocellular carcinoma. Cancer Immunol Immunother. 2019; 68:599-608.

Received August 22, 2022; Revised September 7, 2022; Accepted September 9, 2022.

**Address correspondence to:*
Rui Liao, Department of Hepatobiliary Surgery, The First Hospital Affiliated with Chongqing Medical University, No. 1 Youyi Rd, Chongqing 400016, China.
E-mail: liaorui99@163.com or liaorui@hospital.edu.cn

Released online in J-STAGE as advance publication September 11, 2022.

BST
BioScience Trends

Guide for Authors

1. Scope of Articles

BioScience Trends (Print ISSN 1881-7815, Online ISSN 1881-7823) is an international peer-reviewed journal. *BioScience Trends* devotes to publishing the latest and most exciting advances in scientific research. Articles cover fields of life science such as biochemistry, molecular biology, clinical research, public health, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *BioScience Trends* in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

3. Editorial Policies

For publishing and ethical standards, BioScience Trends follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (*http://www.icmje.org/recommendations*) issued by the International Committee of Medical Journal Editors (ICMJE), and the Principles of Transparency and Best Practice in Scholarly Publishing (*https://doaj.org/bestpractice*) jointly issued by the Committee on Publication Ethics (COPE), the Directory of Open Access Journals (DOAJ), the Open Access Scholarly Publishers Association (OASPA), and the World Association of Medical Editors (WAME).

BioScience Trends will perform an especially prompt review to encourage innovative work. All original research will be subjected to a rigorous standard of peer review and will be edited by experienced copy editors to the highest standards.

Ethics: *BioScience Trends* requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board. For research involving human experiments, a statement that the participants gave informed consent before taking part (or a statement that it was not required and why) should be indicated. Authors should also state that the study conformed to the provisions of the Declaration of Helsinki (as revised in 2013). When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to *BioScience Trends*, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to

the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter prepared by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit: Download Centre (*https://ircabssagroup.com/downcentre*).

Copyright: When a manuscript is accepted for publication in *BioScience Trends*, the transfer of copyright is necessary. A JOURNAL PUBLISHING AGREEMENT (JPA) form will be e-mailed to the authors by the Editorial Office and must be returned by the authors as a scan. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. Please note that your manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Peer Review: *BioScience Trends* uses single-blind peer review, which means that reviewers know the names of the authors, but the authors do not know who reviewed their manuscript. The external peer review is performed for research articles by at least two reviewers, and sometimes the opinions of more reviewers are sought. Peer reviewers are selected based on their expertise and ability to provide high quality, constructive, and fair reviews. For research manuscripts, the editors may, in addition, seek the opinion of a statistical reviewer. Consideration for publication is based on the article's originality, novelty, and scientific soundness, and the appropriateness of its analysis.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in *BioScience Trends*.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in *BioScience Trends* and need assistance before submitting a manuscript. Authors can visit this organization directly at *http://www.iacmhr.com/iac-eso/support.php?lang=en*. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *http://www.ICMJE.org*.

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For articles that are Original Articles, Brief Reports, Reviews, or Policy Forum articles, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *BioScience Trends* could be downloaded at **EndNote** (*https://ircabssagroup.com/examples/BioScience_Trends.ens*).

Examples are given below:

Example 1 (Sample journal reference):

Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):

Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained. Any individually labeled figure parts or panels (A, B, *etc*.) should be specifically described by part name within the legend.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that the symbols and numbers appeared in the figures should be clear. Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g*. mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide *www.bipm.org/en/si/* for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and *BioScience Trends* accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to *BioScience Trends* for review. Please visit Download Centre and download the Submission Checklist file.

6. Online Submission

Manuscripts should be submitted to *BioScience Trends* online at *http://www.biosciencetrends.com*. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at *office@biosciencetrends.com*

7. Accepted Manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned

to the editor (*proof-editing@biosciencetrends.com*) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: Page charges will be levied on all manuscripts accepted for publication in *BioScience Trends* (Original Articles / Brief Reports / Reviews / Policy Forum / Communications: $140 per page for black white pages, $340 per page for color pages; News / Letters: a total cost of $600). Under exceptional circumstances, the author(s) may apply to the editorial office for a waiver of the publication charges at the time of submission.

Misconduct: *BioScience Trends* takes seriously all allegations of potential misconduct and adhere to the ICMJE Guideline (*http://www.icmje.org/recommendations*) and COPE Guideline (*http://publicationethics.org/files/Code_of_conduct_for_journal_editors.pdf*). In cases of suspected research or publication misconduct, it may be necessary for the Editor or Publisher to contact and share submission details with third parties including authors' institutions and ethics committees. The corrections, retractions, or editorial expressions of concern will be performed in line with above guidelines.

(As of February 2022)

BioScience Trends
Editorial and Head Office
Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan.
E-mail: office@biosciencetrends.com