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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our overall goal is to determine if human breast cancer can be prevented from becoming angiogenic when it is still at a microscopic size (< ~ 1 mm3). Some of our key research accomplishments this year are: (i) Heat shock protein-27 (HSP-27) was identified as a gene that was upregulated 27-fold in the angiogenic breast cancer cells MDA-MB-436 as compared to the non-angiogenic dormant breast cancer cells. (ii) Increased expression of HSP-27 was validated by Western blot analysis. (iii) Confirmation of decreased expression of the endogenous angiogenesis inhibitors thrombospondin-1 and endostatin in breast cancer cell lines with a BRCA1 mutation (HCC937). (iv) Demonstration by immunofluorescence of decreased thrombospondin-1 and endostatin expression in sections from human breast tumors with BRCA1 mutations as compared to sections from sporadic breast tumors with wild-type BRCA1. (v) Identification of high levels of prosaposin expression by the non-metastatic breast cancer cell line MDA-MB-231 as compared to the highly metastatic derivatives of this breast cancer cell line that metastasize to bone and lung. (vi) Identification of the secreted protein prosaposin as a regulator of thrombospondin-1 expression in stromal cells. Our studies have potential therapeutic implications to target specific genes that regulate the angiogenic switch, tumor mass expansion, and metastatic disease.

15. SUBJECT TERMS
Tumor angiogenesis, switch to the angiogenic phenotype, thrombospondin-1, VEGF, platelet angiogenesis proteome, dormant, non-angiogenic human breast cancer, BRCA1, reversal of the angiogenic phenotype, stroma.

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"Prevention of the Angiogenic Switch in Human Breast Cancer"
February 2007- February 2008

<u>**INTRODUCTION**</u>:

We have been focused on understanding the mechanisms that regulate the switch to the angiogenic phenotype in human breast cancer cells. Tumor growth is generally initiated by genetic mutations however the expansion of tumor mass requires angiogenesis or new blood vessel growth. Without angiogenesis, tumors remain microscopic, dormant and harmless. However, once breast tumors have undergone an angiogenic switch, they become vascularized, rapidly growing tumors and often metastasize to distant sites which is most often the cause of death in breast caner patients. Our work has been investigating the mechanisms triggering the angiogenic switch, genetic mutations that may regulate expression of angiogenesis factors and control of metastatic disease by downregulation of angiogenesis inhibitors. We are also investigating the consequences of the angiogenic switch in the context of metastasis to determine whether a secondary event is required for this process. Understanding these processes may allow us to target new pathways in the prevention and treatment of breast cancer.

<u>**BODY**</u>:

This past year we have profiled genes that are specifically upregulated in an angiogenic, highly aggressive human breast cancer cell line as compared to a dormant, non-angiogenic breast cancer cell line. We have identified heat shock protein-27 (HSP-27) as a gene that is highly overexpressed in the angiogenic breast cancer cells. Our studies have been focused on identifying whether upregulation of HSP-27 may contribute to the switch to the angiogenic phenotype. Our studies have also been focused on understanding how the breast cancer susceptibility gene (BRCA1) may regulate the expression of endogenous angiogenesis inhibitors. The role of BRCA1 in tumorigenesis is still not clearly defined since it has been implicated in a number of different functions including DNA repair and recombination, cell cycle control and transcription. One mutant BRCA1 copy in the germline predisposes an individual to cancer with the second allele consistently lost in tumors thereby leaving no functional copy of BRCA1. Our work suggests that BRCA1 may normally function to regulate expression of endogenous angiogenesis inhibitors therefore upon genetic loss or mutation, the decrease in endogenous angiogenesis inhibitors may contribute to the switch to the angiogenic phenotype. Finally, our work has also identified the protein prosaposin, which is secreted by benign, non-metastatic breast cancer cells and may regulate expression of the endogenous angiogenesis inhibitor thrombospondin-1 in stromal cells. Intriguingly, prosaposin is dramatically downregulated upon progression to

the metastatic phenotype. We have been investigating whether regulation of thrombospondin-1 in stromal cells may be necessary for the establishment of metastatic disease.

KEY RESEARCH ACCOMPLISHMENTS:

(i) Characterization of the angiogenic switch by breast cancer cells.
• Microarray analysis of the angiogenic and non-angiogenic breast cancer cell line, MDA-MB-436 was performed and analyzed.

• Heat shock protein-27 (HSP-27) was identified as a gene that was upregulated 27-fold in the angiogenic breast cancer cells MDA-MB-436 as compared to the non-angiogenic dormant breast cancer cells. Increased expression of HSP-27 was validated by Western blot analysis.

• Knock-down of HSP-27 by shRNA in the angiogenic breast cancer cells demonstrated a significant decrease in tumor growth upon inoculation into SCID immunocompromised mice.

(ii) Investigation of angiogenesis regulation by the Breast Cancer Susceptibility Gene (BRCA1).

• Confirmation of decreased expression of the endogenous angiogenesis inhibitors thrombospondin-1 and endostatin in breast cancer cell lines with a BRCA1 mutation (HCC937).

• Demonstration by immunofluorescence of decreased thrombospondin-1 and endostatin expression in sections from human breast tumors with BRCA1 mutations as compared to sections from sporadic breast tumors with wild-type BRCA1.

• Preliminary studies indicating that regulation of thrombospondin-1 and endostatin expression by BRCA1 is mediated through the tumor suppressor p53.

• Knock-down of BRCA1 by shRNA in the normal breast epithelial cell line MCF10A to determine whether loss of BRCA1 alone is sufficient for downregulation of thrombospondin-1 and endostatin.

(iii) Investigation of regulation of breast cancer metastasis.

• Identification of high levels of prosaposin expression by the non-metastatic breast cancer cell line MDA-MB-231 as compared to the highly metastatic derivatives of this breast cancer cell line that metastasize to bone and lung.

• Identification of the secreted protein prosaposin as a regulator of thrombospondin-1 expression in stromal cells.

• Preliminary studies suggesting that prosaposin induces p53 expression in stromal fibroblasts, which directly regulates thrombospondin-1 expression.

• Knock-down of prosaposin by shRNA in the non-metastatic breast cancer cells leads to a highly metastatic phenotype of these cells upon orthotopic injection into SCID immunocompromised mice.

REPORTABLE OUTCOMES:

Presentations:

1/25/07	Harvard Medical School Honorary Event Dr. Folkman- lecturer **"How is it helpful for a medical student to understand why the process of angiogenesis may be an organizing principle in biomedicine?"**
2/14/07	University of California San Diego Moores UCSD Cancer Center Director's Seminar Series Dr. Folkman- Lecture **"Angiogenesis: An Organizing Principle in Medicine and Biology"**
3/23- 3/24/07	Symposium honoring Dr. Josh Fidler Dr. Folkman- presentation **"Angiogenesis Regulatory Molecules that Mediate Preferential Tumor Growth in Orthotopic Sites"** Houston, TX
5/12- 5/15/07	Actar Meeting and Nobel Forum lecture Dr. Folkman- minisymposium lecturer **"Anti-angiogenic therapy in treatment of cancer"** Stockholm, Sweden
8/15- 8/16/07	8th World Congress for Microcirculation Dr. Folkman- Keynote Lecturer **"Endogenous Angiogenesis Inhibitors"** Milwaukee, WI
9/14- 9/15/07	Aultman Cancer Center Aultman Cancer Research Institute **"Antiangiogenic Therapy for Cancer: Principles and New Directions"** Northeastern Ohio University

10/13/07 2007 International Conference on Glioma Research and Therapy
 Dr. Folkman- Speaking
 "Clinical Applications of Antiangiogenic Therapy: New Insights"
 Boston, MA

10/20/07 Dr. Folkman- speaker
 "Blocked Angiogenesis as a General Mechanism of Tumor Dormancy"
 San Diego, CA

10/24-
10/26/07 Emory University School of Medicine's Winship Cancer Institute
 Dr. Folkman- keynote address
 "Angiogenesis: A Paradigm for translating from the laboratory to the clinic and back."
 Atlanta, GA

Manuscripts:

Folkman J. Angiogenesis: an organizing principle for drug discovery? **Nat Rev Drug Discov.** 2007; 6: 273-286.

Kaipainen A, Kieran MW, Huang S, Butterfield C, Bielenberg D, Mostoslavsky G, Mulligan R, Folkman J, Panigrahy D. PPARα deficiency in inflammatory cells suppresses tumor growth. **PLoS ONE** 2007; 2:e260.

Cervi D, Yip T-T, Bhattacharya N, Podust VN, Peterson J, Abou-slaybi A, Naumov GN, Bender E, Almog N, Italiano JE Jr., Folkman J, Klement GL. Platelet-associated PF-4 as a biomarker of early tumor detection. **Blood**; 2007; in press

Italiano JE, Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, Ryeom S, Folkman J, Klement GL. Angiogenesis is regulated by a novel mechanism: Pro- and anti-angiogenic proteins are organized into separate platelet granules and differentially released. **Blood**, In press.

Naumov GN, Folkman J. Strategies to prolong the nonangiogenic dormant state of human cancer. In: Davis DW, Herbst RS, Abbruzzese JL, eds. **Antiangiogenic Cancer Therapy**. CRC Press, Boca Raton, FL, 2007; pp 3-21.

Folkman, J. Endostatin finds a new partner: nucleolin. **Blood** 2007; 110(8): 2786 -2787.

Panigrahy D, Kaipainen A, Huang S, Butterfield CE, Barnes CM, Fannon M, Laforme AM, Chaponis DM, Folkman J, Kieran MW. The PPARalpha agonist fenofibrate suppresses tumor growth through direct and indirect angiogenesis inhibition. **Proc Natl Acad Sci USA**; 2008; 105(3):985-990.

Kang, S.Y., Halvorsen, O.J., Lee, J.M., Liu, N.W., Johnston, B.T., Johnston, A.B., Akslen, L.A., and Watnick, R.S. (2007) Prosaposin inhibits tumor metastasis via stimulation of stromal p53 activity. In review, *Cell.*

Patents:

Benny O & Folkman J. (June, 2007) Fumagillol derivative polymersomes for oral administration. *Children's Hospital Boston. Pending*

Fainaru O, Benny O, Folkman J. (June, 2007) Methods and compositions for inhibiting vascular leakage. *Children's Hospital Boston. Pending*

CONCLUSION:

Our work this past year suggests that HSP-27 may play a role in regulating the angiogenic switch in the MDA-MB-231 breast cancer cells. Decreasing expression of HSP-27 in angiogenic breast cancer cells led to a change in tumorigenicity. Disruption of HSP-27 expression in these cells via shRNA technology switched the phenotype of these angiogenic breast cancer cells to that of the dormant non-angiogenic phenotype. After inoculation of these cells into the mammary fat pad of SCID mice, they formed small dormant tumors unlike the parental control cells which formed rapidly growing tumors in these mice. Our future studies include investigating the function of HSP-27 in breast cancer cells. We will determine whether HSP-27 acts as an upstream regulator of the angiogenic switch or if its increased expression is a downstream consequence of the switch to the angiogenic phenotype.

Our studies investigating the regulation of the endogenous angiogenesis inhibitors thrombospondin-1 and endostatin by the breast cancer susceptibility gene, BRCA1 suggests that an important function of BRCA1 may be to maintain high levels of these angiogenesis inhibitors. Our data further implicates that regulation of these inhibitors by BRCA1 may be through the tumor suppressor p53. Since the majority of BRCA-1-null breast tumors demonstrate loss of p53, our future studies include the regulation of p53 by BRCA-1 to determine how loss of BRCA-1 may lead to loss of p53. Further we will examine whether replacement of thrombospodin-1 and/or endostatin may suppress tumor growth of BRCA1-null tumor cells.

Finally our work on the regulation of metastatic disease in breast cancer suggests that highly metastatic breast cancer cells may downregulate expression of a secreted protein called prosaposin. We have found that prosaposin can stimulate the expression of the angiogenesis inhibitor thrombospondin-1 in stromal fibroblasts both at the primary site and in distal organs prior to metastatic dissemination. High levels of thrombospondin-1 may be important in preventing the establishment of microscopic metastases in distal organ sites. Our data also demonstrates that prosaposin regulation of thrombospondin-1 may is mediated through p53 activation. This upcoming year we

will focus on understanding how prosaposin is regulated in non-metastatic tumor cells and we will determine the mechanism by which prosaposin activates p53 in tumor-associated stromal fibroblasts as well as in distal organs.

Taken together, our studies have potential therapeutic implications to target specific genes that regulate the angiogenic switch, tumor mass expansion and metastatic disease.

"SO WHAT SECTION"

By understanding the role of heat shock protein-27 (HSP27) in regulating the switch to the angiogenic phenotype breast cancer cells, it may be possible to target this molecule as a novel therapeutic option for treatment of breast cancer. Similarly, by determining whether regulation of the endogenous angiogenesis inhibitors, thrombospondin-1 and/or endostatin is a critical function of the Breast Cancer Susceptibility Gene -1 (BRCA1), may allow us to treat women with BRCA1-null breast cancer with recombinant endogenous angiogenesis inhibitors.

Additionally, delineating the mechanism and downstream signaling pathway through which Prosaposin stimulates p53 and Tsp-1 in organ fibroblasts at the primary and distal sites holds the promise of identifying new points of intervention for potential anti-metastatic therapies. There are currently available compounds that specifically activate p53 by preventing its degradation by MDM2, ie Nutlin-3. We will test such compounds in our models to determine whether they are more effective at inhibiting tumor metastasis than primary tumor growth.

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None.

APPENDIX:

1. Folkman J. Angiogenesis: an organizing principle for drug discovery? **Nat Rev Drug Discov.** 2007; 6: 273-286.

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OPINION

Angiogenesis: an organizing principle for drug discovery?

Judah Folkman

Abstract | Angiogenesis — the process of new blood-vessel growth — has an essential role in development, reproduction and repair. However, pathological angiogenesis occurs not only in tumour formation, but also in a range of non-neoplastic diseases that could be classed together as 'angiogenesis-dependent diseases'. By viewing the process of angiogenesis as an 'organizing principle' in biology, intriguing insights into the molecular mechanisms of seemingly unrelated phenomena might be gained. This has important consequences for the clinical use of angiogenesis inhibitors and for drug discovery, not only for optimizing the treatment of cancer, but possibly also for developing therapeutic approaches for various diseases that are otherwise unrelated to each other.

The term angiogenesis is generally applied to the growth of microvessel sprouts the size of capillary blood vessels, a process that is orchestrated by a range of angiogenic factors and inhibitors (FIG. 1). Although proliferating endothelial cells undergoing DNA synthesis are a common hallmark of angiogenic microvascular sprouts, extensive sprouts can grow for periods of time, mainly by the migration of endothelial cells[1]. Physiological angiogenesis is distinct from arteriogenesis and lymphangiogenesis and occurs in reproduction, development and wound repair. It is usually focal, such as in blood coagulation in a wound, and self-limited in time, taking days (ovulation), weeks (wound healing) or months (placentation). By contrast, pathological angiogenesis can persist for years. Pathological angiogenesis is necessary for tumours and their metastases to grow beyond a microscopic size and it can give rise to bleeding, vascular leakage and tissue destruction. These consequences of pathological angiogenesis can be responsible, directly or indirectly, for the symptoms, incapacitation or death associated with a broad range of 'angiogenesis-dependent diseases'[2]. Examples of such diseases include cancer, autoimmune diseases, age-related macular degeneration and atherosclerosis (TABLE 1).

The concept of angiogenesis-dependent diseases originated in 1972 with the recognition that certain non-neoplastic diseases, such as the chronic inflammatory disease psoriasis, depend on chronic neovascularization to provide a conduit for the continual delivery of inflammatory cells to the inflammatory site[3–5]. Subsequently, other non-neoplastic diseases were recognized to be in part angiogenesis dependent, for example, infantile haemangiomas[6], peptic ulcers[7], ocular neovascularization[8], rheumatoid arthritis[9] and atherosclerosis[3,10,11]. This led to a more general understanding that the process of angiogenesis itself could be considered as an 'organizing principle'. Organizing principles are common in the physical sciences, and are now starting to be recognized in biology — other examples might be inflammation or apoptosis, which are also aspects of many otherwise unrelated diseases. The heuristic value of such a principle is that it permits connections between seemingly unrelated phenomena. For example, the discovery of a molecular mechanism for one phenomenon might be more rapidly demonstrated for a second phenomenon if one understands *a priori* that the two are connected. Furthermore, when the mechanisms underlying different diseases can be related in this way, the development

of therapeutics for one disease could aid the development of therapeutics for others. Although it remains to be determined to what extent treating pathological angiogenesis in different angiogenesis-dependent diseases will be successful, the recent approval of ranibizumab (Lucentis; Genentech) — an antibody fragment based on the anti-angiogenic cancer drug bevacizumab (Avastin; Genentech) — for age-related macular degeneration suggests that such strategies merit investigation.

Here, I provide an overview of the current state of drug development of angiogenesis inhibitors, as well as certain drugs that have varying degrees of anti-angiogenic activity in addition to their other functions, and highlight examples of anti-angiogenic strategies in unrelated diseases. Furthermore, I discuss burgeoning new directions in angiogenic research, the optimization of anti-angiogenic strategies and how viewing angiogenesis as an organizing principle might uncover fruitful connections for future drug discovery.

A brief history of angiogenesis inhibitors
The attempt to discover angiogenesis inhibitors became possible after my group and others had developed bioassays for angiogenesis during the 1970s. These included the long-term culture of vascular endothelial cells[12], the development of the chick-embryo chorioallantoic-membrane bioassay[13], the development of sustained-release polymers[14] and the implantation of these polymers as pellets in the rabbit[15] and murine[16] cornea to quantify the angiogenic activity of tumour-derived proteins.

The first angiogenesis inhibitors were reported in the 1980s from the Folkman laboratory, during a study that continued over 25 years[17,18] (TIMELINE). No angiogenesis inhibitors existed before 1980, and few scientists thought at that time that such molecules would ever be found. However, the effort to isolate and purify them was driven by preliminary data that led to the 1971 hypothesis that tumour growth is dependent on angiogenesis[19]. This effort was also informed by preliminary data that the removal of an angiogenic sustained-release pellet from the rabbit cornea led to a rapid regression (weeks) of neovascularization that was induced by the pellet[20].

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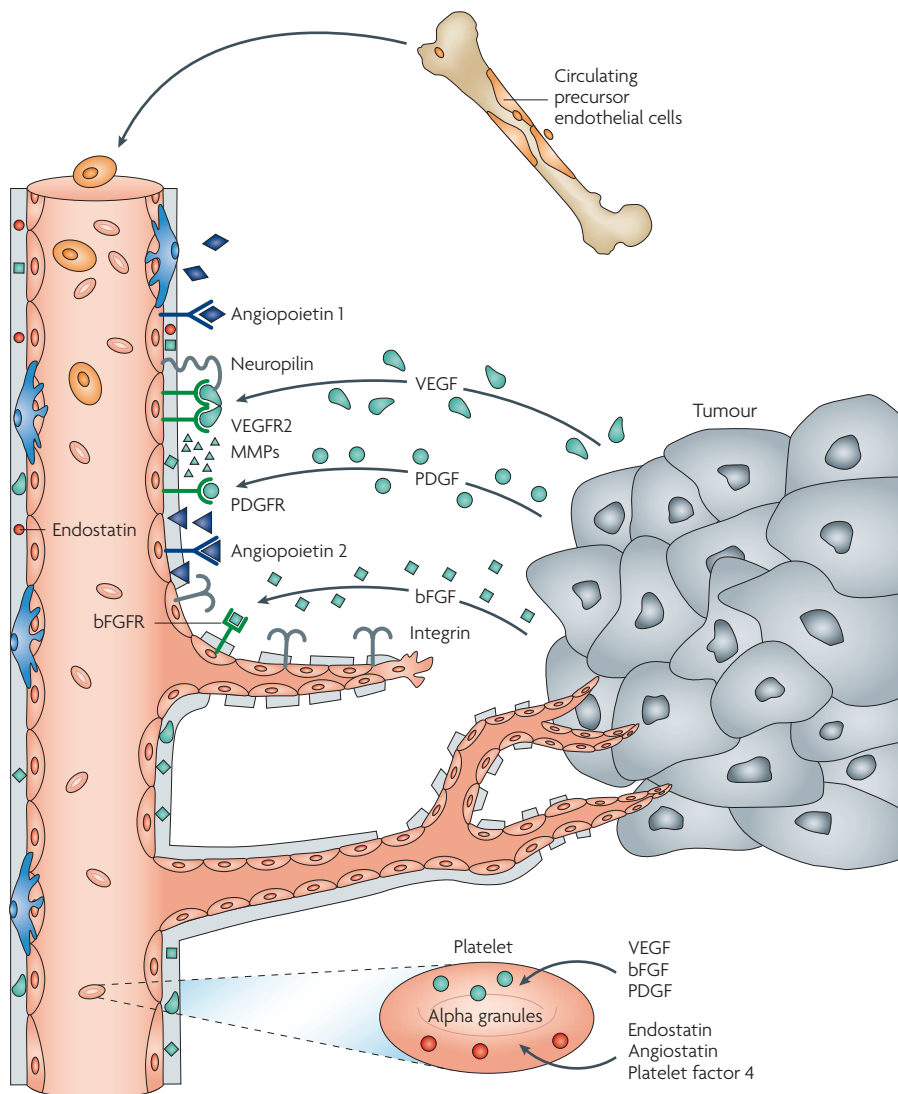
Figure 1 | **Key steps in tumour angiogenesis.** Angiopoietin 1 (ANGPT1), expressed by many cells, binds to the endothelial TIE2 (also known as TEK) receptor and helps maintain a normalized state in blood vessels. Vascular endothelial growth factor (VEGF) is secreted by tumour cells and binds to its receptor (VEGFR2) and to neuropilin on endothelial cells. It is the most common of at least six other pro-angiogenic proteins from tumours. Matrix metalloproteinases (MMPs) are released from tumour cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from stroma, but can also cleave endostatin from collagen 18 in the vessel wall and participate in the cleavage of angiostatin from circulating plasminogen. Tumour cells secrete angio-poietin 2 (ANGPT2), which competes with ANGPT1 for binding to the endothelial TIE2 receptor. ANGPT2 increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. Platelet-derived growth factor (PDGF), an angiogenic protein secreted by some tumours, can upregulate its own receptor (PDGFR) on endothelial cells. Basic fibroblast growth factor (bFGF; also known as FGF2) is secreted by other tumours. Integrins on endothelial cells carry signals in both directions. Integrins facilitate endothelial cell binding to extracellular membranes, a requirement for the cells to maintain viability and responsiveness to growth regulatory proteins. Endothelial cells are among the most anchorage-dependent cells. Certain pro-angiogenic proteins upregulate endothelial integrins and are thought to sustain endothelial cell viability during the intermittant detachments that are required to migrate towards a tumour and to simultaneously increase their sensitivity to growth regulators — both mitogenic (VEGF or bFGF) and anti-mitogenic (endostatin). New endothelial cells do not all originate from neighbouring vessels. A few arrive as precursor bone-marrow-derived endothelial cells. Endothelial growth factors are not all delivered to the local endothelium directly from tumour cells. Some angiogenic regulatory proteins (both pro- and anti-angiogenic) are scavenged by platelets, stored in alpha granules and seem to be released within the tumour vasculature. It was recently discov-ered that pro- and anti-angiogenic proteins are stored in different sets of alpha granules (depicted in green and red respectively)[63].

After the mid-1980s, we and others began to discover additional angiogenesis inhibitors[21–29] (TIMELINE). By the mid-1990s, new drugs with anti-angiogenic activity entered clinical trials. These drugs began to receive Food and Drug Administration (FDA) approval in the United States by 2003. Bevacizumab, which received FDA approval for colorectal cancer in 2004, was the first drug developed solely as an angiogenesis inhibitor[30]. However, certain non-endothelial cells (haematopoietic-derived cells that colonize tumour stroma and some cancer cells, such as those in pancreatic cancer) can also express receptors for vascular endo-thelial growth factor (VEGF; also known as VEGFA), raising the possibility that this drug might also have direct antitumour effects[31,32]. At the time of writing this article, 10 new drugs — in which anti-angiogenic activity is considered to be central to their therapeutic effects — have been approved by the FDA in the United States, and by equiva-lent agencies in 30 other countries, for the treatment of cancer and age-related macular degeneration (TABLE 2). At least 43 other drugs that have varying degrees of anti-angiogenic activity are currently in clinical trials in the United States for different types of cancer, ten of which are in Phase III (TABLE 3). Other FDA-approved drugs revealed anti-angiogenic activity in addi-tion to anticancer activity directed against tumour cells. For example, bortezomib (Velcade; Millennium Pharmaceuticals), approved as a proteasome inhibitor for the treatment of multiple myeloma, was subse-quently demonstrated to also have potent anti-angiogenic activity[33].

As the treatment range of angiogenesis inhibitors covers not only many types of cancer, but also unrelated diseases such as age-related macular degeneration and possibly others, angiogenesis inhibitors, or drugs that have varying degrees of anti-angiogenic activity, might be defined as a class of drugs that specifically target an organizing principle in biomedicine.

Angiogenesis as an organizing principle
Clinical advantages to understanding angio-genesis as an organizing principle.
There are important clinical advantages to viewing angiogenesis as an organizing prin-ciple. For example, if a clinician recognizes that a patient's disease might be partly angio-genesis-dependent, it is conceivable that an angiogenesis inhibitor approved for one type of tumour could be used for a different type of tumour, or even used off-label for a different disease.

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Table 1 | **Angiogenesis-dependent diseases**

Disease	Symptoms
Diabetic retinopathy	Loss of vision
Rheumatoid arthritis[2]	Pain and immobility from destroyed cartilage
Atherosclerotic plaques[3]	Chest pain, dyspnoea
Endometriosis[4,5]	Abdominal pain from intraperitoneal bleeding
Crohn's disease[6]	Intestinal bleeding
Psoriasis[7]	Persistent severe itching
Uterine fibroids	Vaginal bleeding, abdominal pain
Benign prostatic hypertrophy	Urinary retention
Cancer	Bleeding, thrombosis, anaemia, abdominal ascites, bone pain, seizures from cerebral oedema around a tumour and others

An example of the former is the use of bevacizumab in colorectal cancer and also in non-small-cell lung cancer, and an example of off-label use is its use for age-related macular degeneration. Oncologists might also benefit from knowing that certain anticancer drugs (for example, cyclophosphamide) that were originally developed to target cancer cells also have anti-angiogenic activity.

A connection between colorectal cancer and macular degeneration. Bevacizumab is an antibody that neutralizes VEGF and was approved by the FDA for colorectal cancer in 2004 (REFS 30,34). Ranibizumab is a fragment of bevacizumab. In randomized clinical trials, ranibizumab injected into the eye at monthly intervals showed dramatic success in patients with age-related macular degeneration. In patients who were legally blind, with an average visual acuity of ~20/300, approximately 40% recovered their sight and improved to a visual acuity of 20/40 (sufficient for some to drive a car). In ~90–95% of patients, the disease was arrested, and there was no further loss of sight. By contrast, patients who were treated with a placebo continually lost visual acuity over a 12-month period, as was expected[35–40] (FIG. 3a). Pegaptanib (Macugen; OSI Pharmaceuticals), an anti-VEGF aptamer, was the first anti-VEGF drug to be approved by the FDA (2004) for the treatment of age-related macular degeneration. More than 75,000 patients with age-related macular degeneration have been treated with pegaptanib since its approval, and in the past year more than 50,000 patients have been treated with either intravitreal ranibizumab or off-label bevacizumab.

This might be the first time that a relatively non-toxic anticancer drug has been injected into the eye to treat ocular neovascularization. It is rare to treat diseases as diverse as colorectal cancer and age-related macular degeneration with the same agent — with the exception that the target for each was known to be VEGF[41–44].

Discovery of dual roles for cancer drugs. The cancer drugs erlotinib (Tarceva; Genentech, OSI Pharmaceuticals, Roche), cetuximab (Erbitux; Bristol–Myers Squibb, Merck) and vandetanib were originally developed as inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase. For this reason, they are also known as anti-oncoprotein signal-transduction inhibitors[45]. However, they were subsequently found to also inhibit tumour angiogenesis by blocking the VEGF receptor. Cetuximab, an anti-EGFR agent, produces an antitumour effect *in vivo* that is due to the direct blockade of the EGFR-dependent mitogenic pathway and in part to the inhibition of secretion of various pro-angiogenic proteins such as VEGF, basic fibroblast growth factor (bFGF; also known as FGF2) and transforming growth factor-α (TGFα)[46].

Timeline | **Discovery of angiogenesis inhibitors**
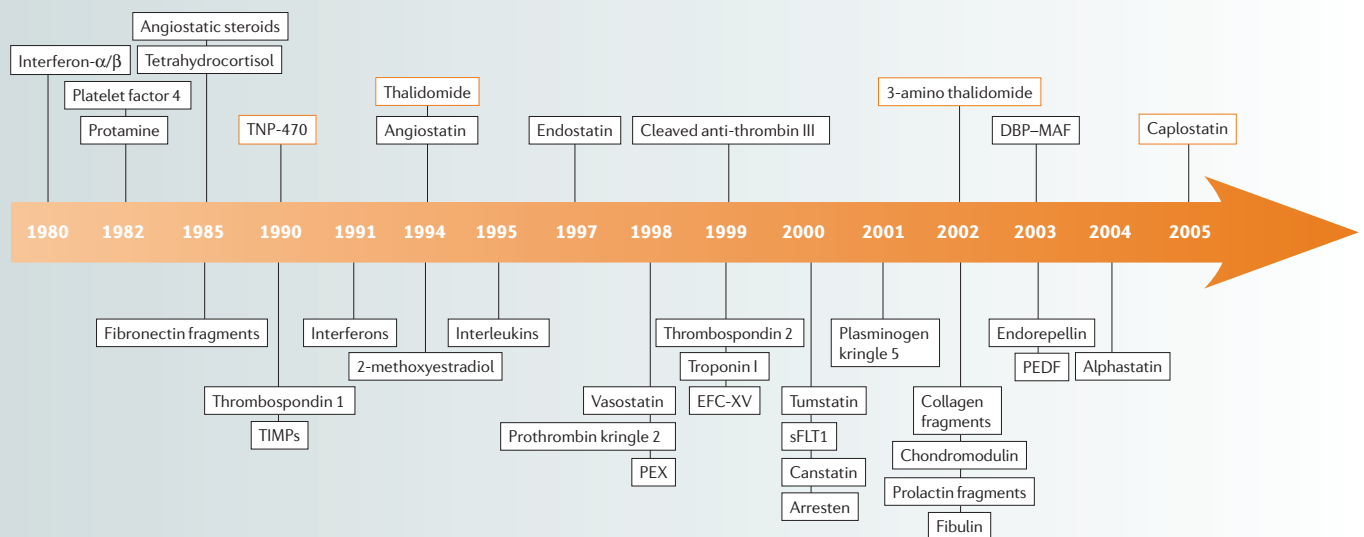
Synthetic angiogenesis inhibitors (orange keyline) and endogenous angiogenesis inhibitors that were identified in the Folkman laboratory are depicted above the timeline. Examples of additional endogenous angiogenesis inhibitors discovered in other laboratories are depicted below the timeline. The first drugs with anti-angiogenic activity were approved in 2003 (TABLE 2). DBP–MAF, vitamin-D-binding protein–macrophage-activating factor; EFC-XV, endostatin-like fragment from type XV collagen; PEDF, pigment epithelium-derived factor (also known as SERPINF1); PEX, haemopexin C domain autolytic fragment of matrix metalloproteinase 2; sFLT1, soluble fms-related tyrosine kinase 1; TIMP, tissue inhibitors of matrix metalloproteinase.

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With this knowledge of their dual role[45], these drugs might be used more effectively by oncologists who could follow guidelines for dose-efficacy of angiogenesis inhibitors, which differ from conventional cytotoxic chemotherapies (see below).

Emerging research directions

The usefulness of recognizing an underlying organizing principle during angiogenesis research is illustrated by several fascinating insights into diverse biological processes. Some examples of these are new insights into platelet biology[47], metastases[22], endothelial control of tissue mass[48,49,72], the concept of oncogene dependence[50] and the surprising discovery that some of the ligand–receptor pairs that mediate axon-pathway finding also mediate

Table 2 | **Anti-angiogenic drugs approved for clinical use and phase of clinical trials for other indications**

Drug (Trade name; company)	Approved*	Phase III	Phase II	Phase I
Bortezomib (Velcade; Millennium Pharmaceuticals)	Multiple myeloma (2003)	NSCLC, multiple myeloma, NHL	Multiple myeloma, NHL, NSCLC, lymphoma, gliomas, melanoma, Waldenstrom's macroglobinaemia, prostate, head and neck, breast, liver, nasopharyngeal, gastric, pancreatic, colorectal, cervical/vaginal cancer, and others	Lymphoma, myelodysplasia, multiple myeloma, NHL, solid tumours, head and neck, cervical, colorectal, ovarian, prostate cancer, and others
Thalidomide (Thalomid; Celgene Corporation)	Multiple myeloma (2003‡)	Multiple myeloma, brain metastases, SCLC, NSCLC, prostate, kidney, ovarian, hepatocellular cancer	Soft tissue sarcoma, multiple myeloma, ALS, melanoma, neuroendocrine tumours, leukaemia, glioma, glioblastomas, paediatric neuroblastoma, NSCLC, NHL, paediatric solid tumours, myelo-fibrosis, myelodysplastic syndrome, AML, CLL, SCLC, Hodgkin's disease, paediatric brain stem, liver, colorectal, kidney, neuroendocrine, endometrial, thyroid, uterine, ovarian cancer, and others	Solid tumours, glioma
Bevacizumab (Avastin; Genentech)	Colorectal cancer (2004), lung cancer (2006)	NSCLC, GIST, diabetic retinopathy, vascular occlusions, retinopathy of prematurity, colorectal, breast, ovarian, peritoneal, pancreatic, prostate, kidney cancer	Glioblastoma, glioma, mesothelioma, NSCLC, AML, CLL, CML, lymphoma, angiosarcoma, melanoma, billary tumours, SCLC, Kaposi's sarcoma, sarcomas, NHL, carcinoid, oesophagogastric, gastric, renal cell, head and neck, rectal, hepatocellular, bladder, pancreatic, gall bladder, breast, neuroendocrine, cervical, ovarian, endometrial cancer, and others	NSCLC, pancreatic, solid tumours, head and neck tumours, VHL, retinal tumours
Erlotinib (Tarceva; Genentech, OSI Pharmaceuticals, Roche)	Lung cancer (2004)	NSCLC, colorectal, pancreatic, ovarian, head and neck, oral cancer	NSCLC, mesothelioma, glioblastoma, glioma, gall bladder, GIST, biliary tumours, bladder cancer prevention, malignant peripheral nerve sheath tumours, endometrial, colorectal, pancreatic, breast, renal cell, prostate, ovarian, head and neck, gastric/oesophageal, liver cancer, and others	NSCLC, glioblastoma, solid tumours, colorectal, pancreatic, head and neck cancer
Pegaptanib (Macugen; OSI Pharmaceuticals)	Age-related macular degeneration (2004)			
Endostatin (Endostar)	Lung cancer (2005§)			
Sorafenib (Nexavar; Onyx Pharmaceuticals)	Kidney cancer (2005)	Kidney, melanoma, hepatocellular cancer	Melanoma, glioblastoma, GIST, SCLC, thyroid, neuroendocrine, mesothelioma, soft tissue sarcoma, NSCLC, CLL, multiple myeloma, cholangiocarcinoma, NHL, kidney, colorectal, prostate, ovarian, peritoneal, pancreatic, breast, gastric, head and neck, uterine, gall bladder, bladder cancer, and others	Solid tumours, melanoma, glioblastoma, NHL, glioma, multiple myeloma, Kaposi's sarcoma, ALL, CML, MDS
Lenalidomide (Revlimid; Celgene Corporation)	Myelodysplastic syndrome (2005)	Multiple myeloma, myelodysplastic syndrome	NSCLC, NHL, multiple myeloma, CLL, myelofibrosis, myelodysplastic syndrome, glioblastoma, ocular melanoma, AML, mantle-cell lymphoma, Waldenstrom's macroglobinaemia, ovarian/peritoneal, thyroid, prostate cancer	Multiple myeloma, prostate cancer, melanoma, myelodysplastic syndrome, solid tumours, paediatric CNS tumours
Sunitinib (Sutent; Pfizer)	GIST, kidney cancer (2006)	Renal cell cancer, GIST	Melanoma, VHL/solid tumour, NSCLC, GIST, hepatocellular, colorectal, prostate, breast, renal cell, gastric, neuroendocrine cancer, and others	Melanoma, solid tumours, colorectal, breast cancer
Ranibizumab (Lucentis; Genentech)	Age-related macular degeneration (2006)			

*Year of first approval by the US Food and Drug Administration, unless stated otherwise. ‡Australia, approved by US Food and Drug Administration in 2006. §China State Food and Drug Administration. ALS, amyotrophic lateral sclerosis (or Lou Gehrig's disease); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CML, chronic myeloid leukaemia; CNS, central nervous system; GIST, gastrointestinal stromal tumour; MDS, myelodysplastic syndromes; NSCLC, non-small-cell lung cancer; NHL, non-Hodgkin's lymphoma; SCLC, small-cell lung cancer; VHL, von Hippel Lindau.

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angiogenesis[51]. Furthermore, genetic variations in the expression of angiogenic proteins between different groups of individuals[52] provide further clues about the role of these angiogenesis-regulatory proteins in different diseases.

Endothelium and neurons share regulatory proteins. In 1998, Klagsbrun and colleagues reported that neuropilin, a cell-surface protein originally identified as a receptor for a signal that guides growing nerves, is also a receptor for VEGF[53,54]. This marked the beginning of a merger between the fields of neural guidance and angiogenesis. It was discovered that various ligand–receptor pairs that mediate axon-pathway finding also mediate angiogenesis[51].

Also, during development, sensory nerves determine the pattern of arterial differentiation in blood-vessel branching in the skin[55]. It was found that in the highly vascular dorsal root ganglia, neuronal VEGF interacts with endothelial cell VEGF receptor 2 (VEGFR2; also known as KDR)[56], which is necessary for endothelial survival. As the interactions of growth and motility proteins for neurons and endothelial cells are gradually uncovered, they might have important roles in drug discovery, for example, for drugs that can repair spinal-cord injuries, reverse Alzheimer's disease or broaden the efficacy of currently approved angiogenesis inhibitors.

New platelet biology. In a review in 2001, my colleagues and I assembled the reports that showed that most of the endogenous angiogenesis-regulatory proteins known at that time were contained in platelets or were on the platelet surface[57]. Several studies subsequently reported that circulating platelets in mice take up and sequester angiogenesis regulatory proteins, such as VEGF, bFGF and connective-tissue-activating peptide, when a microscopic human tumour is present in a mouse[58–60].

The angiogenisis-regulatory proteins are sequestered in alpha granules of platelets at a significantly higher concentration than in plasma. In fact, when radiolabelled VEGF is implanted subcutaneously in a Matrigel pellet in mice, platelet lysates take up virtually all of the radiolabelled VEGF and none is found in plasma[58]. Mouse platelets live for ~3–4 days. Nevertheless, platelets seem to recycle the angiogenesis-regulatory proteins they have scavenged, because the concentration of these proteins increases in the platelets over time (weeks to months), as long as the source of an angiogenesis-regulatory protein is present. Also, a single
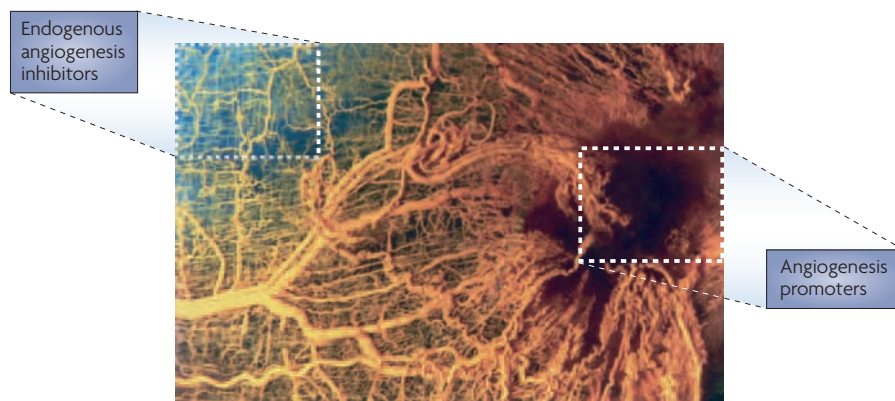

Figure 2 | **Angiogenesis in rat sarcoma.** In this micrograph, blood vessels grow towards a sarcoma (dark area at right) in rat muscle. This contrasts with the normal grid-like pattern of blood vessels that appears at the upper left. (Courtesy of L. Heuser and R. Ackland, University of Louisville, USA)[141].

intravenous injection of thrombospondin 1 (THBS1) (2 μg) into *THBS1*-null mice continues to appear in platelet lysates for weeks (S. Ryeom, personal communication). Furthermore, it was recently reported that in patients with cancer who were receiving bevacizumab, the antibody was taken up by platelets where it was bound to VEGF[61].

This new platelet property, quantifiable by mass spectroscopy of platelet lysates, might permit the development of a biomarker for early detection of tumour recurrence. In tumour-bearing mice, the platelet-angiogenesis proteome detects microscopic tumours at a millimetre size, before they have become angiogenic, but when they are generating angiogenic proteins (VEGF, bFGF and platelet-derived growth factor; PDGF) and anti-angiogenic proteins (endostatin or THBS1)[58,62].

Italiano *et al*. have recently discovered that angiogenesis-regulatory proteins are in fact segregated among two sets of alpha granules in platelets: positive regulators of angiogenesis in one set of alpha granules and negative regulators in the other set[63]. This previously unknown function of platelets links them with the process of angiogenesis. A new opportunity lies ahead to determine whether and how platelets release pro-angiogenic proteins at a wound site and then later release anti-angiogenic proteins. Furthermore, the putative role of platelet release of angiogenesis-regulatory molecules in tumours remains to be elucidated. It might also be possible to develop drugs that selectively release anti-angiogenic proteins from platelets trapped in haemangiomas or in cancer. It is likely that in the future novel angiogenesis-regulatory molecules that could be developed into drugs will be discovered in platelets.

A new mechanism for site specificity of metastasis. It is known that THBS1 is a potent angiogenesis inhibitor[64] that is expressed by fibroblasts and other stromal cells in many tissues. It is also clear that reduction of THBS1 expression in a tumour bed is a necessary prerequisite for induction of neovascularization and for a microscopic tumour to become neovascularized and to grow[65,66]. Watnick and colleagues recently found that certain human tumours produce a novel protein that specifically represses THBS1 in the stromal tissue to which the tumour is subsequently able to metastasize[67]. Suppression of anti-angiogenic activity at the future metastatic site facilitates the initiation of angiogenesis by metastatic tumour cells. If this discovery can be generalized to other tumours, it could be the basis for the development of drugs such as antibodies that could neutralize the THBS1 suppressor protein produced by the primary tumour.

When a new angiogenesis-based metastatic mechanism is uncovered, it is prudent to ask whether the new cancer mechanism could have a physiological counterpart. As in normal tissues, THBS1 is highly expressed under normal conditions in the endometrium[68]. It is not known whether THBS1 is suppressed before the implantation of a fertilized ovum or of a blastocyst, and if so, by what mechanism. This is a topic of current investigation, and there are potential clinical implications. More than 10% of all pregnancies miscarry early in the first trimester. Some women have repeated early miscarriages and are unable to carry a baby to term. *In vitro* fertilization often requires multiple cycles of ovum implantation. Could these problems be the result of insufficient suppression of endometrial THBS1, or of some other endogenous angiogenesis

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Table 3a | **Clinical trials of drugs that have shown anti-angiogenic activity in preclinical models**

Inhibitor (Company)	Target/mechanism	Clinical development
2-methoxyestradiol-EntreMed (EntreMed)	Inhibits HIF1α and tubulin polymerization	• Phase I: Breast cancer and solid tumours • Phase II: Glioblastoma, multiple myeloma, neuroendocrine, renal cell, prostate, ovarian cancer
A6 (Angstrom Pharmaceuticals)	Binds to uPA cell-surface receptor	• Phase II: History of ovarian cancer with rising CA125
Abergrin (MedImmune)	Anti αvβ3 antibody	• Phase I: Melanoma, solid tumours, colorectal cancer • Phase II: Melanoma, prostate cancer, psoriasis, arthritis
ABT-510 (Abbott Laboratories)	Thrombospondin 1 receptor CD36	• Phase I: Head and neck cancer, solid tumours • Phase II: Lymphoma, renal cell, head and neck, NSCLC, soft tissue sarcoma
Actimid (Celgene Corporation)	Downregulates TNF	• Phase II: Prostate cancer (completed)
AG-013736 (Pfizer)	VEGFR, PDGFR	• Phase I: Breast cancer • Phase II: NSCLC, melanoma, thyroid, breast, pancreatic, renal cell cancer
AMG706 (Amgen)	VEGFR, PDGFR, KITR, RETR	• Phase I: Lymphoma, solid tumours, NSCLC, breast, colorectal cancer • Phase II: Solid tumours, NSCLC, gastrointestinal stromal tumours (GIST), breast, thyroid cancer
AP23573 (Ariad Pharmaceuticals)	mTOR, VEGF	• Phase I: Glioma, sarcoma, solid tumours, multiple myeloma • Phase II: Endometrial cancer, prostate cancer, haem malignancies
AS1404 (Antisoma)	Vascular disrupting agent, releases TNF and vWF	• Phase II: Prostate cancer
ATN-161 (Attenuon)	α5β1 antagonist	• Phase II: Renal cell cancer, malignant glioma
AZD2171 (AstraZeneca)	VEGFR1, VEGFR2, VEGFR3, PDGFR	• Phase I: NSCLC, AML, colorectal, head and neck cancer, CNS tumours (child) • Phase II: Solid tumours, NSCLC, glioblastoma, melanoma, mesothelioma, neurofibromatosis, ovarian, CLL, colorectal, breast, kidney, liver, SCLC • Phase III: NSCLC
BMS-275291 (Bristol–Myers Squibb)	MMP inhibitor	• Phase I: Kaposi's sarcoma • Phase II: Kaposi's sarcoma, prostate cancer, NSCLC • Phase III: NSCLC
CCI-779 (Wyeth)	mTOR, VEGFR	• Phase I: Solid tumours, prostate cancer, CML, and others • Phase II: CLL, melanoma, glioblastoma, multiple myeloma, GIST, SCLC, NHL, NSCLC, neuroendocrine tumours, breast, pancreatic, endometrial cancer, and others
CDP-791 (Imclone Systems)	VEGFR2, KDR	• Phase II: NSCLC
Celecoxib (Pfizer)	Increases endostatin	• Phase I: NSCLC, pancreatic, prostate cancer, solid tumours • Phase II: Head and neck cancer prevention, breast cancer prevention, lung cancer prevention, NSCLC, paediatric solid tumours, Ewing's sarcoma, glioma, skin cancer prevention, basal cell nevus syndrome, Barrett's oesophagus, hepatocellular, oesophogael, prostate, cervical, colorectal, head and neck, breast, thyroid, nasopharyngeal cancer, and others • Phase III: Colon, prostate, bladder cancer, NSCLC, and others
Cilengitide (EMD Pharmaceuticals)	αvβ3 and 5 antagonist	• Phase I: Solid tumours, lymphomas, paediatric brain tumours • Phase II: Glioblastoma, gliomas
Combretastatin (Oxigene)	VE-cadherin	• Phase I: Solid tumours • Phase II: Solid tumours, anaplastic thyroid cancer
E7820 (Eisia Medical Research Inc.)	Inhibits integrin α2 subunit on endothelium	• Phase I: Lymphoma • Phase II: Colorectal cancer
Everolimus (Novartis)	VEGFR, mTOR	• Phase I: Breast cancer, solid tumours, lymphoma • Phase II: NSCLC, melanoma, AML, ALL, CML, lymphoma, glioblastoma, prostate, colorectal, neuroendocrine, breast, endometrial, kidney cancer, paediatric tumours, solid tumours • Phase III: Islet cell pancreas II/III, and others
Genistein (National Cancer Institute (NCI), USA)	Suppresses VEGF and neuropilin and MMP9 in tumour cells, upregulates CTAP	• Phase I: Melanoma, kidney, prostate, bladder, breast cancer
Homoharringtonine (ChenGenex Therapeutics)	Downregulates VEGF in leukaemic cells	• Phase II: CML, APML • Phase III: CML
IMC-1121b (Imclone Systems)	VEGFR2, KDR	• Phase I: Solid tumours
INGN 241 (Introgen Therapeutics)	MDA7, VEGF	• Phase II: Melanoma

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Table 3b | **Clinical trials of drugs that have shown anti-angiogenic activity in preclinical models**

Inhibitor (Company)	Target/mechanism	Clinical development
Interleukin-12 (NCI)	Upregulates IP10	• Phase I: Solid tumours, melanoma, paediatric neuroblastoma, kidney, breast cancer • Phase II: Melanoma, NHL, multiple myeloma, breast, ovarian, peritoneal, prostate cancer
Enzastaurin (Eli Lilly and Company)	VEGF	• Phase I: Solid tumours, gliomas • Phase II: Gliomas, lymphoma, brain tumours, NSCLC, pancreatic, colorectal cancer • Phase III: Lymphoma prevention, glioblastoma
Neovastat (Aeterna Zentaris)	MMP inhibitor	• Phase II: Multiple myeloma • Phase III: Kidney, NSCLC
NM-3 (Genzyme Corporation)	Inhibits VEGF expression by tumour cells, inhibits endothelial proliferation	• Phase I: Solid tumours
NPI-2358 (Nereus Pharmaceuticals)	β-tubulin	• Phase I: Solid tumours
Phosphomannopentaose sulphate (Progen Industries, Medigen Biotechnology)	bFGF, stimulates release of TFP1	• Phase II: Melanoma, NSCLC, prostate, hepatocellular cancer
PKC412 (Novartis)	VEGFR2	• Phase I: AML • Phase II: Mast-cell leukaemia
PPI-2458 (Praecis)	METAP2	• Phase I: Solid tumours, NHL
Prinomastat (Agouron Pharmaceuticals)	MMP inhibitor	• Phase II: Glioblastoma
PXD101 (CuraGen Corporation)	HDAC inhibitor	• Phase I: Solid tumours, haem malignancies • Phase II: Multiple myeloma, myelodysplastic syndrome, lymphoma, AML, NHL, ovarian/peritoneal, liver cancer
Suramin (NCI)	IGF1, EGFR, PDGFR, TGFβ, inhibits VEGF and bFGF	• Phase I: Bladder, breast, kidney cancer • Phase II: Glioblastoma, breast, kidney, adrenocortical cancer • Phase III: Prostate cancer
Tempostatin (Collard Biopharmaceuticals)	Extracellular matrix proteins	• Phase I: Solid tumours • Phase II: Kaposi's sarcoma
Tetrathiomolybdate (Sigma–Aldrich)	Copper chelator	• Phase II: Prostate, oesophageal, breast, colorectal cancer • Phase III: Psoriasis
TKI-258 (Novartis, Chiron Corporation)	FGFR3, VEGFR	• Phase I: Multiple myeloma, AML, melanoma
Vatalanib (Novartis)	VEGFR1,2, PDGFR	• Phase I: Solid tumours, NSCLC, gynaelogic tumours • Phase II: GIST, AML, CML, solid tumours, NSCLC, VHL, haemangioblastoma, mesothelioma, breast, prostate, pancreatic, neuroendocrine cancer, glioblastoma, meningioma, myelodysplastic syndrome, multiple myeloma, age-related macular degeneration • Phase III: Colorectal cancer
VEGF Trap (Regeneron Pharmaceuticals)	VEGF	• Phase I: NHL, age-related macular degeneration, diabetic macular oedema • Phase II: Kidney, ovarian cancer, NSCLC, age-related macular degeneration • Phase III: Ovarian cancer
XL184 (Exelixis)	MMET, VEGFR, RTK, FLT3, TIE2	• Phase I: Solid tumours
XL880 (Exelixis)	C-met, RTK	• Phase I: Solid tumours • Phase II: Papillary renal cell carcinoma
XL999 (Exelixis)	VEGFR, PDGFR, EGFR, FLT3, Src	• Phase I: Solid tumours • Phase II: Multiple myeloma, colorectal, ovarian, renal cell cancer, AML, NSCLC
ZD6474 (AstraZeneca)	VEGFR2, EGFR	• Phase I: Glioma • Phase II: Breast cancer, NSCLC, SCLC, thyroid, gliomas, multiple myeloma • Phase III: NSCLC

AML, acute myeloid leukaemia; APML, acute promyelocytic leukaemia; bFGF, basic fibroblast growth factor; CML, chronic myeloid leukaemia; CLL, chronic lymphocytic leukaemia; CNS, central nervous system; CTAP, connective tissue activation peptide; EGFR, epidermal growth factor receptor; FGFR3, fibroblast growth factor receptor 3; FLT3, fms-related tyrosine kinase 3; HDAC, histone deacetylase; HIF1, hypoxia-inducible factor 1; IGF1, insulin-like growth factor 1; IP10, inducible protein 10; KDR, kinase insert domain receptor; MDA7, interleukin-24; METAP2, methionyl aminopeptidase 2; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; NHL, non-Hodgkin's lymphoma; NSCLC, non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; RETR, ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease) receptor; SCLC, small-cell lung cancer; TFP1, transferrin pseudogene 1; TGFβ, transforming growth factor-β; TNF, tumour-necrosis factor; uPA, urokinase-type plasminogen activator; VE-cadherin, vascular/endothelial-cadherin; VEGF(R), vascular endothelial growth factor (receptor); vWF, von Willebrand factor.

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inhibitor in the endometrium? If so, could this condition be diagnosed by the measurement of THBS1 in the vaginal fluid? Could endometrial THBS1 then be suppressed, for example, by a vaginal suppository containing a putative short-acting THBS1-suppressor protein? Another intriguing finding is that haemangiomas, benign tumours of infancy, have the gene signature of cells of the fetal placental endothelium, implying that they might originate from the fetal placenta[69,70] (BOX 1). As haemangiomas usually regress spontaneously, they might reveal important clues about the molecular mechanisms of spontaneous regression of new blood vessels.

Endothelial cell control of tissue mass. When approximately 70% of the liver is removed in a rat (hepatectomy), the original mass regenerates completely in approximately 10 days[71]. Hepatocyte proliferation and endothelial cell proliferation are initiated the day after surgery. At approximately day 8, there is a wave of endothelial cell apoptosis, following which hepatocyte proliferation ceases[48]. The liver stops growing at ~10 days. However, if an angiogenic protein, such as VEGF or bFGF, is administered systemically, endothelial cells continue to proliferate and the liver continues to grow beyond its normal size. By contrast, if a specific inhibitor of endothelial proliferation is administered, liver regeneration is prevented and the liver remains at 30% of its normal size. Discontinuation of the endothelial inhibitor is followed immediately by liver regeneration that is complete by 10 days[48]. These experiments indicate that normal tissue and organ regeneration are controlled in part by the microvascular endothelium.

Growth and regression of fat is controlled by endothelial proliferation or apoptosis, respectively[49]. Leptin-deficient mice gain up to approximately 1 gram per day, mainly in fat. Adipocyte enlargement and proliferation is accompanied by endothelial proliferation that is restricted to fat. Systemic administration of an angiogenesis inhibitor (TNP-470 or endostatin) specifically induces endothelial apoptosis and a decrease in fat accompanied by rapid weight loss. When the normal weight for age is reached, weight loss stops. A similar result is obtained when endothelial cells in fat are specifically targeted by a genetically regulated inhibitor of proliferation[72]. Growth of normal prostate is also under endothelial control[73], and so is bone growth[74]. Therefore, it seems that microvascular endothelial cells can control tissue mass, regardless of whether the cells in this mass have a normal genome or a cancer
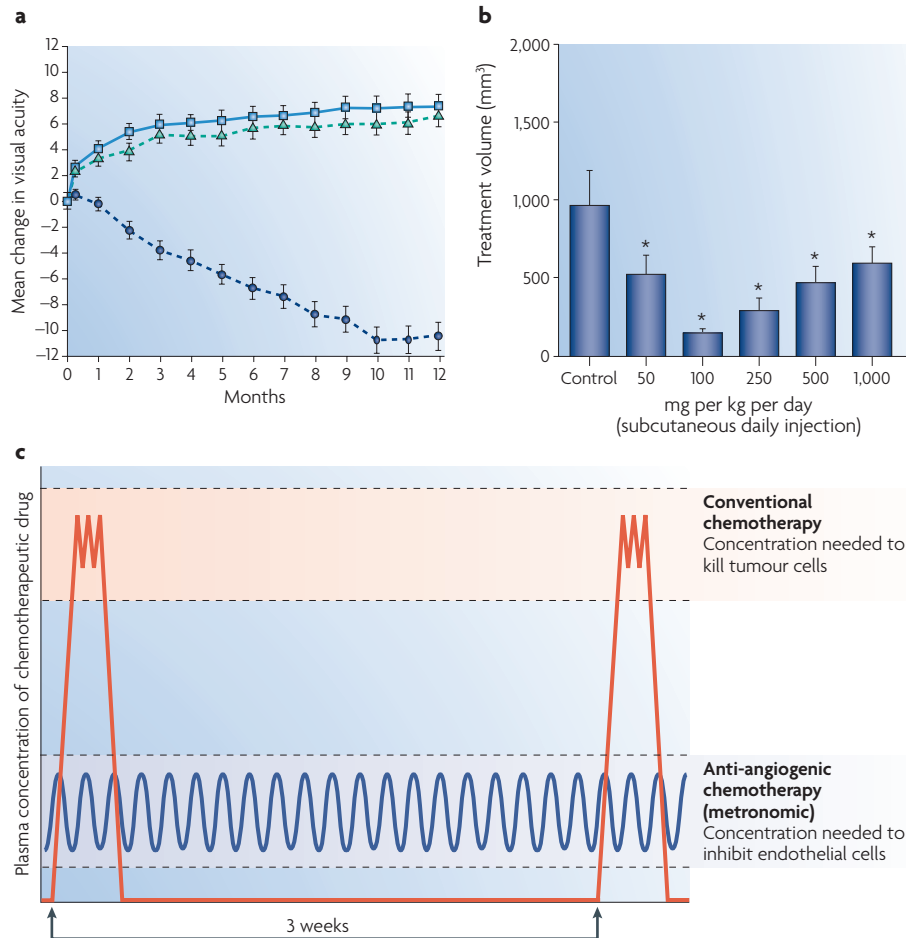
genome[75]. This raises a provocative question: Is there some type of set-point or feedback mechanism in endothelial cells that tells them when a normal organ, such as liver, has reached its normal mass? If so, do tumour cells override this mechanism, and how?

What are the implications of this general principle for drug discovery? There is the possibility that specific endothelial inhibitors might be used to control obesity[72], as well as overgrowth of other tissues, such as uterine fibroids, overgrowth of bone caused by lymphangiogenesis and ectopic bone growth (fibrodysplasia ossificans progressiva)[76]. Specific endothelial inhibitors might also be used to control vascular malformations that grow rapidly after puberty or after attempts at surgical excision, and for which there are currently no drugs. The endocrine-specific angiogenesis-regulatory proteins, such as

Bombina variegata peptide 8 kDa protein[77] (Bv8; also known as PROK2; testicular-cancer-specific), are of particular interest.

Is oncogene dependence angiogenesis dependent? The recognition that endothelial cells control tumour mass is crucial for a more complete understanding of how oncogenes initiate tumour growth. The conventional wisdom is that oncogene activation in a cell leads directly to the formation of large lethal tumours in mice. This concept is reinforced by experiments in which *Ras* or *Myc* oncogenes, under the control of the doxycycline promoter, induce rapid tumour growth when the oncogene is activated, leading to rapid tumour regression when the oncogene is inactivated[78–83]. This phenomenon is called oncogene dependence or oncogene addiction[84].

Figure 3 | **Examples of anti-angiogenic therapy. a** | Phase III clinical trial of Lucentis (ranibizumab; Genentech), a fragment of Avastin, an antibody to vascular endothelial growth factor (VEGF). Lucentis is used for intra-ocular injection in patients with age-related macular degeneration[37]. **b** | A biphasic (U-shaped) dose-efficacy curve for human pancreatic cancer in immunodeficient mice treated with endostatin. The tumour cells are also deficient in p53 (adapted from REF. 119). **c** | Dosing schedule differences between conventional chemotherapy (red) and anti-angiogenic (metronomic) chemotherapy (blue) (adapted from REF. 131 and from discussions with R. Kerbel).

However, my group has found that during oncogene-induced tumour growth there is intense tumour angiogenesis associated with suppression of THBS1 in the tumour bed. When an oncogene is inactivated, the expression of THBS1, a potent angiogenesis inhibitor, is increased in the tumour bed, leading us to propose that oncogene addiction is angiogenesis dependent[83]. This hypothesis has now been supported by the deletion of THBS1 in the tumour and the host. In these mouse models, an activated oncogene induces more rapid tumour growth than in wild-type mice, but tumours do not regress after the inactivation of the onco-gene[82]. Restoration of THBS1 expression in the tumour results in tumour regression upon oncogene inactivation[50].

How could this change in thinking about oncogene addiction provide new oppor-tunities in drug discovery? Conventional wisdom (FIG. 4) suggests that the develop-ment of drugs targeted against oncogenes should be sufficient to control cancer. Imatinib (Gleevec; Novartis), which targets the product of the *BCR–ABL* oncogene, has demonstrated proof-of-concept by its success in the treatment of chronic myeloid leukaemia. Furthermore, imatinib targets the product of the oncogene *cKIT*, and has also proved successful in treating gastrointestinal stromal tumours in which this protein has a key role[85,86]. However, many patients eventually develop drug resistance[87], and there are numerous other oncogenes that could be responsible for inducing expres-sion of redundant growth factors in these tumours. The imatinib experience also suggests that drugs will need to be developed against combinations of many oncogenes. A single angiogenesis inhibitor, especially a broad-spectrum angiogenesis inhibitor such as endostatin[28] or caplostatin, or a combina-tion of angiogenesis inhibitors might block the effect of a large family of oncogenes, as the blockade of angiogenesis can prevent tumour growth downstream of oncogene activation. Analysis of 15 of the most studied oncogenes revealed that the majority of them increase the expression of VEGF (and/or bFGF) and decrease the expression of THBS1 in tumour cells[88,89].

Genetic regulation of angiogenesis. Although we all carry endogenous angiogenesis inhibi-tors in our blood and tissues (at least 29 at the time of writing)[24,25,90], different individuals reveal distinct genetic differences in their angiogenic response to a given stimulus. For example, individuals with Down syndrome are protected against diabetic retinopathy,

> **Box 1 | Are infantile haemangiomas metastases from the placenta?**
>
> Haemangiomas are benign tumours made of capillary blood vessels that appear in 1 out of 100 newborns and usually begin to undergo spontaneous regression at approximately the end of the first year[6]. Some haemangiomas can be life threatening if they occur in the brain, airway or liver. The mechanism of haemangioma regression is unclear. Haemangiomas provide the possibility that they might reveal a clue about molecular mechanisms of spontaneous regression of new blood vessels. It was recently reported that all infantile haemangiomas express the glucose receptor GLUT1 (also known as SLC2A1), and that this receptor is also found on the endothelium of the placenta[69]. This observation led to a gene array analysis of endothelial cells from haemangioma and other tissues, which revealed that gene expression of haemangioma endothelium is identical to gene expression of fetal placental endothelium, but not to any other tissue analyzed[70]. The implication is that haemangiomas might be metastases from the fetal placenta. A further implication is that putative endogenous angiogenesis inhibitors that control the regression of placental vasculature at term might also be involved in the regression of haemangiomas. This speculation remains to be tested, but it illustrates how viewing a given process as part of an organizing principle can be useful.

although they have a similar incidence of diabetes as individuals without Down syn-drome[52,91]. They also have higher levels of cir-culating endostatin (~1.6-fold) than normal individuals because of an extra copy of the gene for the endostatin precursor (collagen XVIII) on chromosome 21 (REFS 91,92). Interestingly, they seem to be among the most protected of all humans against cancer. Although testicular cancer and a meg-akaryocytic leukaemia have been reported for individuals with Down syndrome, they have the lowest incidence of the other ~200 human cancers compared with age-matched controls[52,91]. Conversely, individuals with a polymorphism in endostatin (specifically arginine substituted for alanine at N104) have a significantly higher risk of breast cancer[93]. The correlation between endostatin levels and cancer susceptibility was demon-strated in mice. Mice that were engineered to genetically overexpress endostatin to mimic individuals with Down syndrome have tumours that grow 300% slower[94], and in mice that had THBS1 deleted, tumours grow approximately 300% more rapidly, and more quickly still if two angiogenesis inhibitors are knocked out (tumstatin and THBS1)[94].

Another interesting finding is that African Americans rarely develop the 'wet' form of age-related macular degenera-tion. They usually do not have intravitreal haemorrhages and do not go blind from the 'dry' form of this disease[95]. By contrast, African Americans have a similar incidence of diabetic retinopathy. In age-related macular degeneration, neovascularization is in the choroidal layer that is surrounded by melanocytes containing melanin. In diabetic retinopathy, neovascularization arises from the retina. The retinal pigmented epithelial cells contain a lighter form of oxidized melanin, which differs from melanin in

the choroid or in the skin. Also, African American infants rarely develop cutaneous haemangiomas compared with white infants.

These correlations suggest that factors linked to pigmentation and melanin are producing an inhibitory influence on the ang-iogenic balance in the melanin-rich tissues. However, there is no melanin in prostate or breast tissue, and African Americans are not protected from cancer of these organs.

This hypothesis was examined in animal experiments. When the gene for tyrosinase (in the melanin pathway) was deleted from mice, the albino relatives C57Bl/6J-Tyr[c-2J] showed intense iris neovascularization and haemorrhage (hyphaema) compared with weak neovascularization and no haemor-rhage in the pigmented iris of wild-type mice. The amount of corneal neovasculariza-tion was not significantly different between these two strains because the cornea is not a pigmented tissue[96,97].

Genetic variations of angiogenic factors have important consequences for the clini-cal treatment of angiogenesis-dependent
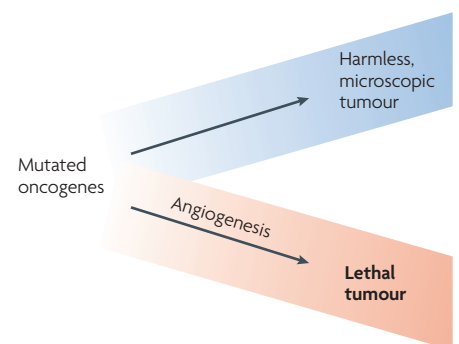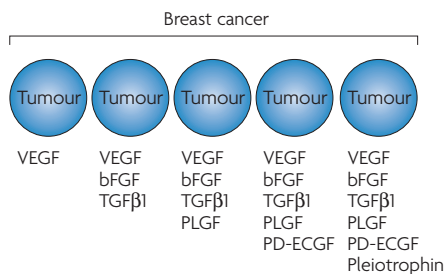

Figure 4 | **Oncogene addiction is angiogenesis dependent.** An oncogene-induced tumour that cannot recruit new blood vessels will remain as a harmless microscopic tumour in experimental animals[50,83].

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Figure 5 | **Angiogenic proteins in breast cancer.** Human breast cancer can cause the expression of at least six different angiogenic proteins (adapted from REF. 142). bFGF, basic fibroblast growth factor (also known as FGF2); PD-ECGF, platelet-derived endothelial cell growth factor (also known as ECGF1); PLGF, placental growth factor (also known as PGF); TGFβ1, transforming growth factor-β1; VEGF, vascular endothelial growth factor.

diseases. For example, some tumours that seem poorly vascularized and have a low microvessel density will be inhibited by a significantly lower dose of an angiogenesis inhibitor than is required for a highly vascularized tumour with a significantly higher microvessel density[98]. This may be counter-intuitive to clinicians who might inform a patient that their tumour is not very vascular and therefore will not respond to anti-angiogenic therapy. In fact, these tumours might be expressing their own angiogenesis inhibitors[99,100] and might respond to a lower therapeutic dose of angiogenesis inhibitor than would be required for a highly vascularized tumour.

The genetic heterogeneity of the angiogenic response is another reason for the pressing need to develop blood or urine biomarkers[101] to optimize the dosing of anti-angiogenic therapy. Furthermore, when mice are used for preclinical studies of angiogenesis inhibitors, it is crucial to know the genetic background of the mice in regards to their angiogenic responsiveness.

Optimizing anti-angiogenic therapy

Insights into the molecular mechanisms and significance of angiogenesis in different biological contexts are creating exciting new opportunities for drug discovery. However, as in some cases including cancer, anti-angiogenic therapies can also be used in combination with existing drugs. It is important to understand the difference between anti-angiogenic and cytotoxic drugs to optimize efficacy.

Anti-angiogenic therapy and cytotoxic chemotherapy. In February 2004, when the FDA approved bevacizumab for colorectal cancer, M. McClellan, then FDA Commissioner, said: "Anti-angiogenic therapy can now be considered the fourth modality for cancer treatment."[102]. It is a different modality because there are certain notable differences about chemotherapy that do not always readily transfer to anti-angiogenic therapy.

Importantly, anti-angiogenic therapy primarily targets the activated microvascular endothelial cells in a tumour bed rather than the tumour itself. It can accomplish

this directly by preventing endothelial cells from responding to angiogenic proteins, as endostatin[28] and caplostatin[103,104] do. Anti-angiogenic therapy can also inhibit endothelial cell proliferation and motility indirectly by suppressing a tumour's production of angiogenic proteins, as erlotinib does[105], or by neutralizing one of these proteins, as bevacizumab does.

Also, although chemotherapy is usually more effective on rapidly growing tumours than on slowly growing tumours, the opposite is often true of anti-angiogenic therapy. More rapidly growing tumours can require higher doses of anti-angiogenic therapy[98]. Furthermore, chemotherapy is optimally given at a maximum tolerated dose, with off-therapy intervals of 1–3 weeks to rescue bone marrow and intestine. Anti-angiogenic therapy might optimally require that endothelial cells be exposed to steady blood levels of the inhibitor[100]. Therefore, daily dosing is optimal for those angiogenesis inhibitors with a short half-life. However, certain antibodies such as bevacizumab can be administered every 2 weeks because of long-lasting antibody levels in plasma, and perhaps because of neutralization of VEGF in platelets by bevacizumab that enters the platelets and binds with VEGF[61]. Zoledronate (Zometa; Novartis) is an amino-bisphosphonate that has been shown to inhibit angiogenesis[106] by targeting matrix metalloproteinase 9 (MMP9)[107], by reducing circulating levels of pro-angiogenic proteins in the circulation[108] or by suppressing multiple circulating pro-angiogenic factors in patients with cancer[109]. It accumulates in bone and can therefore be administered every month. However, after prolonged use, zoledronate may need to be administered less frequently to avoid osteonecrosis of the jaw.

Another important difference concerns the side effects of anti-angiogenic therapy compared with chemotherapy. Bone-marrow suppression, hair loss, severe vomiting and diarrhoea, and weakness are less common with anti-angiogenic therapy, and endostatin has shown minimal or no side effects in animals[110] and in humans[111]. It has to be noted though, that certain angiogenesis inhibitors increase the incidence of thrombotic complications, such as thalidomide (Thalomid; Celgene)[112] and bevacizumab. The risk of thrombosis is increased when these angiogenesis inhibitors are administered together with conventional chemotherapy[113]. Other side effects of inhibitors of VEGF include hypertension, intratumoural bleeding and bowel perforation, especially

Table 4 | **Three types of angiogenesis inhibitors**

Mechanism	Drug	Action
Type 1		
Blocks one main angiogenic protein	Avastin (Avastin; Genentech)	Blocks VEGF
	VEGF Trap (Regeneron Pharmaceuticals)	Blocks VEGF
Type II		
Blocks two or three main angiogenic proteins	Sutent (Sutent; Pfizer)	Downregulates VEGF receptor 2, PDGF receptor, cKIT receptor
	Tarceva (Tarceva; Genentech, OSI Pharmaceuticals, Roche)	Downregulates VEGF production, bFGF production, TGFα by tumour cell
Type III		
Blocks a broad range of angiogenic regulators	Endostatin	Downregulates VEGF, bFGF, bFGF receptor, HIF1α, EGF receptor, ID1, neuropilin Upregulates thrombospondin 1, maspin, HIF1α, TIMP2
	Caplostatin	Broad anti-angiogenic and anticancer spectrum

bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; HIF1α, hypoxia-inducible factor 1α; ID1, inhibitor of DNA binding 1, dominant negative helix–loop–helix protein; PDGF, platelet-derived growth factor; TIMP2, tissue inhibitor of metalloproteinase 2; TGFα, transforming growth factor-α; VEGF, vascular endothelial growth factor.

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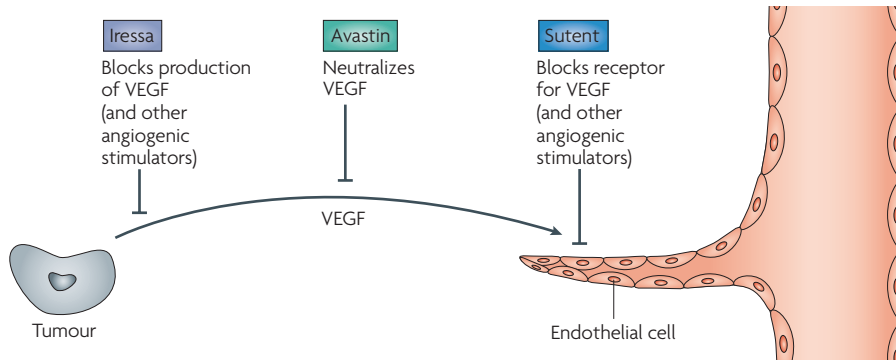
Figure 6 | **Three general mechanisms of angiogenesis inhibitors currently approved by the FDA.** Iressa blocks tumour expression of an angiogenic factor. Avastin blocks an angiogenic factor after its secretion from a tumour. Sutent blocks an endothelial cell receptor. VEGF, vascular endothelial growth factor.

in cases in which the intestine contains a tumour. Thalidomide has a slightly higher incidence of thromboembolic complications, as well as constipation and peripheral neuropathy — these are usually reversible upon discontinuation of thalidomide. Lenalidomide (Revlimid; Celgene), an FDA-approved derivative of thalidomide, has significantly reduced side effects. Side effects need to be carefully considered, especially when anti-angiogenic and cytotoxic medications are combined. So far, there are almost no data that allow a direct comparison of clotting risk for anti-angiogenic therapy alone, compared with cytotoxic therapy or combination therapy.

However, there can also be unexpected benefits from combining angiogenesis inhibitors, or drugs that have varying degrees of anti-angiogenic activity, with conventional chemotherapy. For example, Jain has shown that bevacizumab, by decreasing vascular leakage in a tumour, can lower intratumoral-tissue pressure and increase delivery of chemotherapy to a tumour[114]. In other words, anti-angiogenic therapy might 'normalize' tumour vessels[115]. Teicher et al. showed that anti-angiogenic therapy could decrease intratumoural pressure, which resulted temporarily in increased oxygenation to a tumour with subsequent increased sensitivity to ionizing radiation[116].

Biphasic dose efficacy of anti-angiogenic therapy. Dose efficacy is generally a linear function for chemotherapy. By contrast, several angiogenesis inhibitors have been reported to follow a biphasic, U-shaped dose-efficacy curve (known as hormesis)[117]. For example, interferon-α (IFNα) is anti-angiogenic at low doses, but not at higher doses[118]. Similarly, rosiglitazone (Avandia;

GlaxoSmithKline), a peroxisome proliferator-activated receptor-γ (PPARγ) ligand, as well as endostatin protein therapy[119] (FIG. 3b) and endostatin gene therapy[120] inhibit angiogenesis with a U-shaped dose-efficacy curve[121]. Before the U-shaped dose-efficacy response was recognized for anti-angiogenic gene therapy, my group had observed that gene therapy of endostatin could produce such high blood levels that all anti-angiogenic activity was lost[122]. It is now clear that blood levels of certain angiogenesis inhibitors (such as endostatin) that are too high or too low will be ineffective, and that the biphasic dose-efficacy curve offers the best explanation for why endostatin gene therapy of murine leukaemia failed[123,124].

Even the effect of endostatin on the gene expression (for example, hypoxia-inducible factor 1α; HIF1α) of fresh human endothelial cells *in vitro* reveals a U-shaped dose-efficacy pattern[28]. This is important information for drug discovery. For example, in the ranibizumab trial for age-related macular degeneration, a higher dose did not increase efficacy over a lower dose.

Anti-angiogenic therapy and drug resistance. Tumours might become refractory to anti-angiogenic therapy, especially if a mono-anti-angiogenic therapy targets only one angiogenic protein (for example, VEGF)[124]. Endothelial cells seem to have a lower probability for developing resistance to anti-angiogenic therapy, even though mouse endothelial cells in a tumour bed can become genetically unstable[80,125]. Although VEGF is expressed by up to 60% of human tumours, most tumours can also express five to eight other angiogenic proteins — for example, human breast cancers can express up to six angiogenic proteins (FIG. 5). High-grade brain tumours might express more

angiogenic proteins than other tumours. When the expression of one angiogenic protein is suppressed for a long period, the expression of other angiogenic proteins might emerge[126]. The mechanism of this 'compensatory' response is unclear. Some angiogenesis inhibitors target up to three angiogenic proteins, whereas others target a broad range of angiogenic proteins (TABLE 4). Certain tumours, such as high-grade giant-cell tumours and angioblastomas, produce bFGF as their predominant angiogenic protein and do not seem to deviate from this. For this reason, low-dose daily IFNα therapy for 1–3 years is sufficient to return abnormally high levels of bFGF in the urine of these patients to normal. IFNα has been reported to suppress the production of bFGF by human cancer cells[118]. This treatment regimen has produced long term complete remissions (up to 10 years) without drug resistance (at the time of writing; see REFS 127–129 and L. Kaban, personal communication).

Currently, the majority of FDA-approved angiogenesis inhibitors, as well as those in Phase III clinical trials, neutralize VEGF, target its receptor or suppress its expression by tumour cells (FIG. 6). When drug resistance develops to some of these inhibitors, they are often perceived to represent the whole class of angiogenesis inhibitors. It remains to be seen if broad-spectrum angiogenesis inhibitors will develop less drug resistance than angiogenesis inhibitors that target against a single angiogenic protein. In experimental tumours, TNP-470, a synthetic analogue of fumagillin and caplostatin, its derivative[103,130], did not induce drug resistance when administered to mice for prolonged periods of time[104].

Anti-angiogenic chemotherapy (metronomic therapy). Browder et al. first reported that when murine tumours were made drug resistant to cyclophosphamide and then cyclophosphamide was administered on a conventional chemotherapy maximum-tolerated dose schedule, all mice died of large tumours[131]. However, if cyclophosphamide was administered more frequently at a lower dose, the tumours were potently inhibited because of endothelial apoptosis. If an angiogenesis inhibitor (TNP-470)[104] was added, which by itself could only inhibit the tumours by 50%, the drug-resistant tumours were eradicated[131]. This experiment demonstrated a new principle: a cytotoxic chemotherapeutic agent could be redirected to an endothelial target by changing its dose and frequency of
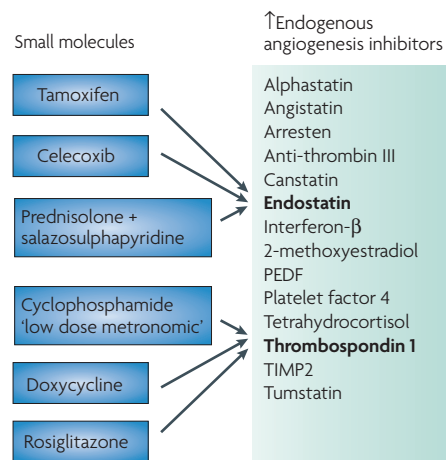
Figure 7 | **Small molecules to increase endogenous angiogenesis inhibitors.** Examples of small molecules that are orally available and might induce increased levels of endogenous angiogenesis inhibitors in the blood or joint fluid. PEDF, pigment epithelium-derived factor; TIMP2, tissue inhibitor of matrix metalloproteinase 2 (REF. 124).

administration. Browder *et al.* called this regimen anti-angiogenic chemotherapy. Klement *et al.* confirmed this approach with a different chemotherapeutic agent[132]. Bocci *et al.*[133] further showed that anti-angiogenic chemotherapy increased circulating THBS1, and that deletion of the *THBS1* gene in mice completely abrogated the antitumour effect of this anti-angiogenic therapy. These results suggested that THBS1 acts as a mediator of anti-angiogenic chemotherapy[133]. The optimization of chemotherapy to treat vascular endothelium in the tumour bed is also called 'metronomic' therapy[134] (FIG. 3c) and has entered clinical trials for brain tumours and other tumours that were refractory to conventional chemotherapy. Kieran *et al.* recently studied 20 children with different types of brain tumours refractory to surgery, radiotherapy and chemotherapy, who were treated for 6 months with daily oral thalidomide and celecoxib (Celebrex; Pfizer), plus daily low-dose oral cyclophosphamide alternated every 21 days with daily low-dose oral etoposide[135]. Twenty-five percent of the patients were progression free more than 2.5 years from starting therapy. Forty percent of patients completed the 6 months of therapy, resulting in prolonged or persistent disease-free status. Sixteen percent of patients showed a radiographic partial response. Only elevated THBS1 levels in the blood correlated with prolonged response. This is consistent with the elevated circulating THBS1 levels observed in tumour-bearing

mice treated with anti-angiogenic (metronomic) cyclophosphamide[133]. It is possible that angiogenesis inhibitors, such as bevacizumab, might be augmented by low dose anti-angiogenic (metronomic) chemotherapy with fewer side effects than conventional dosing of chemotherapy.

New pharmacology: oral drugs that increase endogenous angiogenesis inhibitors. The clinical finding that individuals with Down syndrome have an elevated circulating level of endostatin approximately 1.6-fold higher than normal individuals[91] is provocative. It suggests that small elevations of one or more endogenous angiogenesis inhibitors in the blood might protect against recurrent cancer, or might prevent the switch to the angiogenic phenotype in women at high risk for breast cancer. It is also possible that other genes on chromosome 21 have anti-angiogenic activity.

It has been found that certain orally available small molecules can upregulate expression of specific endogenous anti-angiogenic proteins, opening the way for a new field of pharmacology (FIG.7). Endostatin is increased by tamoxifen[136], celecoxib[137] and (in joint fluid) by prednisolone plus salazosulphapyridine[138]. THBS1 is upregulated by low dose cyclophosphamide[133], doxycycline[139] and rosiglitazone[121]. This unifying concept points to future drug discovery in which the known endogenous angiogenesis inhibitors could be screened for small-molecule inducers that would increase the circulating level of one or more of them.

Outlook

Angiogenesis inhibitors are now being approved and introduced into medical practice throughout the world. At the same time, a need for molecular biomarkers is being met by an expanding worldwide research effort to develop gene-based and protein-based molecular signatures in blood, platelets and urine for very early diagnosis of recurrent cancer. One can speculate that if these two fields intersect, it might someday be possible to diagnose microscopic tumours at a millimetre size, at about the time of the angiogenic switch but perhaps years before they are symptomatic, or before they can be visualized by any conventional methods.

For example, today most individuals with the diagnosis of colon cancer are operated on. At least 50–60% of these patients are cured by the surgery. In the other patients, cancer will recur in approximately 4–6 years. Physicians are helpless to do

anything until symptoms (such as pain and jaundice) occur, or until the recurrent cancer can be located by ultrasound, magnetic resonance imaging or CAT (computed axial tomography) scan. However, sensitive and specific molecular biomarkers that are being developed today could be used in the future to diagnose the presence of a microscopic recurrent tumour even before it could be anatomically located. Once these biomarkers are validated in clinical trials, then physicians could 'treat the rising biomarker' with non-toxic angiogenesis inhibitors until the biomarker returns to normal. A paradigm shift would be that recurrent cancer would be treated without waiting to see it, when it is still relatively harmless with low or no metastatic potential (that is, before the switch to the angiogenic phenotype). It might also be possible to use angiogenesis-based biomarkers to monitor the progression or regression of certain angiogenesis-dependent diseases that are non-neoplastic. These could include atherosclerosis, endometriosis, Crohn's disease and rheumatoid arthritis, among others.

There might be an analogy in the history of the treatment of infection. Before 1930, there were virtually no drugs for any infection, and most infections progressed to abscesses. Surgeons had to wait until the abscess was large enough to be located by X-rays so that the abscess could be surgically drained. The surgical textbooks of that era instructed surgeons how to locate an abscess: above the liver, behind the liver, in the mastoid, and so on. The term 'laudable' pus was commonly used to mean that if a surgeon could successfully drain an abscess the patient might live. After 1941, when antibiotics were introduced, it was no longer necessary to precisely locate an infection. Today the treatment of most infections is simply guided by blood tests (white-blood-cell count or blood cultures). As we continue to gain insight into angiogenesis and the role of angiogenic factors in seemingly unrelated diseases, the consequent potential of angiogenic modulators could see P. Carmeliet's prediction in the December 2005 issue of *Nature*[140] becoming prophetic: "Angiogenesis research will probably change the face of medicine in the next decades, with more than 500 million people worldwide predicted to benefit from pro- or anti-angiogenesis treatments"[140].

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PPARα Deficiency in Inflammatory Cells Suppresses Tumor Growth

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Inflammation in the tumor bed can either promote or inhibit tumor growth. Peroxisome proliferator-activated receptor (PPAR)α is a central transcriptional suppressor of inflammation, and may therefore modulate tumor growth. Here we show that PPARα deficiency in the host leads to overt inflammation that suppresses angiogenesis via excess production of the endogenous angiogenesis inhibitor thrombospondin-1 and prevents tumor growth. Bone marrow transplantation and granulocyte depletion show that PPARα expressing granulocytes are necessary for tumor growth. Neutralization of thrombospondin-1 restores tumor growth in PPARα-deficient mice. These findings suggest that the absence of PPARα activity renders inflammatory infiltrates tumor suppressive and, thus, may provide a target for inhibiting tumor growth by modulating stromal processes, such as angiogenesis.

INTRODUCTION

Non-neoplastic "host" cells, such as endothelial, stromal and inflammatory cells, play a critical role in tumor growth; and genes prognostic for cancer outcome may be expressed in the non-neoplastic tissue compartment [1]. While tumor angiogenesis has been intensely studied for more than two decades and has become an accepted target in cancer therapy, it is only in the last few years that inflammation has entered center stage of investigations into non-cell autonomous processes in cancer.

Chronic inflammation in the tumor stroma has long been known to contribute to tumor progression. Increased infiltration of innate immune cells to the tumor, such as macrophages, mast cells and neutrophils, correlates with increased angiogenesis and poor prognosis [2,3]. In contrast, lymphocytic/monocytic inflammatory infiltrates are sometimes associated with tumor inhibition and a more favorable prognosis [3–5]. Recently, NF-κB, a central positive regulator of inflammation, has emerged as a molecular link between inflammation and cancer growth. NF-κB promotes tumor growth not only in a cancer cell-autonomous manner by transactivating anti-apoptotic genes, but it also stimulates inflammatory processes in the microenvironment that lead to the production of tumor-promoting cytokines [6].

Conversely, PPARα, a ligand-activated nuclear receptor/ transcription factor, is a key negative regulator of inflammation. Activation of PPARα by ligands inhibits inflammation [7] whereas PPARα deficient mice exhibit enhanced inflammation [8]. Despite PPARα's role in suppressing inflammation, it appears to be necessary and sufficient for rodent tumorigenesis [9]. In fact, prolonged PPARα activation by peroxisome proliferators induces hepatocarcinogenesis in rodents; conversely PPARα KO mice are resistant to tumorigenesis induced by PPARα agonists [10,11]. This may be due in part to cell-autonomous effect of PPARα, because it is expressed in many tumor cell lines [12,13]. Another possibility is that in PPARα deficient mice, stromal processes, such as inflammation, inhibit tumor growth, which results in microscopic-sized tumors that remain dormant. The role of PPARα in inflammation has been extensively studied in normal physiological processes (wound healing) and cardiovascular diseases (atherosclerosis) [14,15]; but the effect of PPARα mediated suppression of

inflammation on tumors has not been characterized. Here we show that overt inflammation in the absence of PPARα in the host tissue prevents tumor growth. This indicates that in contrast to the emerging notion that inflammatory infiltrates promote tumors, the specific nature of the inflammatory process must be considered when linking inflammation to tumorigenesis.

RESULTS

Deletion of PPARα in Host Tissue inhibits Tumor Growth and Metastasis

We used several murine models to determine how the increased inflammatory response observed in the absence of PPARα affects tumor growth and metastasis. Fi rst, we stably transformed mouse embryonic fibroblasts (MEF) with SV40 large T antigen and H-ras [16] to obtain isogeneic tumorigenic cell lines that were either wild type (PPARα(+/+)MEF/RS) or lacked PPARα (PPARα(−/−) MEF/RS). These two tumorigenic cell lines allowed us to distinguish between the tumor cell- autonomous role and the host tissue role of PPARα. We found that the growth of these isogeneic tumors derived from both cell lines was almost completely suppressed in KO host mice that lacked PPARα, but not in WT

animals, p<0.0001 (Figures 1A and 1B). Although tumors derived from MEFs deficient of PPARα were partially suppressed in WT animals (by 41%), indicating a cell-autonomous role of PPAR in tumor growth, a drastic effect in tumor suppression was observed when the host was PPARα deficient both in the case of PPARα(+/+) tumors (87% suppression) as well as PPARα(−/−) tumors (97% suppression) (Figure 1C). These results suggest that the presence of PPARα gene in the host animals is essential for tumor growth.

To examine the role of established tumor murine models we first used WT and KO mice derived from WT (S1)×KO (S4) crossmating. The growth of B16-BL6 tumor was almost completely inhibited in the PPARα KO (S1/S4) host, but was not affected in PPARα WT (S1/S4) animals, p<0.0001 (Figure 1D). This result suggests that presence of the PPARα gene in the host tissue is essential to support tumor growth.

Given that the above results clearly suggest that the status of the PPARα locus in the host affects tumor growth, we next evaluated the growth of three PPARα-positive murine tumor models in PPARα KO (S4) animals, including Lewis lung carcinoma (LLC), metastatic B16-F10/GFP melanoma, and B16-BL6 melanoma, p<0.001 (Figure 1E–G). LLC tumors have been reported to grow aggressively at similar rates in the Sv129, C57BL/6 and Sv129/C57BL/6 strains without evidence of transplantation immunity. This suggests that disparity in either minor or major immuno-histocompatibility genes does not affect tumor growth in these models [17] (Figure S1). Macroscopic growth of LLC and B16-F10/GFP tumors was completely suppressed in PPARα KO mice, even when mice were monitored for more than 100 days post implantation (Figure 1E–G). Similarly, tumor metastasis was also suppressed in PPARα KO mice. When B16-F10/GFP melanoma
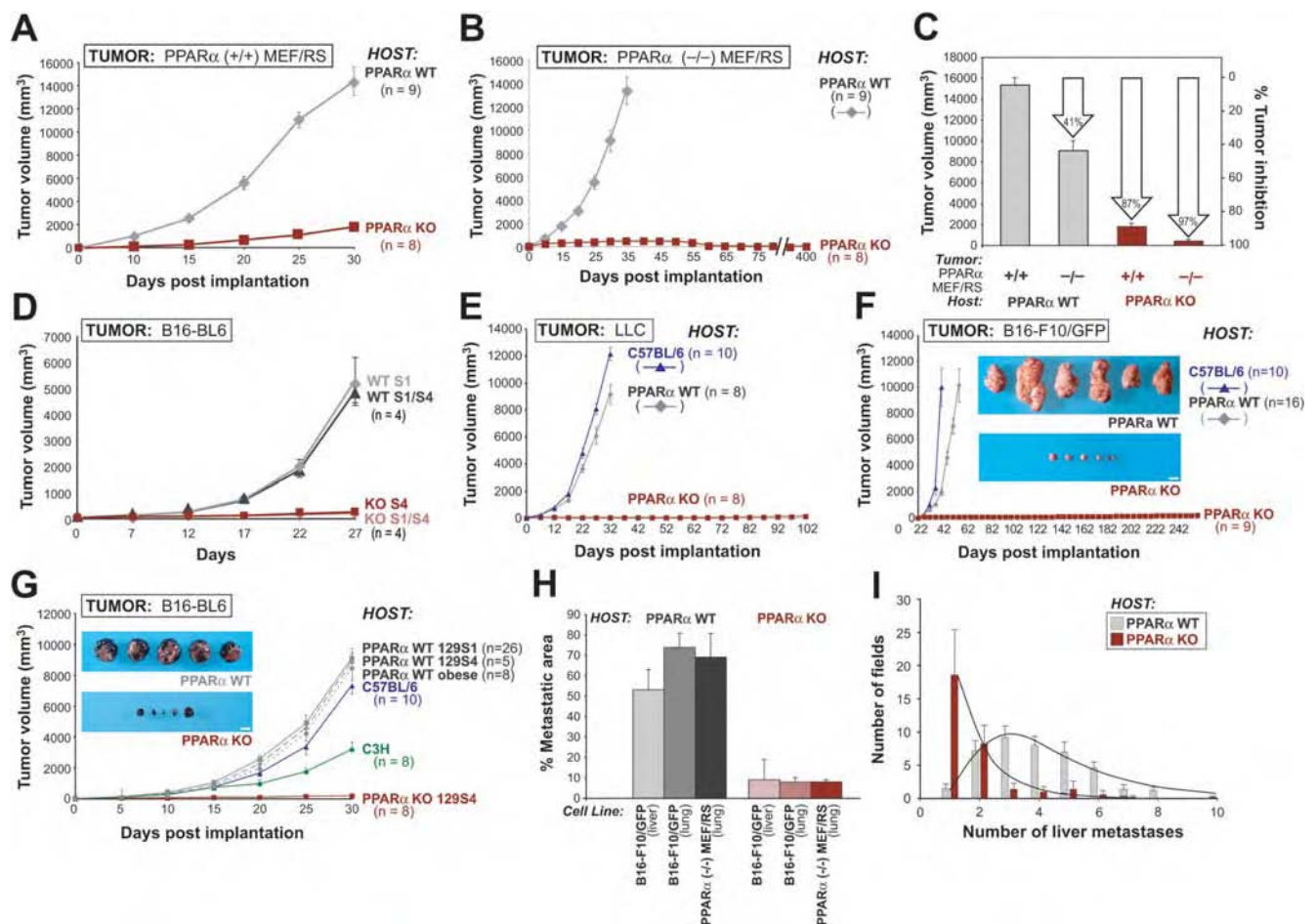

Figure 1. Tumor growth and metastasis are inhibited in PPARα knockout (KO) mice. PPARα wild type (WT) and PPARα KO mice were injected subcutaneously or intravenously with various tumor cell lines; (n) = number of mice/group. **(A–C)** The growth of engineered PPARα(+/+) MEF/RS and PPARα(−/−) MEF/RS tumors in PPARα WT and KO mice. **(A)** PPARα(+/+)MEF/RS tumor growth in PPARα WT (◆ gray) and KO (■ brown) mice. **(B)** The growth curves of PPARα(−/−)MEF/RS in PPARα WT (◆ gray) and KO (■ brown) mice.**(C)** Columns summarize the inhibitory effect of PPARα (−/−) tumor and host cells at day 30 post implantation (average±standard error of the mean). **(D–F)** The growth of different murine tumors in different mouse strains. **(D)** The growth of B16-BL6 melanoma was compared in WTS1 (◆ gray), WTS1/S4 (▲gray), KOS4 (■ brown) and KO S1/S4 (◆ pink) strains. WT S1/S4, PPARα WT second generation littermates from PPARα WT 129/S1 and KO 129/S4; KO S1/S4, PPARα KO second generation littermates from PPARα KO 129/S4. **(E)** Lewis lung carcinoma growth in PPARα WT (◆ gray), PPARα KO (■ brown) and C57BL/6 (▲ blue) mice. **(F)** B16-F10/GFP tumor growth in PPARα WT, PPARα KO and C57BL/6 mice, blue insets demonstrate representative B16-F10/GFP tumors in PPARα WT and KO mice on day 30 post implantation. Scale bar, 1 cm. **(G)** B16-BL6 melanoma was implanted in mice of indicated genetic backgrounds. Representative B16-BL6 tumors in PPARα WT (◆ gray) and PPARα KO (■ brown) mice on day 30 post implantation are shown (blue insets). Scale bar, 1 cm. **(H–I)** Metastasis in PPARα WT and KO mice. **H:** Metastatic areas of B16-F10/GFP and PPARα(−/−)MEF/RS tumor cells at day 21 post-injection in lung and liver of PPARα WT (◆ gray) and KO mice (■ brown). **I:** Number of liver metastases in PPARα WT (■ gray) and KO (■ brown) mice injected with B16-F10/GFP tumor cells (average±standard deviation).
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cells and engineered PPARα deficient tumor cells, PPARα (−/−) MEF/RS (see below) were injected via tail vein, 21 out of 21 PPARα wild-type (WT) mice died of lung and/or liver metastasis by day 21. In contrast, the PPARα KO hosts suppressed metastatic growth in lung and liver, reducing the infiltration of the tumor cells from 50–70% of normal organ tissue area in the WT hosts to less than 10% tissue area in PPARα KO animals (Figure 1H). Furthermore, the incidence of metastasis, as measured by the number of histologically identified metastatic foci, was strongly suppressed in PPARα KO mice. The majority of microscopic fields of liver sections in PPARα KO mice revealed only one or two metastases compared to 4–5 foci in livers of WT hosts (Figure 1I). Together these findings support the importance of PPARα expression in host cells for tumor development.

The non-growing PPARα(−/−)MEF/RS tumors in PPARα KO mice prompted us to investigate whether these tumors were just a mass of connective tissue or viable dormant microtumors, a state in which tumor cell proliferation is balanced by cell death [18,19]. Analysis of the small (<2 mm), non-growing lesions at the

injection site identified viable PPARα(−/−) MEF/RS large T antigen expressing and proliferating tumor cells (Figure 2A). When re-transplanted to PPARα WT mice, these tumors grew rapidly to over 10,000 mm³ (Figure 2A) indicating that PPARα in the host can rescue PPARα −/− tumor cells. Although these findings suggest that the presence of PPARα both in the tumor cells as well as in the host is necessary for unabated tumor growth, they also demonstrate that PPARα in tumor cells is not necessary for tumor cell viability. Conversely, the results underscore the importance of PPARα in the host tissue to sustain tumor growth.

Histological examination revealed a pronounced leukocyte infiltration (based on CD45-positive staining) in the non-necrotic stroma of all tumors grown in PPARα KO mice (Figure 2B). In contrast, PPARα WT animals exhibited the usual leukocytic infiltrate that was limited to necrotic areas (Figure 2B). Moreover, PECAM-1 staining performed to visualize blood capillaries revealed a decreased microvessel density in tumors from PPARα KO hosts when compared to tumors from WT hosts of the same size at day 7 (data not shown), as well as at day 30 post
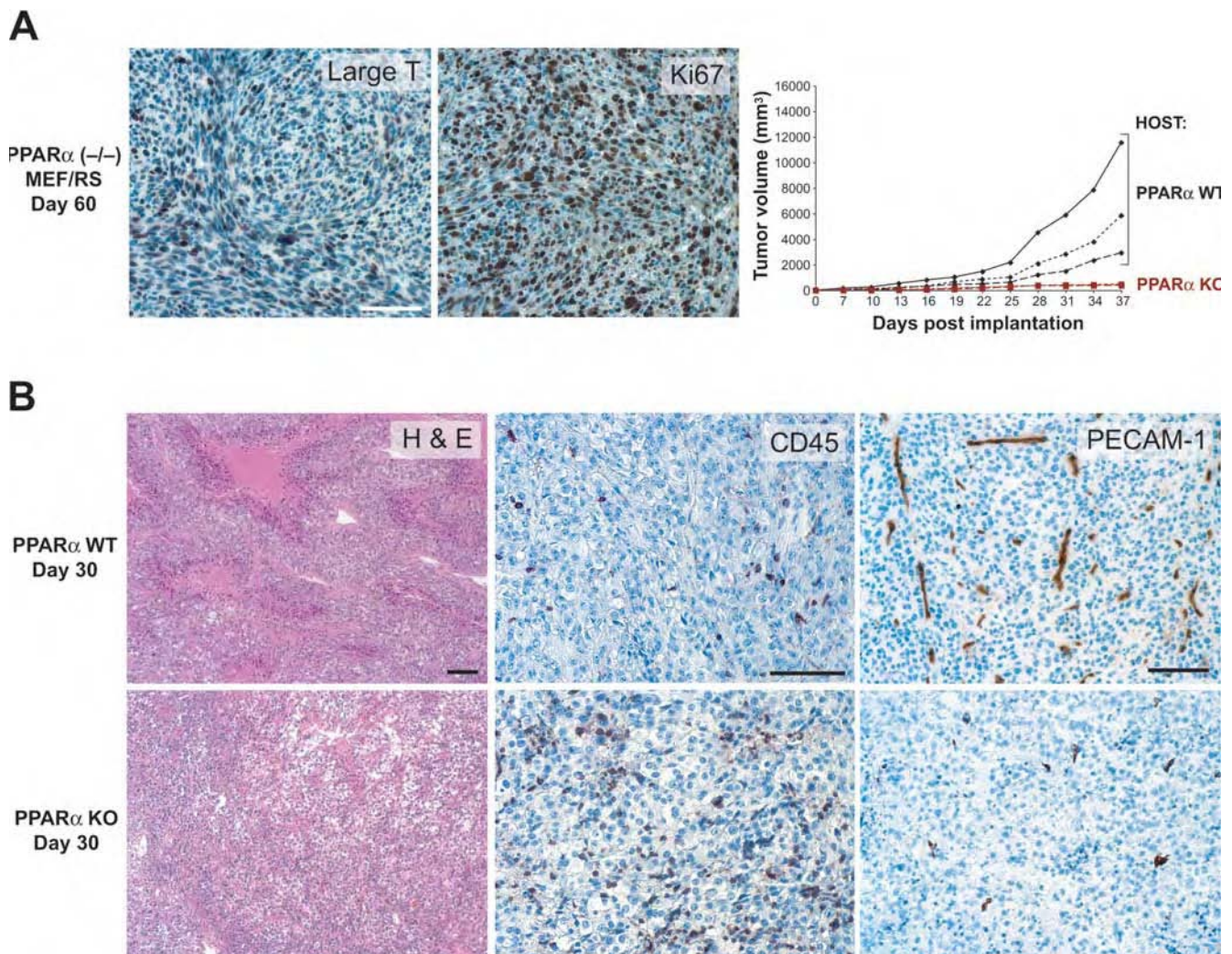
Figure 2. Immunohistological analysis of dormant tumors in PPARα KO mice. The dormant tumors contain viable and proliferating cells, and show decreased microvessel (PECAM1) and increased leukocyte (CD45) staining. **(A)** Dormant PPARα(−/−)MEF/RS tumors in PPARα KO mice from day 60 post-tumor implantation revealed abundant SV40 large T-antigen staining and proliferation (Ki-67). Dormant PPARα(−/−)MEF/RS tumors on day 60 were implanted as pieces (1 mm³) into PPARα WT and KO mice (3 mice in each group). **(B)** Immunohistochemical analysis of subcutaneous B16-F10/GFP tumors (H&E, CD45/brown color, PECAM-1/brown color) from day 30 post-implantation in PPARα WT mice and KO mice. Scale bars, 100 μm.
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implantation (Figure 2B). Therefore, the absence of PPARα in the stromal tissue of the host appears to have two major consequences: an increase in inflammation and a decrease in tumor angiogenesis.

## Loss of Host PPARα Inhibits Corneal Neovascularization and Permeability

Decreased microvessel density may reflect direct or indirect antiangiogenic effects caused by the lack of PPARα activity. Because all tumors used here are known to produce the angiogenic cytokine VEGF, we first investigated whether PPARα plays a role in VEGF signaling in the host cells. We employed two different *in vivo* VEGF-activity assays: VEGF-mediated, FGF2-induced corneal neovascularization, and VEGF-induced vascular permeability. Implantation of pellets containing 20 ng of FGF2 into the corneas of mice promotes the extravasation of leukocytes and stimulates VEGF-dependent corneal neovascularization [20,21]. PPARα KO mice exhibited >50% inhibition of vessel length when compared to WT animals, while the initial sprouting (reflected in clock hours of the neovascularized area) was not affected (Figure 3A). Complete abrogation of angiogenesis in the WT mice in the presence of soluble VEGF-receptor-1 (VEGFR1) confirmed that angiogenesis in these WT animals was mediated by VEGF (Figure 3A), consistent with previous studies [20]. In our second approach, we evaluated whether host PPARα affected VEGF-induced vascular permeability, a standard test of *in vivo* VEGF activity [22,23]. In response to VEGF, WT mice displayed

Evans blue extravasation into the subcutaneous skin and ears (Figure 3B) that was 300–400% greater than that of PPARα KO mice (Figure 3B). Together, these results indicate that host PPARα is indispensable for VEGF-dependent signaling.

PPARα Deficiency in Bone Marrow Cells Inhibits Tumor Growth

Given the observation that the tumor bed of PPARα KO mice exhibited an increased inflammatory response, we performed reciprocal bone marrow transplantations between WT and KO mice to determine whether the hematopoietic compartment of PPARα deficient mice plays a role in the inhibition of tumor growth. Bone marrow cells from WT mice were capable of restoring the "wild-type" tumor growth pattern of B16-BL6 tumors in PPARα deficient hosts (Figure 4A). Conversely, PPARα-deficient bone marrow cells, when transplanted into WT hosts, conferred the tumor-suppressing phenotype of PPARα KO mice, p<0.0001 (Figure 4A). It is important to note that in the bone marrow transplantation protocol used, >90% of the hematopoietic system of the recipient was derived from the donor marrow (Figure S2A); this argues against the possibility that PPARα KO bone marrow cells have a direct, "dominant-negative" effect that overrides a tumor promoting effect of WT bone marrow cells. Instead, the result strongly suggests that the influence of host PPARα on tumor growth is conveyed solely by PPARα activity in bone marrow derived cells, because in these reciprocal trans-
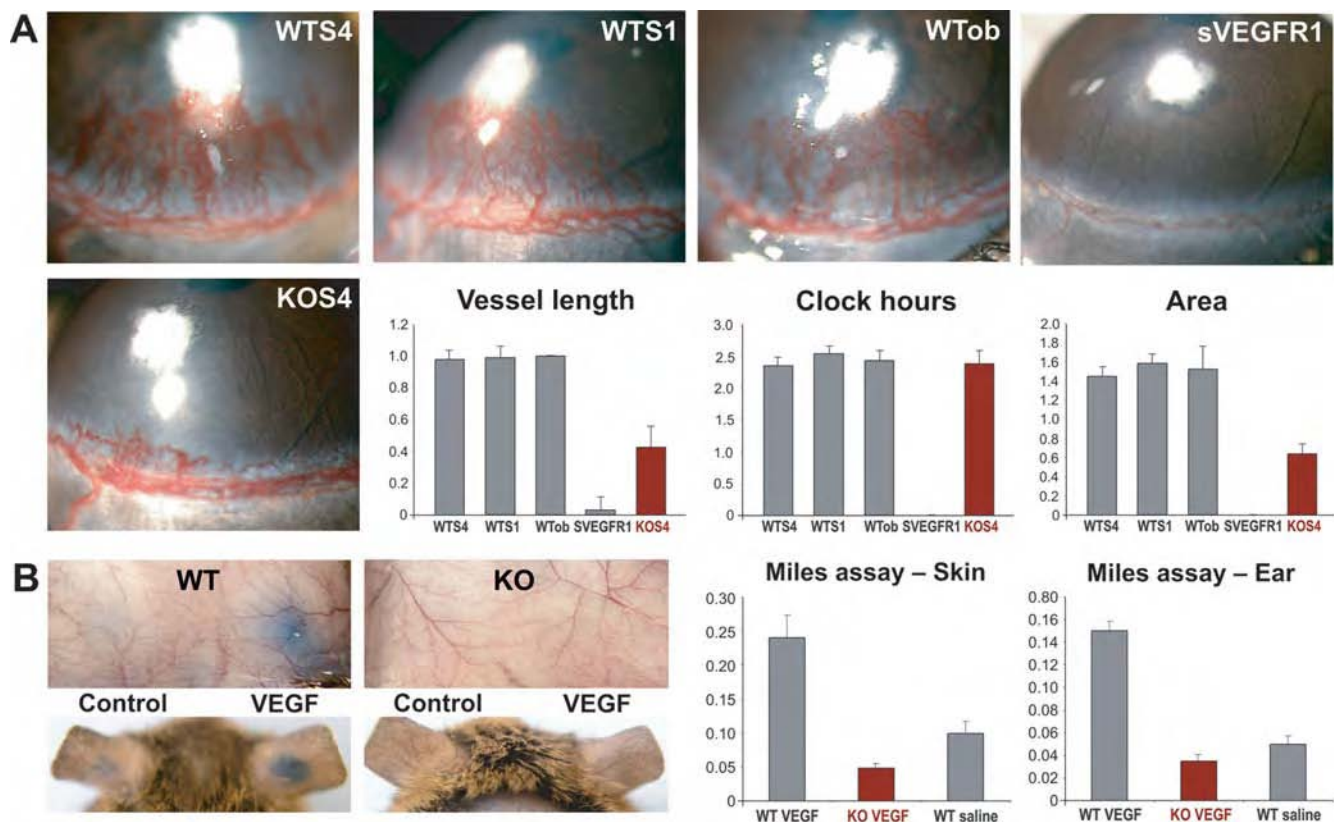
Figure 3. FGF2-induced corneal neovascularization and VEGF-induced vascular permeability are inhibited in PPARα KO mice. **(A)** FGF-2 (20 ng) stimulates corneal neovascularization in WT 129S4/SvJae strain, WT 129S1/SvIMJ strain and obese WT (129S1/SvJae) mice. Soluble murine VEGFR1 completely inhibits FGF2-induced angiogenesis in WT mouse (sVEGFR1). FGF2-induced corneal neovascularization is potently suppressed in PPARα KO mouse (KOS4). Vessel length, clock hours, and area of neovascularization in PPARα WT and KO mice are represented in bar graphs (average±standard deviation). **(B)** Evans blue dye leakage in dorsal skin and ears after injection with VEGF or saline in PPARα WT and KO mice (n = 6 mice/group). Spectrophotometric analysis of extravasated Evans blue of skin and ear is represented in bar graph (average±standard deviation).
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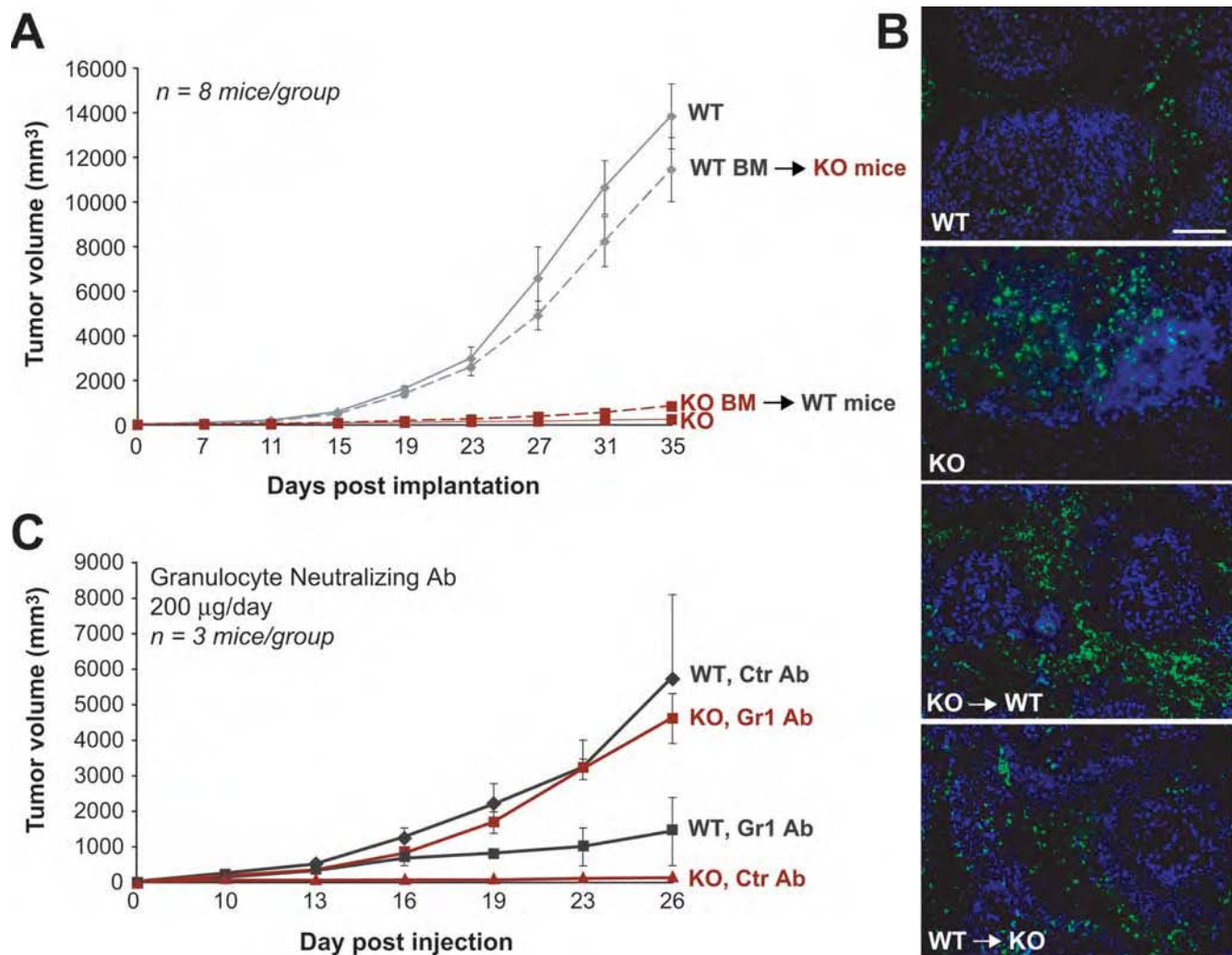
Figure 4. The inhibitory effect of PPARα resides in the hematopoietic compartment. **(A)** B16-BL6 melanoma growth in WT mice receiving KO bone marrow (KO BM →WT mice) compared to PPARα KO mice receiving WT bone marrow (WT BM →KO mice). WT bone marrow "rescues" tumor growth in PPARα KO mice. **(B)** Subcutaneous B16-BL6 tumors on day 28 post-implantation show abundant CD45 staining in PPARα WT mice receiving KO bone marrow (KO →WT). In B16-BL6 tumors in KO mice receiving WT bone marrow (WT→KO) CD45 staining (shown in green) was markedly reduced. Hoechst staining of nuclei is blue. Scale bar, 100 μM. **(C)** Effect of granulocyte depletion using Gr-1 antibody or control antibody (Ctr Ab, IgG2b) on B16-BL6 melanoma growth rate in PPARα KO and WT mice.
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plantation experiments the PPARα status of the transplanted bone marrow cells recapitulates the tumor phenotype of the host. However, it cannot be excluded that the suppressor activity carried by PPARα-deficient bone marrow cells overrides a potential tumor stimulatory contribution of PPARα in other, non-bone marrow derived host cells, such as from the local stroma.

Depletion of Granulocytes in the PPARα KO Mice Restores Tumor Growth

Immunohistological analysis of B16-BL6 tumors in WT mice transplanted with PPARα-deficient bone marrow cells showed an intense increase in leukocyte staining, mimicking the intratumoral leukocyte profile of tumors grown in PPARα KO mice (Figure 4B). This pronounced leukocyte infiltration in WT mice transplanted with PPARα-deficient bone marrow cells suggests that the presence of PPARα within the inflammatory cells prevents an overt inflammatory response to tumors. Histological and immunohistological analysis of the dormant tumors in PPARα knockout

mice revealed that the leukocyte population was predominantly composed of granulocytes, mainly neutrophils (Figure S2B). To corroborate an active role of these PPARα-deficient granulocytes in tumor suppression, we depleted them in the host animals. Flow cytometry analysis confirmed that the granulocyte-specific neutralizing antibody GR1 completely depleted neutrophils (Figure S2C). The anti-granulocyte antibody GR1 restored tumor growth rate in the PPARα KO mice almost completely by day 26 (Figure 4C). In PPARα KO mice that received the control antibody (IgG2b), tumor growth remained inhibited. Conversely, in WT mice the GR1 antibody suppressed tumor growth (Figure 4C vs. 4A), confirming the previous reports that neutrophils are necessary for tumor growth [2,3]. However, tumor inhibition was even stronger in WT animals whose bone marrow had been replaced with that of PPARα KO mice (Figure 4A) as well as in PPARα deficient hosts (Figure 4A and 4C), again suggesting that not only is PPARα necessary for tumor growth, but that its absence confers a tumor suppressor activity on neutrophils.

The Inhibitory Role of TSP-1 on Tumor Growth

We next asked why are tumor growth and angiogenesis inhibited by PPARα-deficient leukocytes? Activated inflammatory cells promote angiogenesis, tumor cell proliferation and metastasis through the production of angiogenic mediators, growth factors, chemokines and proteases [2,24–26]. A connection between the positive and negative mediators of the inflammatory response, NF-κB and PPARα, has recently been suggested, because PPARα has been shown to repress NF-κB activity/expression [27]. However, this model disagrees with our result that PPARα-mediated suppression of inflammation is permissive for tumor growth rather than inhibitory. Therefore, our finding suggests that PPARα regulates an aspect of inflammation that is different from that controlled by NF-κB and hence, PPARα modulation of inflammation affects tumor growth independently of NF-κB. While NF-κB exerts its tumor-promoting effect by induction of cytokines, we investigated whether PPARα deficiency suppresses tumor growth by increasing the expression of the matrix protein thrombospondin-1 (TSP-1) which inhibits angiogenesis and stimulates granulocyte migration [28].

In fact, TSP-1 was elevated in the plasma and tumor tissue of PPARα KO mice (Figure 5A and Figure S2D). Because TSP-1 can be expressed in several cell types, including tumor cells, endothelial cells and fibroblasts, we next determined the cellular origin for TSP-1 in the tumors of PPARα deficient mice. B16-BL6 and B16-F10/GFP melanomas in PPARα KO mice contained high levels of TSP-1 protein (Figure 5A), despite that these tumor cells do not express TSP-1 [29]. TSP-1 was found in tumors in PPARα WT mice only when the mice received bone marrow from PPARα KO animals. In contrast, little or no TSP-1 was detected in the tumors in PPARα KO mice whose bone marrow cells had been replaced by those from PPARα WT animals (Figure 5B). Moreover, in B16-BL6 tumors from PPARα KO mice treated with GR1 antibody, little or no TSP-1 was detected (Figure 5C). Purified peripheral blood leukocytes from tumor-bearing PPARα deficient mice expressed high levels of TSP-1 while WT leukocytes express very little if any TSP-1 (Figure 5D). Taken together, these findings suggest that in this model system, TSP-1 was produced predominantly by the inflammatory cells, and not by resident stromal cells.

To corroborate the role of TSP-1 in angiogenesis in PPARα deficient animals, we performed the corneal neovascularization assay in the presence of neutralizing anti-TSP-1 antibody. Suppression of vessel length (endothelial cell migration and invasion) in PPARα KO mice was partially reversed by inactivation of TSP-1 function (Figure 5E). There was no effect on the contiguous circumferential zone of the limbal vessel sprouting as measured by clock hours (Figure 5E). In contrast, in the WT mice, corneal neovascularization was not affected by the TSP-1 antibody (Figure 5E).

Provided that neovascularization is a valid marker for tumor angiogenesis, these results are in agreement with the established role of TSP-1 in tumor inhibition [30]. However, we found that the neutralizing TSP-1 antibody did not completely restore tumor growth in PPARα KO mice to the level of that in WT mice, p<0.02 (Figure 5F). This may be either due to the limited access of TSP-1 antibody to the tumor bed or suggests that other endogenous inhibitors of angiogenesis may be involved. In fact, endostatin and IL-12 levels were significantly higher in PPARα KO mice (data not shown). Unexpectedly, we found that in WT animals neutralization of TSP-1 also had an inhibitory (rather than promoting) effect on the tumor, suppressing tumor growth by approximately 71% when compared to control antibody-treated mice, p<0.02 (Figure 5F). This suggests a complex, dualistic role of TSP-1 as a regulator of tumor growth.

DISCUSSION

In this study we identified the cellular basis for the tumor suppressing phenotype of PPARα deficient mice. Thus, PPARα pathway represents a new link between inflammation, angiogenesis, and tumorigenesis. Absence of PPARα in host granulocytes leads to inhibition of tumor growth, as demonstrated by: (1) transplantation of bone marrow cells from PPARα KO mice to PPARα WT mice and (2) by depletion of granulocytes by the neutralizing antibody, Gr1. Interestingly, PPARα deficient granulocytes carried TSP-1, a protein that inhibits angiogenesis, leukocyte migration and tumor growth. When TSP-1 was depleted by neutralizing antibody in PPARα KO mice, tumor growth was partially reversed.

PPARα is best known as a critical regulator of lipid metabolism and inflammation [31], and is expressed in tissues that catabolize fatty acids such as the liver, as well as in various cell types including smooth muscle cells, monocyte/macrophages, lymphocytes, and endothelial cells [31]. PPARα is the molecular target of the fibrate class of lipid-lowering drugs, which have been widely used for decades in the treatment of dyslipidaemia. Upon activation by PPARα ligands, PPARα heterodimerizes with retinoic acid receptor (RXR) regulating target gene expressions. PPARα ligands act as PPARα agonists. In addition to controlling lipid levels, they also function as potent anti-inflammatory agents in diseases such as atherosclerosis, colitis, and dermatitis [32–35]. Accordingly, PPARα KO mice exhibit significant reduction of atherosclerotic lesions, delayed wound healing, and delayed liver regeneration [14,15,36], due to overt inflammatory processes. PPARα deficiency also results in a prolonged inflammatory response to lipid mediators [8]. These findings collectively suggest that PPARα has a physiological role in suppressing inflammation [7].

PPARα agonists have been reported to induce liver tumors in rodents, but not in humans [10,37,38]. The mechanism for this species difference is still unclear. Accordingly, PPARα KO mice are totally resistant to liver tumors induced by PPARα ligands such as WY-14643 and clofibrate. This indicates that PPARα is required for ligand-induced peroxisome proliferation and hepatocarcinogenesis in rodents in a cell-autonomous manner [9]. It is unclear to what extent this requirement of PPARα for tumor growth is due to tumor cell-autonomous effects or its role in the host compartment of tumors, as shown by our current findings. In our experimental model the suppression of tumor growth in PPARα KO mice is mediated by leukocytes, mainly neutrophils. PPARα deletion is a second example for suppression of tumor growth by ablation of a gene in inflammatory cells; deletion of IKKβ in myeloid cells inhibits epithelial cell tumor growth [26]. However, our model does not exclude a contribution by cell-autonomous tumor promoting effects of PPARα. In fact, we found that deletion of PPARα in the tumor cell itself potentiated the tumor suppressing effect of PPARα-deficiency in the host tissue (Figure 1G and H), in agreement with the earlier reports of the requirement for PPARα in PPARα agonist induced liver tumors [9]. Therefore, PPARα, in addition to NF-κB, may represent another example of an oncogenic protein with a dual role in cancer by controlling essential functions both in cancer cell-autonomous processes as well as processes in the tumor bed, such as inflammation and angiogenesis. Oncogenes and NF-κB have been shown to stimulate tumor cell proliferation and angiogenesis by modifying cytokine expression profiles [25]. Therefore, PPARα does not simply suppress inflammation, acting in opposition to NF-κB, but it does so in a qualitatively different manner in that
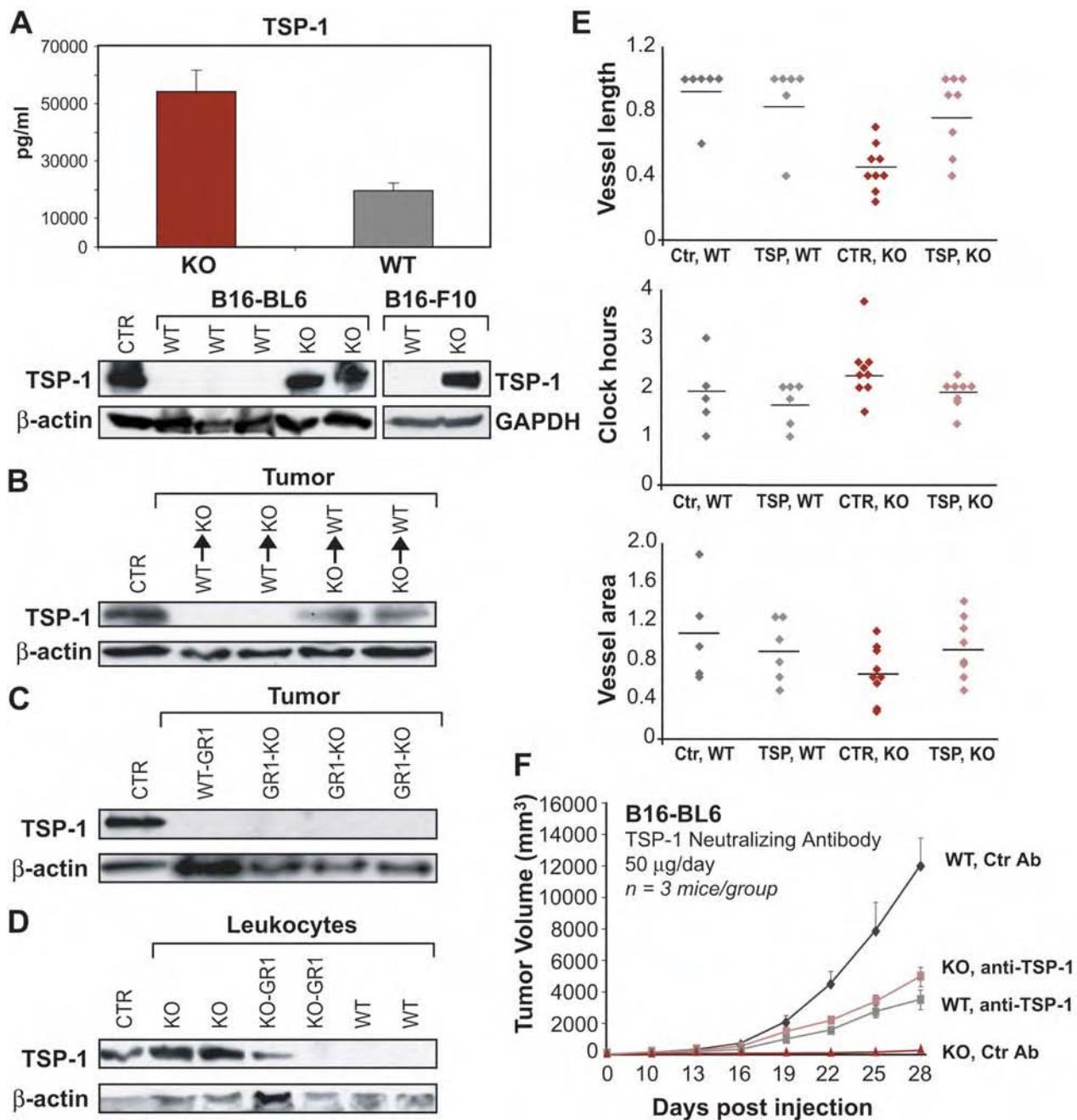
Figure 5. Effects of thrombospondin-1 (TSP-1) on angiogenesis and tumor growth in the PPARα-deficient state. **(A)** First panel demonstrates TSP-1 levels in plasma of PPARα KO and WT mice (ELISA); second panel shows TSP-1 levels in B16-BL6 and B16-F10/GFP tumor lysates at day 30 grown in PPARα WT and KO mice (western blotting); positive CTR for TSP-1, proliferating HUVECs. **(B)** Western blot analysis of TSP-1 protein in B16-BL6 tumor lysates from PPARα KO mice receiving WT bone marrow (WT BM→KO), and PPARα WT mice receiving KO bone marrow (KO BM→WT); positive CTR for TSP-1, proliferating HUVECs. **(C)** TSP-1 protein expression is lost in B16-BL6 tumor lysates from PPARα KO mice depleted of granulocytes (GR-1 antibody); positive CTR for TSP-1, proliferating HUVECs. **(D)** Western blot analysis of TSP-1 expression from isolated leukocytes from tumor-bearing PPARα KO mice; positive CTR, proliferating HUVECs. Levels of β-actin demonstrate protein loading. **(E)** Effect of TSP-1 neutralizing antibody and control antibody (IgM) on vessel length (n = 6–9 eyes), clock hours (n = 5–9 eyes) and vessel area (n = 5–9 eyes) in the corneal neovascularization assay. **(F)** B16-BL6 melanoma growth in KO and WT mice treated with TSP-1 neutralizing or control antibody (IgM).
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cellular infiltrates that do not express PPARα, actively suppress rather than stimulate tumor growth.

PPARα-deficient leukocytes produce TSP-1, a potent inducer of leukocyte migration and inhibitor of angiogenesis. Thrombospondin-1 (TSP-1) is a trimeric glycoprotein (450kD) that has several functional domains with different binding affinities. It binds to

several cell surface receptors (CD36, integrins αVβ3, α3β1, α4β1, α5β1, heparan sulfate proteoglycans) and also binds calcium and extracellular proteins, such as plasminogen, fibrinogen, fibronectin and urokinase [30,39]. This multitude of binding partners may explain the diversity of TSP-1 functions: TSP-1 modulates cell adhesion, migration, proliferation and differentiation regulating

processes such as inhibition of angiogenesis (through CD36 and β1- integrin) and stimulation of neutrophil migration [28,40,41]. TSP-1 is expressed in several cell types in the host: platelets, neutrophils, monocytes, fibroblasts, pericytes, endothelial cells, and tumor cells [42]. Through its role as an activator of TGF-β, it also modulates inflammatory reactions which may contribute to the lethality of TSP-1 KO mice [43]. TSP-1 inhibits tumor growth in mice when overexpressed, putatively via suppression of angiogenesis [40,44,45]. However, TSP-1 may also act as a promoter of tumor growth, because anti-TSP-1 receptor antibody inhibited breast tumor growth [46]. Moreover, in vitro TSP-1 has been shown to promote tumor cell invasion and chemotaxis [47–49]. In addition, further complicating the picture, in human plasma and tumor stroma the levels of TSP-1 have been correlated with both good and poor cancer prognosis [50–56]. This conflicting influence of TSP-1 is recapitulated in our animal model: TSP-1 delivered by leukocytes inhibited tumor growth. However, in the WT animals neutralization of TSP-1 also strongly inhibited tumor growth (Figure 5C). A possible explanation for this apparent paradox is that TSP-1 may have a biphasic effect on angiogenesis and leukocyte migration so that low doses (as found physiologically in WT animals) stimulate and high doses (present in PPARα KO mice) inhibit these processes [57]. Such a "U-shape" dose-effect curve has been reported for many cytokines and bioactive molecules, such as interferon-α, PPARγ ligands and endostatin which all exhibit a biphasic effect on angiogenesis [58–62]. Therefore, in WT mice, TSP-1 may operate in the dose-effective window of promoting inflammation which in turn stimulates angiogenesis and tumor growth. In contrast, in PPARα KO mice where TSP-1 is constitutively high, it would act as an inhibitor of tumor growth, perhaps through its antiangiogenic effects. Another possibility, technical rather than biological, is that the activity of TSP-1 is always inhibitory under the conditions studied, but the TSP-1 antibody itself generates the biphasic effect. High levels of TSP-1 in KO mice in the presence of TSP-1 antibodies may promote formation of large antigen - antibody complexes that facilitate TSP-1 clearance, while at low levels, as in WT mice, TSP-1 may be stabilized by the antibody [63].

Given the accumulating findings pointing to the importance for tumor growth of processes in non-cancer host tissues, such as angiogenesis, inflammation and other functions mediated by residual stroma and infiltrating bone marrow cells, our results add a new element to the emerging paradigm that tumor formation is not only a cell-autonomous process. Hence, the action of genes involved in tumor formation must be seen in the broader context of host and tumor [64]. While several pro-inflammatory factors stimulate tumor growth, we report a new molecular link between inflammation and cancer, in that abnormal inflammatory processes can inhibit tumor growth and angiogenesis - thus broadening the spectrum for anticancer therapies that aim at interfering with stromal processes.

MATERIALS AND METHODS

Tumor Xenograft Studies

All the animal studies were reviewed and approved by the animal care and use committee of Children's Hospital Boston. Three to six-month old male PPARα knockout mice (129S4/SvJae), corresponding age-matched WT mice (129S1/SvIMJ, C57BL/6), obese WT mice (129S1/SvIMJ-retired breeders), C3H/HeJ and Balb/cJ mice were obtained from Jackson laboratories (Bar Harbor, ME). Retired WT breeders (35–40 gram) were used to control for weight as PPARα KO mice become obese with age [65]. WT mice (129S4/SvJae) were provided by Dr. John

Heymach, Children's Hospital, Boston. PPARα WT and KO littermates were F2 generation. For tumor studies, PPARα negative (−/−) and PPARα positive (+/+) tumors were developed by transforming mouse embryonic fibroblasts (embryonic day 11) isolated from PPARα KO and WT mice, respectively, with SV40 large T-antigen and H-ras (generous gift from Dr. William Hahn). Tumor cells were injected subcutaneously (1×10^6 cells in 0.1 ml PBS). B16-BL6 melanoma cells were implanted directly from tissue culture; the growth of LLC and B16-F10/GFP tumors was achieved in 129 strains as follows: LLC and B16-F10/GFP cells were first grown in C57BL/6 mice and transplanted as pieces (1 mm^3) subcutaneously into PPARα WT mice. When tumors were 1000–2000 mm^3, they were serially passaged from mouse to mouse as 1 mm^3 pieces and then grown in culture [59]. For experiments, LLC and B16-F10/GFP tumor cells were injected subcutaneously into the 129S PPARα WT and PPARα KO mice either from culture or from mouse to mouse as a cell suspension as described [59]. Tumors were measured every 3–5 days, and the volume was calculated as width^2×length×0.52. For metastasis studies, 500,000 cells in 0.1 ml PBS were injected via tail vein (n = 15 mice/group). On day 21, when the PPARα WT mice died, all remaining mice were euthanized. Histological sections of livers were quantified for liver metastasis (n = 34–53 fields). For corneal tumor studies, tumor pieces (1 mm^3) were implanted into the cornea, and the angiogenic response was recorded; photos were taken weekly using a slit-lamp microscope. For granulocyte depletion studies, GR-1 or control antibody (IgG2b) at 300 µg/mouse (Biolegend, San Diego, CA) was administered intraperitoneally two days prior to B16-BL6 melanoma implantation in PPARα WT and PPARα KO mice, and every 3 days post-implantation. Granulocyte depletion was confirmed by flow cytometry using phycoerythrin conjugated Ly-6G (GR-1) antibody (Biolegend, San Diego, CA).

For neutralizing antibody experiments the A4.1 anti-TSP-1 monoclonal antibody (Lab Vision, Fremont, CA) (CSVTCG/CD36) or control antibody (IgM) at 50 µg/mouse were administered intraperitoneally daily to PPARα WT and KO mice in the corneal neovascularization and B16-BL6 melanoma experiments.

Immunohistochemistry

Tumor samples were processed and immunohistochemical stainings were performed according to standard protocols [59]. For rat anti-mouse PECAM1 (BD Biosciences, San Jose, CA) staining, sections were treated with 40 µg/ml proteinase K (Roche Diagnostics Corp.) for 25 minutes at 37°C. Detection of PECAM1 staining was completed using the tyramide amplification system according to the manufacturer's instructions (PerkinElmer, Boston, MA). For mouse monoclonal thrombospondin-1 (clone A6.1, Lab Vision, Fremont, CA) staining, sections were pretreated with pepsin for 15 minutes at 37°C (Biomeda, Foster City, CA). For rat anti-mouse CD45 (BD Biosciences, San Jose, CA), and mouse monoclonal NP57 neutrophil elastase (Lab Vision, Fremont, CA) stainings no pretreatments were needed, and stainings were performed using Innogenex IHC kit (San Ramon, CA).

Angiogenesis Assays

Corneal neovascularization assays were performed. Vessel length was the length of the vessels from the limbal vessel to the pellet. Vessel sprouting was measured as clock hours, the contiguous circumferential zone of the neovascularization, using a 360° reticule (where 30° of arc equals one clock hour). Vessel area was determined using the formula 0.2π×vessel length×clock hours of vessels [66].

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For *in vivo* Miles permeability assay, PPARα WT and KO mice received an intravenous injection with 0.5% Evans blue dye (100 μl) retro-orbitally. After ten minutes, the mice were given intradermal injections (50 μl) into the dorsal skin or ear at 2 different sites, consisting of vehicle control or VEGF (50 ng; R&D Systems Inc., Minneapolis, MN). Twenty minutes later the dorsal skin and/or ears were harvested for densitometric analysis to quantify dye leakage. Columns represent mean±standard deviation (n = 6 mice per group; experiments were performed three times).

Transplantation of Bone Marrow Stem Cells

PPARα WT and KO recipient mice were lethally irradiated with 14 Gy (in a split dose, 4 hours apart) 24 hours before bone marrow transplantation (BMT). Bone marrow cells (1×10^6) were injected retro-orbitally into recipient mice under isoflurane anesthesia. Neomycin sulfate antibiotic (2 mg/ml) was administered for two weeks post BMT in the drinking water. Mice recovered for a minimum of 2–3 months prior to tumor implantation.

Western Blot Analysis

For preparation of tumor lysates from PPARα WT and KO mice, B16BL6 tumors were homogenized with protease inhibitor (Roche, Germany). Total protein extracts (50 μg) were analyzed on blots incubated with primary mouse monoclonal TSP-1 (Ab-11, Lab Vision, Fremont, CA) and HRP-conjugated secondary antibodies (Amersham Biosciences Corp. Piscataway, NJ). A positive control for TSP-1 was obtained from exponentially growing HUVECs. For isolation of leukocytes, peripheral blood of PPARα WT and KO mice was obtained by retro-orbital bleeding under isoflurane anesthesia, red cells were cleared by incubating samples for 30 minutes on ice in red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO). Leukocytes were lysed in 100 μl of a solution consisting of 20 mmol/L imidazole hydrochloride, 100 mmol/L KCl, 1 mmol/L MgCl, 1 mmol/L EGTA, 1% Triton X-100, 10 mmol/L NaF, 1 mmol/L sodium molybdenate, 1 mmol/L EDTA and protease inhibitor cocktail [67].

TSP-1 ELISA

TSP-1 was measured by ELISA (Cytimmune, Rockville, MD) in blood plasma collected from non-tumor bearing PPARα WT and KO mice. Blood was collected via retro-orbital puncture.

Statistical Analyses

Statistical ananlyses were performed by Student's *t* test. The results were considered statistically significant for $p < 0.05$.

SUPPORTING INFORMATION

Figure S1 Tumor angiogenesis is inhibited in the cornea of PPARα KO mice. PPARα WT and KO host mice were implanted with tumor pieces (1 mm3) as indicated. (A) Comparison of PPARα(+/+)MEF/RS and PPARα(−/−)MEF/RS in WT mice day 9 and day 16. (B) PPARα(+/+)MEF/RS and PPARα(−/−)MEF/RS in PPARα KO day 9 and day 16. The angiogenic

response of PPARα(−/−)MEF/RS in PPARα KO mice regressed by day 16. (C) Lewis Lung Carcinoma (LLC) in PPARα WT and KO, C3H/HeJ and Balb/cJ on day 12. LLC tumors induced tumor angiogenesis independent of host haplotype. Therefore, major histo-incompatibility (MHC) does not prevent tumor-induced neovascularization and tumor growth. In contrast, LLC tumors failed to trigger any angiogenic response in PPARα KO host. (D) B16-BL6 melanoma in PPARα WT and KO on day 16. (E) Histology of B16-BL6 melanoma in the cornea of PPARα WT and KO mice. Scale bars, 500 μm (left) and 100 μm (right) (F) Leukocyte (CD45, brown) staining of LLC tumors in the cornea of PPARα WT and KO mice. Scale bar, 100 μm.
Found at: doi:10.1371/journal.pone.0000260.s001 (8.59 MB AI)

Figure S2 (A) FACS analysis demonstrates % of CD45.1 host cells. In our bone marrow transplantation protocol, >90% of the hematopoietic system of the host was derived from the donor marrow (as proved by using CD45.1 mice as recipients and PPARα KO mice that are CD45.2 as donors). (B) Panleukocyte (CD45, brown) and neutrophil elastase (red) staining in PPARα(−/−)MEF/RS tumors in PPARα WT (day 25) and PPARα KO mice (day 55). Scale bar, 500 μm. (C) FACS analysis demonstrates granulocyte depletion in PPARα KO mice. (D) TSP-1 expression (brown) in B16-F10 (day 30) and PPARα(−/−)MEF/RS (day 60) tumors in PPARα KO and WT mice as determined by immunohistochemical staining. Scale bars, 100μm and 500 μm, respectively.
Found at: doi:10.1371/journal.pone.0000260.s002 (5.12 MB TIF)

Text S1 Genetic Background and Transplantation Immunity.
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Author Contributions

Conceived and designed the experiments: SH DP AK. Performed the experiments: SH DP AK. Analyzed the data: SH DP AK. Contributed reagents/materials/analysis tools: SH. Wrote the paper: SH DP AK. Other: Participated in designing the studies and data analysis and participated in the intellectual input and discussions with AK and DP, and contributed to the writing of the paper: JF. Participated in designing the studies and data analysis and participated in the intellectual input and discussions with AK and DP and contributed to the writing of the paper: SH. Participated in designing all bone marrow transplantation and antibody depletion studies and performed the and contributed to the writing of the paper: GM. Participated in data analysis and the intellectual input and discussions with AK and DP: MK. Participated in designing the corneal assays and performed them: CB. Participated in designing all the metastasis assays and performed them: DB. Participated in designing the bone marrow transplantation experiments and the intellectual input and discussions with GM: RM.

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Platelet-associated PF-4 as a biomarker of early tumor growth.

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Running Foot: PF-4 as a marker for early tumor detection

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<u>Abstract</u>.

Early tumor detection and intervention are important determinants of survival in patients with cancer. We have recently reported that the 'platelet angiogenesis proteome' may be used to detect microscopic tumors in mice. We now present evidence that changes in platelet-associated platelet factor-4 (PF-4) detect malignant growth across a spectrum of human cancers in mice. A deregulated expression of an 8206 Da protein was observed by SELDI-ToF MS proteomic comparison of platelets from normal and tumor-bearing mice. The differentially expressed protein band was identified as PF-4 by tandem mass spectrometry and ProteinChip immunoassay using anti-PF-4 antibody. The platelet-associated PF-4 appeared to be up-regulated in early growth of human liposarcoma, mammary adenocarcinoma, and osteosarcoma. A 120 day follow-up study of liposarcoma revealed a sustained $\geq$2-fold increase of platelet-associated PF-4 at 19, 30, and 120 days. In contrast, only an insignificant change of PF-4 was observed in the plasma of mice bearing the different human tumor xenografts, and throughout the 120 days of the liposarcoma study. We conclude that platelet-associated PF-4, but not its plasma counterpart, may represent a potential biomarker of early tumor presence.

Introduction

The identification of biomarkers of early tumor recurrence, growth, and therapeutic response has been of great interest in oncology. Considerable effort is currently focused on methods for early tumor detection, including those involving: detection of specific proteins or proteomic profiles in the serum [1-4], DNA in stool samples [5-8], and gene expression profiles in lesional biopsies [9-12]. The realization that angiogenesis is a critical part of tumor progression of *solid* [13] and *liquid* [14;15] tumors led, over the past few decades, to numerous attempts to correlate plasma and serum levels of angiogenic proteins with disease progression [16;17]. While helpful in the identification of patients with disseminated disease, the reliability of serum and plasma levels of VEGF, bFGF, or other angiogenesis regulatory proteins in early-stage tumors remains uncertain [18;19].

Our previous report that platelets may serve as a reservoir of biomarkers [20] introduced the finding that the platelet protein content may reflect the presence of a tumor. Further proteomic analysis of platelets from tumor-bearing and non-tumor bearing mice revealed that the majority of differentially expressed proteins were angiogenesis regulators, rather than the more abundant proteins such as albumin and fibrinogen. The levels of albumin and fibrinogen contained in the platelets were equal in tumor-bearing and non-tumor-bearing mice. This finding suggests a very selective sequestration of angiogenesis regulating proteins by platelets. We also showed that the enhanced sequestration of angiogenesis regulators in the platelet may enable us to detect tumors as small as 1 mm^3 in mice.

Platelets may sequester these proteins and protect them from plasma proteolytic enzymes. As a result of this sequestration, platelet-associated proteins, and PF-4 in particular, may be more reliable in detecting early cancer growth than their respective plasma or serum counterparts.

Platelet factor-4 (PF-4) is a tetrameric, lysine-rich member of the CXC chemokine family produced almost exclusively by megakaryocytes. Under physiological conditions, only a small amount of platelet factor-4 is taken up by circulating platelets, therefore the bulk of the PF-4 protein originates in megakaryocytes. PF-4 was originally cloned from a human erythroleukemia cell line [21], and its genetic mapping and polymorphisms were discovered soon thereafter [22;23]. PF-4 is stored within the α-granules of platelets and secreted at high concentrations in the vicinity of injured blood vessels following platelet activation [24]. Platelet factor-4 was discovered to inhibit angiogenesis in 1982 [25]. By 1990, it was shown to inhibit tumors in mice [26]. In 1995,

platelet factor-4 was reported to bind preferentially to vascular endothelium *in vivo* [27] and to bind selectively to regions of active angiogenesis *in vivo* [28]. By 1998, PF-4 was revealed to be a marker of new vessel formation in xenografts of human breast cancer [29].

In the absence of any known receptor for PF-4, its antiangiogenic effect [30;31] is presumed to be due to its ability to bind stimulatory chemokines like IL-8 [32;33] and to compete with other growth factors for heparin binding [34;35]. The heterodimer of IL-8 and PF-4 enhances the anti-proliferative activity of PF-4 and attenuates the stimulatory effects of IL-8 [32]. PF-4 also modulates the effect of pro-angiogenic growth factors. It binds with high affinity to vascular endothelial growth factor ($VEGF_{165}$), preventing the interaction of $VEGF_{165}$ with its receptor (VEGFR-2) thus inhibiting angiogenesis [34;36]. A PF-4 derivative generated by peptide bond cleavage between Thr-16 and Ser-17 exhibits a 30- to 50-fold greater growth inhibitory activity on endothelial cells than PF-4 itself [37]. The anti-tumor effect of PF-4 is also revealed by a decrease in the number and size of lung metastases of B16F10 melanoma [38] and a decrease in growth of HCT-116 human colon carcinoma [39]. PF-4 modifies the mitogenic effect of bFGF on fibroblasts [40], inhibits the proliferation of activated human T cells [41] and tumor infiltrating lymphocytes, and inhibits cytokine release by tumor stroma [42].

Here we present new data demonstrating that changes in the platelet concentration of PF-4 may be used as a novel biomarker to detect human tumor xenografts that range from microscopic to macroscopic size. These tumors in mice include human liposarcoma, mammary adenocarcinoma, and osteosarcoma.

Materials and Methods

Human-tumor xenografts.

All of the cancer cell lines used exhibit either non-angiogenic (microscopic, dormant tumors) or angiogenic (rapidly growing tumors) phenotypes in immuno-deficient mice. The non-angiogenic and angiogenic cell lines have been previously described by Folkman *et al.* [43-45]. For each of the three parent cell lines [liposarcoma (SW872), osteosarcoma (KHOS-24OS), and mammary adenocarcinoma (MDA-MB-436)] two phenotypes exist, a non-angiogenic tumor and an angiogenic one.

All cell lines were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 1% antibiotics (penicillin, streptomycin) and 0.29 mg/mL L-glutamine in

a humidified 5% CO_2 incubator at 37°C. For injections into mice, 80-90% confluent tumor cells were rinsed in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO), briefly trypsinized and suspended in serum-free DMEM. Five million viable cells from each of the tumor cell lines were suspended in 200 µL of serum-free media and implanted subcutaneously (SW872 and KHOS-24OS cell lines) into the flanks of six to eight weeks old male SCID mice. For the human breast adenocarcinoma (MDA-MB-436) cell line, 1 million viable cells were suspended in 50 µL of serum-free media and implanted in the mammary fat pad through a 0.75-1 cm incision. The corresponding sham operation was 0.75-1.0 cm incision. The mice were terminally bled under isofluorane anesthesia at 30 days post implantation to collect the platelets. The mice were obtained from the Massachusetts General Hospital (MGH), Boston, MA. Animals and tumors were monitored daily as per institutional guidelines. Data was analyzed using Student t-test to determine the p values for differences between means, the graphs in figure 5 and 6 are therefore expressed as means +/- SEM.

Platelet and plasma processing for SELDI-ToF Mass Spectroscopy.

Blood samples were processed according to standard methods for platelet collection. Briefly, mice were anaesthetized using 2% isofluorane/1L O_2 flow system. One milliliter of whole blood was collected by terminal cardiac bleed into 105 mM sodium citrate, pH 5 anticoagulant at a ratio of 1:9 (v/v), buffer to blood. The first centrifugation step at 180 g for 20 minutes at room temperature allowed for the collection of platelet rich plasma (PRP). A second centrifugation at 900 g for 30 minutes at room temperature separated the platelets and the upper phase, platelet poor plasma (PPP). This resulted in two separate phases for processing and analysis by SELDI-ToF MS technology (Ciphergen, Fremont, California), platelet pellets and PPP. The pellets and 20 µL of PPP from each mouse were processed in 25 µL and 40 µL, respectively, of U9 buffer (2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propansulfonate), 50 mM Tris-HCl, pH 9 (Ciphergen) for 1 hour at room temperature. Platelet lysates were then centrifuged at 10,000 g for 1 minute at 4°C. Both platelet extracts and PPP were fractionated by anion-exchange chromatography modified after the Expression Difference Mapping (EDM) Serum Fractionation protocol (Ciphergen, Fremont, CA). The fractionation was performed in a 96-well format filter plate on a Beckman Biomek® 2000 Laboratory Work Station equipped with a DPC® Micromix 5 shaker. An aliquot of 20 µL of the platelet and 60 µL of denatured plasma diluted with 100 µL of 50 mM Tris-HCl pH9 was transferred to a filter bottom

96-well microplate pre-filled with BioSepra Q Ceramic HyperD F sorbent beads re-hydrated and pre-equilibrated with 50 mM Tris-HCl, *pH 9*. All liquids were removed from the filtration plate using a multiscreen vacuum manifold (Millipore, Bedford, MA) into respective wells of 96-well microtitre plates with the capture of the initial flow-through as fraction 1. This step was repeated for subsequent incubations with 2 x 100 µL of the following buffers: *pH 7.5* (1 M urea, 0.1% CHAPS, 50 mM NaCl, 2.5% acetonitrile, 50 mM Tris-HCl (50 mM HEPES)); *pH 5* (1 M urea, 0.1% CHAPS, 50 mM NaCl, 2.5% acetonitrile 50 mM NaAcetate); *pH 4* (1 M urea, 0.1% CHAPS, 50 mM NaCl, 2.5% acetonitrile 50 mM NaAcetate); *pH 3* (1 M urea, 0.1% CHAPS, 500 mM NaCl, 2.5% acetonitrile 50 mM NaCitrate), which yielded the respective fractions 2, 3, 4 and 5. A final organic wash with 33% isopropanol / 16.7% acetonitrile / 8% formic acid represents fraction 6.

Expression difference mapping (EDM) on ProteinChip arrays was carried out using weak cationic exchange chromatography protein arrays (WCX2 and CM10 ProteinChip arrays; Ciphergen, Fremont, CA) by loading sample fractions onto a 96-well bioprocessor, and equilibrating with 50 mM sodium acetate 0.1% octyl glucoside (Sigma, St. Louis, MO), *pH 5*. A further dilution of 40 µL anion exchange chromatography fraction into 100 µL of the same buffer on each array spot was incubated for 1 hr. Array spots were washed for 3 minutes with 100 µL 50 mM sodium acetate 0.1% octyl glucoside *pH 5*. After rinsing with water, 2 x 1 µL of sinapinic acid matrix solution was added to each array spot. For protein profiling, all fractions were diluted 1**:**2.5 in their respective buffers used to pre-equilibrate ProteinChip arrays. This step was followed by readings using the Protein Biology System II (PBSII) and Protein Ciphergen System 4000 (PCS4000) SELDI-ToF mass spectrometer (Ciphergen, Fremont, CA) and processed with the ProteinChip Software Biomarker Edition[®], Version 3.2.0 (Ciphergen, Fremont, CA). After baseline subtraction, spectra were normalized by means of a total ion current method. Peak detection was performed by using Biomarker Wizard software (Ciphergen, Fremont, CA) employing a signal-to-noise ratio of 3.

For immunocapture experiments, anti-PF-4 antibody (rabbit, affinity purified polyclonal antibody, R&D, Minneapolis, MN) was immobilized on pre-activated ProteinChip array (RS100, Ciphergen, Fremont, CA). After blocking and washing of excess antibody, platelet extract diluted in BSA Triton X100 PBS was incubated with the immobilized antibody. After washing with PBS containing urea and CHAPS, the captured proteins were detected by SELDI. To

confirm a full capture of the protein, the mobile phase was also incubated on a preactivated spot for a second time to verify immunodepletion. A final confirmation of the protein identity was obtained using murine PF-4 ELISA (data included as Supplemental Figure 2).

Protein identification.

Candidate protein biomarker was purified by affinity chromatography on beads with immobilized IgG spin columns and by reverse phase chromatography. The purity of each step was monitored by employing Normal Phase (NP) ProteinChip arrays. The enriched fractions were reduced by 5 mM DTT in TRis-HCL buffer, pH 9 and alkylated with 25 mM iodoacetamide. The alkylated preparation was finally purified using 16 % Tricine SDS-PAGE. The gel was stained by Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA). Selected protein bands were excised, washed with 200 µL of 50% methanol/10 % acetic acid for 30 min, dehydrated with 100 µl of acetonitrile (ACN) for 15 minutes, and extracted with 70 µL of 50 % formic acid, 25 % ACN, 15% isopropanol, and 10 % water for 2 hr at room temperature with vigorous shaking. The candidate biomarkers in extracts were again verified by analysis of 2 µL on a Normal Phase ProteinChip array (NP20). The remaining extract was digested with 20 µL of 10 ng/µL of modified trypsin (Roche Applied Science, Indianapolis, IN) in 50mM ammonium bicarbonate (*pH 8*) for 3 hr at 37°C. Single MS and MS/MS spectra were acquired on a QSTAR mass spectrometer equipped with a Ciphergen PCI-1000 ProteinChip Interface. A 1 µL aliquot of each protease digest was analyzed on an NP20 ProteinChip Array in the presence of CHCA matrix (Ciphergen). Spectra were collected from m/z values of 900 to 3000 in single MS mode. After reviewing the spectra, specific ions were selected and subjected to collision-induced-dissociation (CID). The CID data were submitted to the database-mining tool Mascot (Matrix Sciences) for identification (Table 1 and Supplemental Figure 1).

<u>**Results**</u>

Identification of the differentially expressed PF-4 in tumor-bearing mice.

Platelet lysates from healthy non-tumor-bearing mice and those bearing non-angiogenic or angiogenic xenografts of human liposarcoma, mammary adenocarcinoma, and osteosarcoma for a minimum of thirty days, were subjected to a standard biomarker discovery protocol. Among the several unknown differentially expressed proteins, elevation was observed in the platelet content of a polypeptide with an apparent molecular weight of 8206 Da (Figure 1A and Figure

2). This protein was later found to be consistently elevated across three repeated experiments, and across several tumor types.

A careful analysis was therefore performed to identify the protein of interest. The 8206 Da protein was purified using chromatography and SDS-PAGE. Gel-purified protein was digested with trypsin and unique tryptic fragments were analyzed by tandem MS (data in Supplemental Figure 1). The ion with m/z of 1350.75 was identified as Cys-carbamidomethylated peptide HCAVPQLIATLK with Mowse score [46] of 60 (ion score >17 indicated significant homology, >22 indicated identity or extensive homology). The ion with m/z of 1677.90 was identified as Cys-carbamidomethylated peptide HCAVPQLIATLKNGR with Mowse score of 14 (ion score >13 indicated significant homology, >15 indicated identity or extensive homology). Both peptides corresponded to unique tryptic fragments of mouse PF-4 (SwissProt accession # Q9Z126) previously identified [47-49]. Theoretical molecular weight of mouse PF-4 is 8210.71 Da, however considering two Cys-Cys bridges in the polypeptide molecule, the expected MW is 8206.71 Da. The latter value is very close to the observed experimental molecular weight of the candidate biomarker.

Confirmation of identity of platelet-derived PF-4 by ProteinChip immunoassay using anti-PF-4 antibody.

Further validation of this candidate biomarker, was obtained by immunoprecipitation using rabbit anti-PF-4 antibody. Figure 2 represents the spectral read-out obtained from arrays coated with an immobilized anti-PF-4 antibody prior to incubation with the platelet extracts from mice. The presence of a thick protein peak at 8206 Da (arrow) validates both the presence and theoretical mass of PF-4 (Figure 2). The identity of the differentially expressed protein was further confirmed by immunocapture/immunodepletion of the protein. The protein captured using the PF4 specific antibody has a peak identical to that of the recombinant protein (upper two panels of Figure 3) and the peak is absent in the mobile phase of the spotted lysate (Figure 3).

Validation of PF-4 as a surrogate marker of tumor presence.

Platelets of non-angiogenic or angiogenic human liposarcoma xenografts, SW872, exhibited a seven-fold elevation of platelet-derived PF-4 when compared to non-tumor-bearing controls at 30 days post implantation (Figure 4A) without a corresponding increase of PF-4 in the plasma. In this model, platelets of mice bearing the non-angiogenic xenografts (tumors less than 1 mm) contained PF-4 levels comparable to its angiogenic counterpart. Platelets of mice

bearing the angiogenic mammary adenocarcinoma, MDA-MB-436 (Figure 4B) or the angiogenic osteosarcoma, KHOS-24OS (Figure 4C) revealed similar trends at 4- and 2-fold up-regulation, respectively. The non-angiogenic xenografts did not show an elevation of PF-4 in platelets. The platelet-associated PF-4 content was consistently found to be higher than that of the corresponding plasma, even though the degree of elevation varied based on tumor type (Figure 4A-C). Angiogenic tumors were associated with the greatest differences between platelet-derived PF-4 versus that of plasma, even though platelets of mice bearing the non-angiogenic tumors of liposarcoma also showed PF-4 elevation. Liposarcoma xenografts exhibited the greatest platelet content of PF-4, while the increases in PF-4 for mammary adenocarcinoma and osteosarcoma were not as large (Figure 4A-C).

Platelet PF-4 in early tumor detection.

To test whether platelet content of angiogenesis regulators can be utilized in detection of early tumor growth, we explored the ability of PF-4 to predictably detect non-angiogenic (dormant) microscopic tumors in mice over an extended period of time. We conducted a time-course analysis of platelet-associated PF-4 in mice bearing a subcutaneous xenograft of the non-angiogenic (dormant) clone of human liposarcoma (SW872). The malignant progression of liposarcoma has been previously described [43], and it is known that the non-angiogenic (dormant) clone undergoes a spontaneous switch to the angiogneic phenotype, begins to grow, and becomes detectable by gross examination at a median of approximately 133 days post implantation. We show that PF-4 remains significantly elevated throughout a period of 120 days of observation of the non-angiogenic (dormant) state. Even without a palpable tumor, at 19 days, the median level of PF-4 in platelets is 1.7 fold higher than baseline without a corresponding increase in plasma level of the protein (Figure 5A&B). The plasma and platelet levels of PF-4 were similar at the time of implantation. However, while platelet PF-4 rose in the first two weeks of tumor growth, and remained elevated for the duration of the 120 days of the experiment, plasma PF-4 continued to decline (Figure 5B). The size of the tumor did not exceed 1 mm for the duration of the experiment.

Discussion

Numerous angiogenesis regulatory proteins are present in platelets [50]. While the relative concentrations of these proteins remain stable under physiologic conditions, their levels change

significantly in the presence of a tumor. There are other reports documenting angiogenesis regulatory factors in platelets of cancer patients [51-53], and continuing controversy persists as to whether serum or plasma levels of angiogenesis factors are more accurate for the measurement of angiogenesis-related diseases [54]. In a study of paired serum and plasma samples [55;56], VEGF levels correlated with platelet count in 116 patients with colorectal cancer, but not in controls. Support can be found for both serum or plasma measurements [55;57;58].

The diagnostic use of angiogenic proteins such as VEGF, bFGF, or PF-4 in early disease has been hindered in part by the minute levels of the proteins and their short half-lives in the circulation. The finding of platelet sequestration of angiogenesis regulatory proteins suggests a new modality for early detection of human cancer. We propose that angiogenesis regulators are not released from platelets into the circulation. Instead, these proteins are exchanged locally at sites of platelet adhesion and aggregation, where they remain bound to glycosaminoglycans such as heparan sulphate in tissues. As such, the levels of these proteins in plasma or serum increase significantly only in the presence of a large tumor load that generates sufficient angiogenesis regulatory proteins to saturate the mass of circulating platelets. We provide evidence that at least one of these platelet proteins, PF-4, can reliably predict the presence of a microscopic, non-angiogenic (dormant) human tumor in mice and circulates predominantly in platelets early in the disease process. Its relative absence in plasma may explain why the proteomic search for plasma and serum markers of patients with various cancers [59-61] did not identify this marker. We emphasize that while only PF-4 is being presented here, other angiogenesis regulatory proteins sequestered in platelets may have the same diagnostic capacity and remain to be identified.

We introduce PF-4 as one of the platelet-associated angiogenesis regulators that may serve as an early tumor biomarker. We show that platelet associated PF-4 can be detected as early as 19 days post implantation, and that a steady elevation of the protein can be observed throughout 120 days (Figure 5). While this manuscript does not provide sufficient data to support a functional role of PF-4 in tumor angiogenesis, there is sufficient published evidence that PF-4 is an angiogenesis suppressor and a tumor growth suppressor [25;26;38;39;62-64]. PF-4 may modulate tumor growth by modifying VEGF effects [35] or by binding and neutralizing heparin [65] and related sulfated glycosaminoglycans [42] required for the binding of pro-angiogenic factors [30;40;66]. The binding and neutralization of heparin down-regulates angiogenesis mainly by preventing the binding of other angiogenesis regulators to heparan sulphate in tissues and by interfering with

VEGF and bFGF signaling pathways [67]. The high levels of VEGF and bFGF secreted by the non-angiogenic clone of SW872 liposarcoma [43] may be counterbalanced by PF-4 leading to tumor quiescence and dormancy. The increase in platelet-associated PF-4 in dormant (non-angiogenic) tumors may therefore be reflective of the functional inhibition of angiogenesis in liposarcoma, which secretes large amounts of VEGF and bFGF [43]. A feedback loop may exist in animals bearing tumors, such that increased VEGF and bFGF induce megakaryocyte synthesis of PF-4. This is supported by the finding that tumors which do not secrete large amounts of VEGF and bFGF, such as the non-angiogenic clones of MDA-MB-436 mammary adenocarcinoma and the KHOS-24OS osteosarcoma [45], do not manifest a marked elevation of PF4 (Figure 4). These tumors may use other means of tumor growth suppression. PF-4 appears to be a marker of angiogenesis and was present in the platelets of all of the tested angiogenic tumor models.

The changes in platelet-associated PF4 may have the potential to convey valuable clinical information about the angiogenic potential of the tumor, and a serial measurement of platelet PF-4 levels may provide us with the ability to detect tumor progression in an otherwise healthy subject.

Numerous reports have suggested an active role of platelets in cancer progression [68;69] and in tumor growth and metastasis [70-72]. Most investigators assume that platelets act as a reservoir of angiogenic proteins which are released into the sera [53;73]. However, we show here that platelets actively sequester select proteins in tumor-bearing animals, and that this process is distinct from the non-specific uptake of proteins such as albumin. One of the main reasons previous proteomic analysis on platelets [74-78] may not have detected the differential expression of angiogenesis related proteins was because these studies analyzed normal platelets and not platelets of cancer patients. We report for the first time, to our knowledge, the changes in the "platelet angiogenesis proteome" in response to the presence of a tumor in experimental animals.

Platelet-associated PF-4 may be a potential tumor biomarker. Platelet-associated PF-4 should be explored in other mouse models of cancer and in high risk patient populations predisposed to early tumor progression due to mutations in APCC, p53, PTEN, or BRCA1. If validated, it may improve our ability to intervene very early in recurrent cancer, keep cancers in a dormant stage, and possibly convert cancer into a chronic, more manageable disease [79;80]. As biologic modifiers, including angiogenesis inhibitors, that are relatively less toxic, become available for cancer therapy, early treatment may be much more possible than it has been in the

past. A long-term goal would be to "treat the biomarker" until it returns to normal, before the onset of symptoms of recurrent tumor and before the tumor can be anatomically located.

Legends

Table 1: Identification of the 8 206Da protein as PF-4. Single MS spectra were acquired on a QSTAR mass spectrometer equipped with a Ciphergen PCI-1000 ProteinChip Interface from m/z values of 900 to 3000. After reviewing the spectra, specific ions were selected and subjected to collision-induced-dissociation (CID). The CID data were submitted to the database-mining tool Mascot (Matrix Sciences) for identification. Following further collision induced dissociation (CID) of the predominant tryptic fragments with m/z of 1350.75 (A) and 1677.90 (B), amino acid sequencesre obtained. A Mowse Score of 60 and 14 revealed these sequences to match with high fidelity to the sequence of murine PF-4.

Figure 1: Identification of the platelet- and plasma-derived candidate proteins. Platelets were harvested from mice bearing non-angiogenic or angiogenic xenografts of human liposarcoma at 30 days post tumor implantation and compared to those of their littermate controls using a standard SELDI-ToF biomarker discovery method. A spectral read-out from SELDI-ToF MS is presented here in gel view format, and groups are color-coded for clarity. Grey represents protein content of platelets from control animals, blue from mice bearing the nonangiogenic dormant clone, and red from mice bearing the angiogenic clone. A differentially expressed protein was observed at 8206 Da. The candidate peptide (arrows) was later analyzed and identified as PF-4. Each horizontal strip represents an individual mouse sample (n=5), and the color intensity corresponds to the height of the protein peak. The experiment was reproduced on two independent occasions for a total of 15 mice per group.

Figure 2: Validation of candidate biomarker by immunoprecipitation. Spectral read-out in gel view format obtained from arrays prepared with an anti-PF-4 antibody prior to incubation with platelet extracts from mice within the indicated groups. The labeled arrow indicates both the presence and theoretical mass of PF-4.

Figure 3: Confirmation of the PF-4 identity by immunocapture and immunodepletion. For immunocapture experiments, anti-PF-4 antibody was immobilized on a pre-activated ProteinChip array, followed by incubation with platelet extracts derived from mice bearing the

dormant clone of liposarcoma. Comparison of a profile generated by the recombinant PF-4 (first panel) with that generated by platelet lysates of dormant liposarcoma-bearing mice reveals an identical molecular weight and isoelectric point of the protein in question (second panel). Immunodepletion of the PF-4 protein is confirmed by the absence of its respective peak from the mobile phase (fourth panel).

Figure 4: PF-4 in platelets of mice bearing human-tumor xenografts. Platelets of mice bearing xenografts of SW872 liposarcoma (Panel A), MDA-MB-436 mammary adenocarcinoma (Panel B), or KHOS-24 osteosarcoma (Panel C) were analyzed using SELDI-ToF. The blue bars represent whole platelet extracts and brown bars represent plasma. The platelets of non-tumor-bearing control mice, served as a reference for the endogenous levels of platelet and plasma derived PF-4. The control group is shared by all three experiments. Platelets of non-angiogenic or angiogenic human liposarcoma xenografts, SW872, exhibited a seven-fold elevation of platelet-derived PF-4 when compared to non-tumor-bearing controls at 30 days post implantation (Panel A) without a corresponding increase of PF-4 in the plasma. Platelets of mice bearing the angiogenic mammary adenocarcinoma, MDA-MB-436 (Panel B) also had significant elevation of platelet, but not plasma PF-4. In the case of angiogenic osteosarcoma, KHOS-24OS (Panel C) a similar trend at 4- and 2-fold up-regulation can be observed, but the value did not reach significance. Each bar represents the mean peak intensities corresponding to the level of the protein (+/-SEM) of 5-10 mice per experiment. Student t-test was used to compare means of the groups. Each experiment was repeated twice.

Figure 5: Elevation of platelet-derived PF-4 correlates with the presence of microscopic tumors. Platelets and plasma from mice bearing a non-angiogenic subclone of the human liposarcoma (SW872) were analyzed at the indicated times using SELDI-ToF. The relative levels of PF-4 protein in platelets of non-tumor-bearing mice at time 0 (●), i.e. before the implantation of the tumors, were compared to platelet-associated PF-4 on day 19(■), day 30 (▲) and day 120 (▼). At 19 days, without a palpable tumor, the median level of PF-4 in platelets is 1.7 fold higher than baseline without a corresponding increase in plasma level of the protein. Even though the elevation has not reached significance, the trend was maintained throughout the length of the

experiment. The red symbols within the cluster analysis represent the median peak intensity of 5-6 mice +/-SEM.

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Table 1

m/z	Amino acid sequence	Mowse score[a]	Significant homology score[b]	Identity or extensive homology score[c]
Peptide I 1350.75	HCAVPQLIATLK + CAM[d]	60	>17	>22
Peptide II 1677.90	HCAVPQLIATLKNGR + CAM	14	>13	>15

[a] Ion score is -10*Log(P), where P is probability that the match is a random event.
[b] Individual ion scores higher than a number displayed in the column indicate significant homology
[c] Individual ion scores higher than a number displayed in the column indicate identity or extensive homology (p<0.05)
[d] Carbamidomethyl cysteine

Platelet

Plasma

Control

Non
Angiogenic

Angiogenic

7500 8000 8500 9000

7500 7500 8000 8500 9000

PF4

PF4

Figure 1

Anti-*PF4* SELDI-ToF

Control

Non Angiogenic

Angiogenic

7500   8000   8500   9000

8206 Da
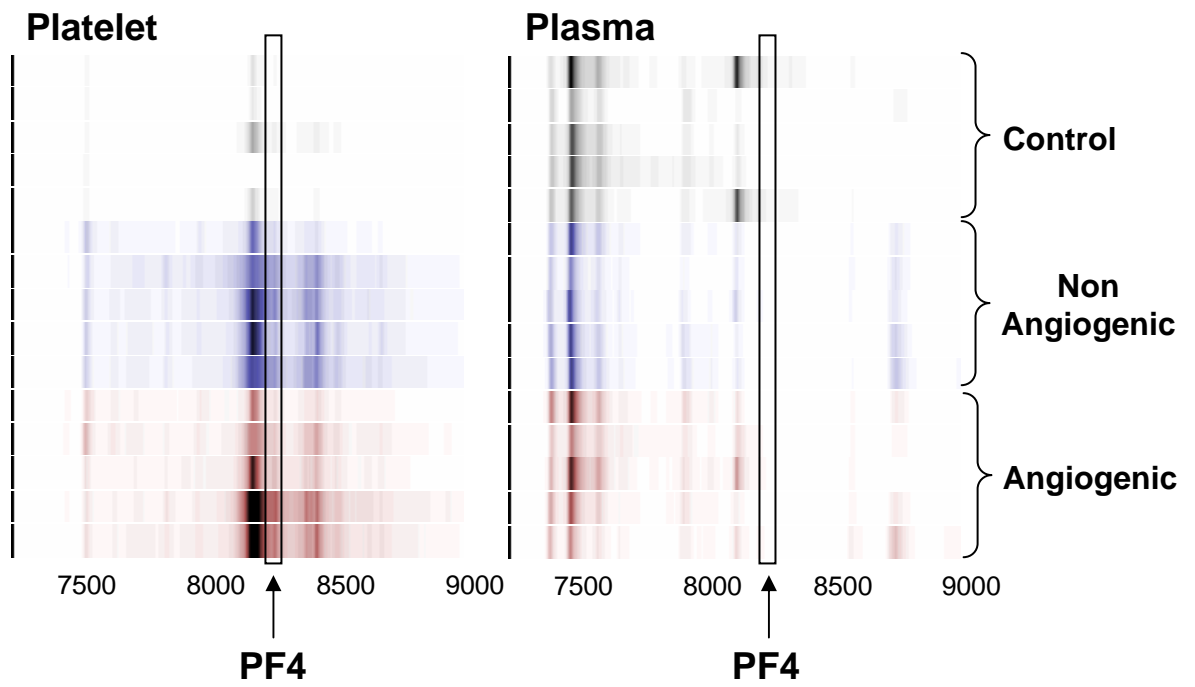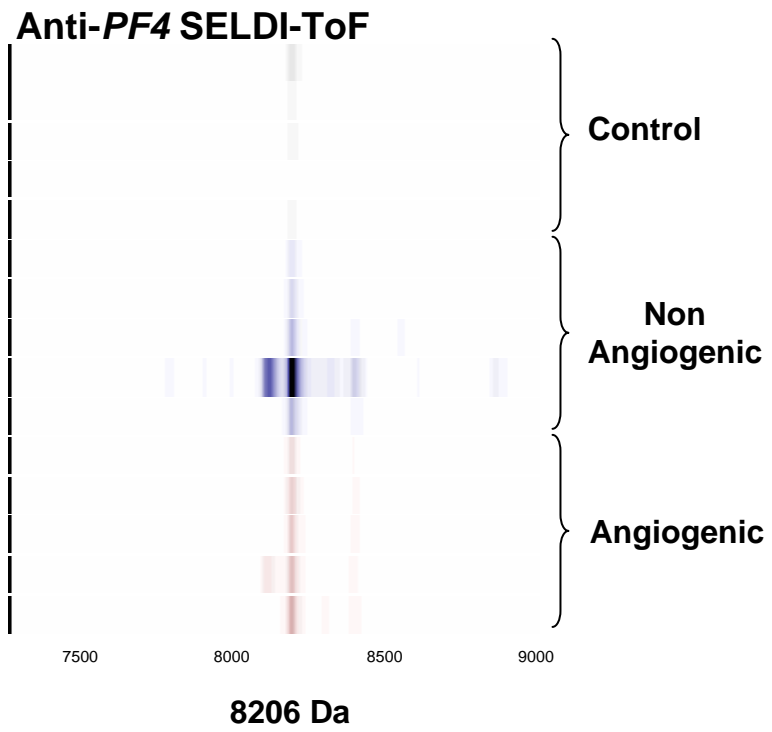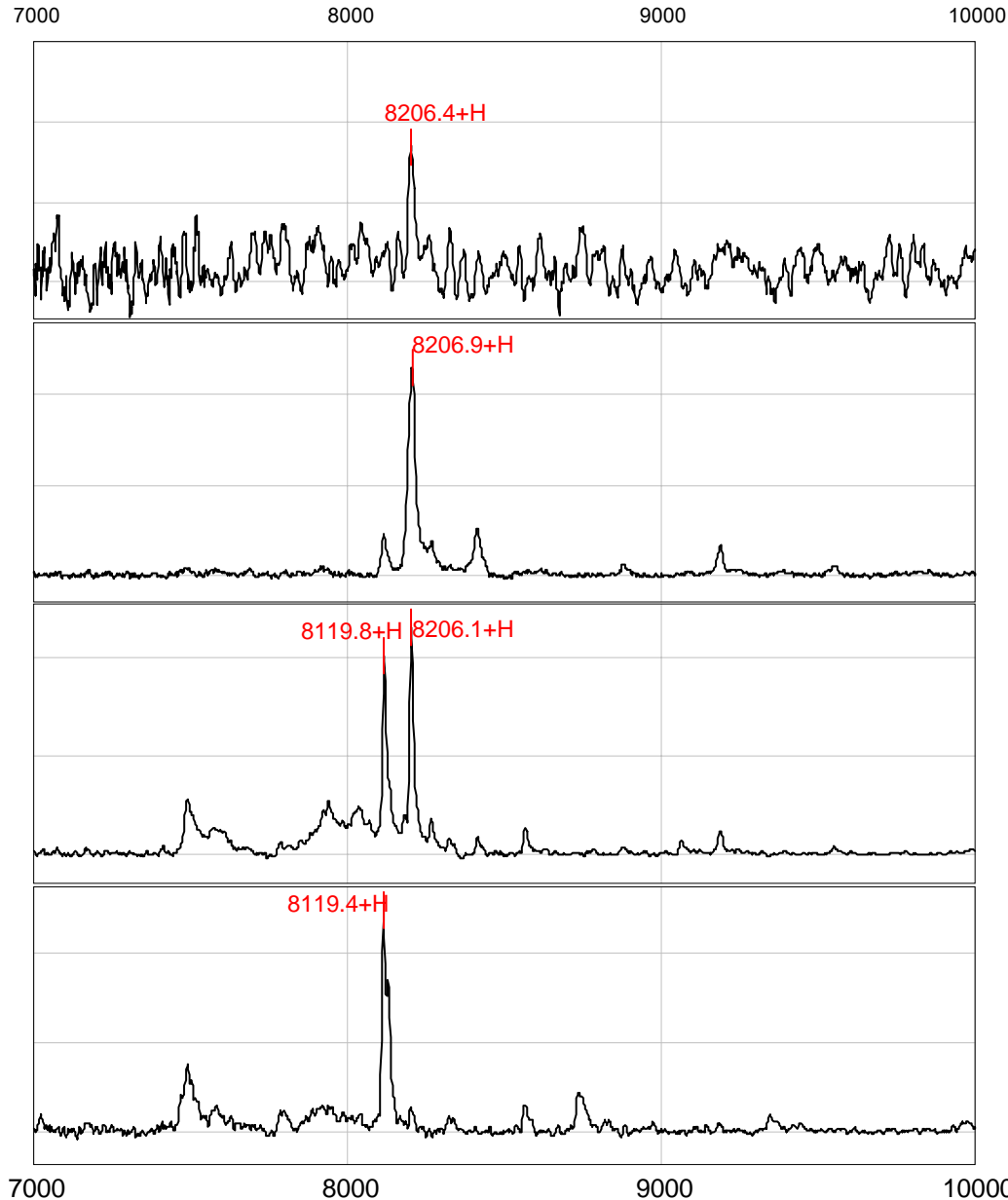
Figure 2

Immunocapture of Recombinant PF4

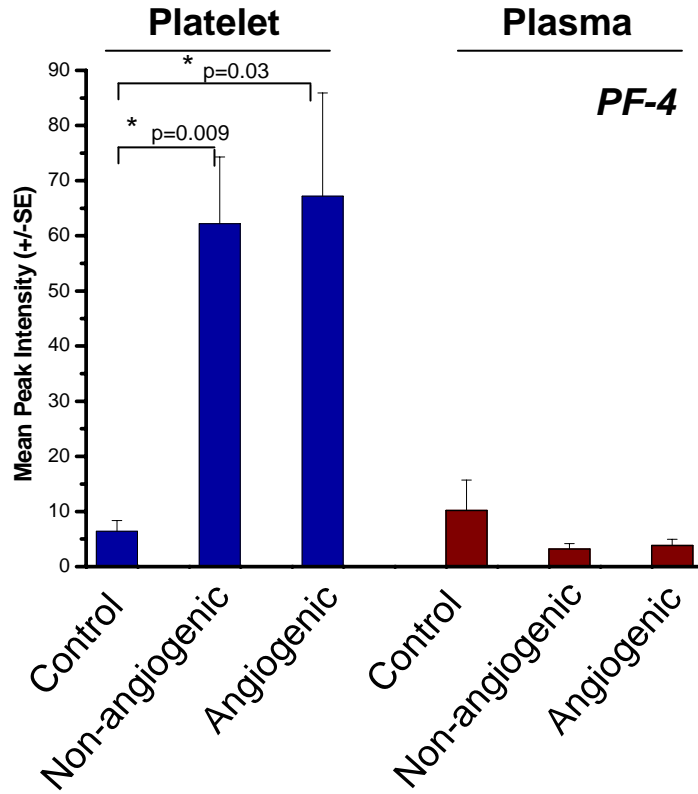**Representative Profile of Immunocapture of PF4 from Platelets from mice bearing Dormant Tumors**

Representative Profile of Dormant Tumor Platelet

Profile after Immunodepletion of PF4

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Figure 3

A

Liposarcoma (SW872)

Platelet   Plasma

PF-4

Mean Peak Intensity (+/-SE)

* p=0.03
* p=0.009

Control, Non-angiogenic, Angiogenic, Control, Non-angiogenic, Angiogenic

B

Breast Cancer (MDA-436)

Platelet   Plasma

PF-4

Mean Peak Intensity (+/-SE)

* p=0.037

Control, Non-angiogenic, Angiogenic, Control, Non-angiogenic, Angiogenic

Figure 4

C

Platelet   **Plasma**

**Osteosarcoma
(KHOS-24OS)**

PF-4

Mean Peak Intensity (+/-SE)

30

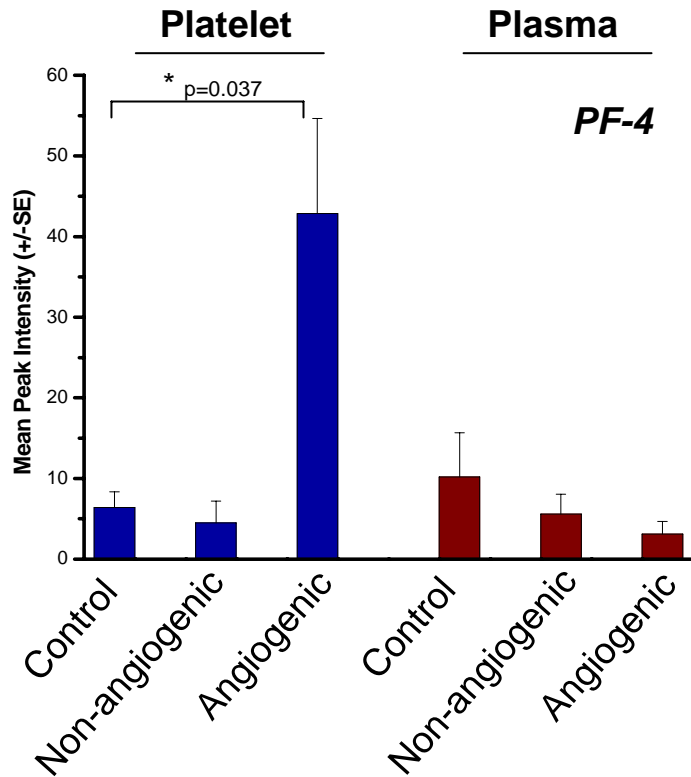20

10

0

Control   Non-angiogenic   Angiogenic   Control   Non-angiogenic   Angiogenic
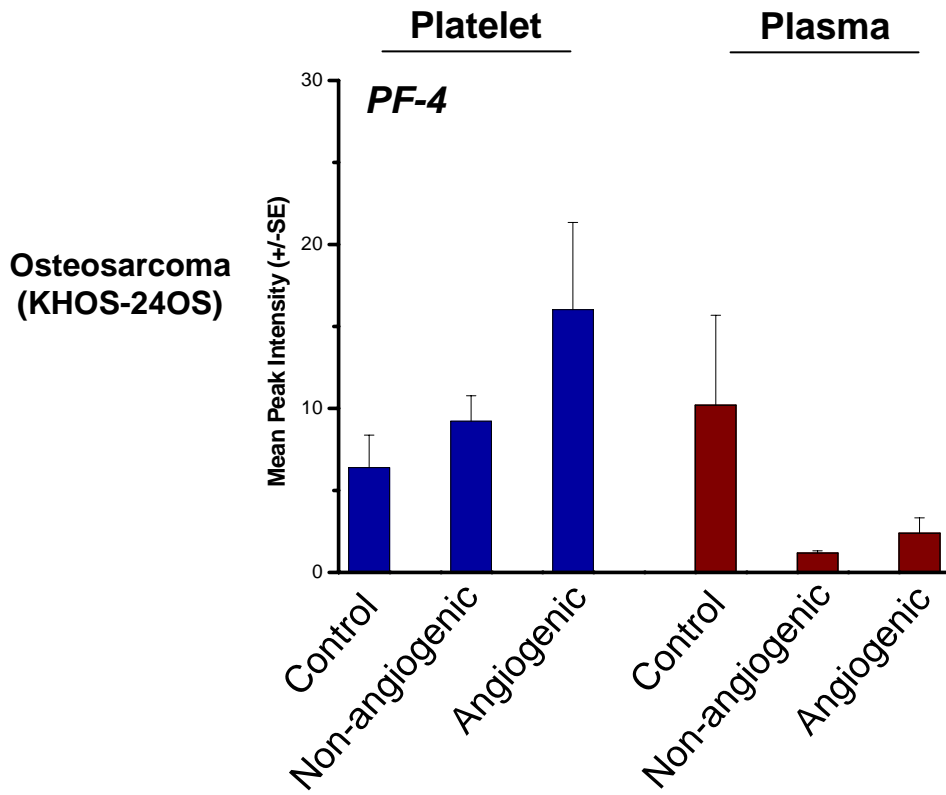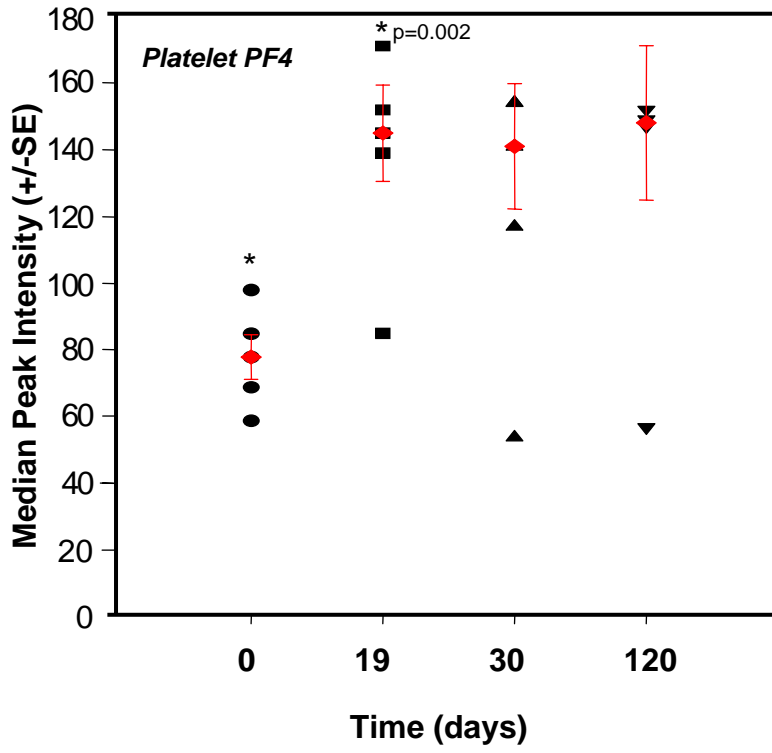
A

B

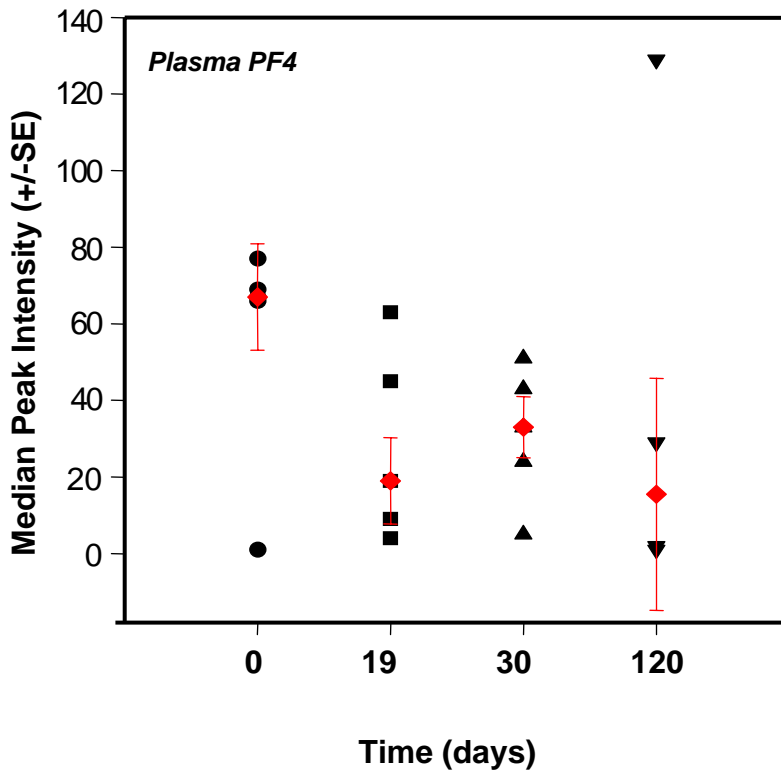Figure 5

Angiogenesis is regulated by a novel mechanism: Pro- and anti-angiogenic proteins are organized into separate platelet α-granules and differentially released

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Abstract:

Platelets, in addition to their function in hemostasis, play an important role in wound healing and tumor growth. Because platelets contain both angiogenesis stimulators and inhibitors, the mechanisms by which platelets regulate angiogenesis remain unclear. As platelets adhere to activated endothelium, their action can enhance or inhibit local angiogenesis. We therefore suspected a higher organization of angiogenesis regulators in platelets. Using double immunofluorescence and immunoelectron microscopy we show that pro- and antiangiogenic proteins are separated in distinct subpopulations of α-granules in both platelets and megakaryocytes. Double immunofluorescence labeling of VEGF (an angiogenesis stimulator) and endostatin (an angiogenesis inhibitor), or for thrombospondin-1 and basic FGF, confirms the segregation of stimulators and inhibitors into separate and distinct α-granules. These observations motivated the hypothesis that distinct populations of α-granules could undergo selective release. The treatment of human platelets with a selective PAR-4 agonist (AYPGKF-NH$_2$) resulted in release of endostatin-containing granules, but not VEGF-containing granules, while the selective PAR-1 agonist (TFLLR-NH$_2$) liberated VEGF, but not endostatin-containing granules. We conclude that the separate packaging of angiogenesis regulators into pharmacologically and morphologically distinct populations of α-granules in megakaryocytes and platelets may provide a mechanism by which platelets can locally stimulate or inhibit angiogenesis.

Introduction

Angiogenesis, the process of new vessel development, plays an essential role in embryogenesis, but postnatal angiogenesis is limited to sites of abnormal vascular surface. An activated vascular endothelium can be induced by tissue injury or wound healing, by hormonal cycling such as in pregnancy and ovulation, or by tumor-induced vessel growth. In all of these circumstances, platelets act as the initial responder to vascular change, and provide a flexible delivery system for angiogenesis related molecules[1-4]. The process of postnatal angiogenesis is regulated by a continuous interplay of stimulators and inhibitors of angiogenesis, and their imbalance contributes to numerous inflammatory, malignant, ischemic, and immune disorders [5]. There is a revived interest in the overlap between angiogenesis and platelets[6] because a number of clinical trials have now shown that anticoagulation can improve cancer survival[7,8] beyond the benefit derived from the treatment of deep vein thrombosis alone.

It is known that platelets stimulate endothelial cells in culture and can promote the assembly of capillary-like structures in vitro[9,10]. Platelets may modulate angiogenesis by releasing promoters such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), and matrix metalloproteinases (MMPs) [1,6,11-18]. The repertoire of angiogenesis inhibitors contained within platelets includes endostatin, platelet factor-4, thrombospondin-1, alpha-2-macroglobulin, plasminogen activator inhibitor-1, and angiostatin [19,20]. Although platelets contain three types of secretory granules (α-granules, dense granules, and lysosomes), most angiogenic regulatory proteins have been localized to 〈-granules. α-granules are 200-500 nm in size, and contain proteins that enhance the

adhesive process, promote cell-cell interactions, and stimulate vascular repair. By adhering to the endothelium of injured organs and tissues, and then secreting the contents of their α-granules, platelets may be capable of depositing high concentrations of angiogenesis regulatory proteins in a localized manner.

A body of experimental data and clinical investigations suggests that platelets are major regulators of angiogenesis[21]. However, because platelets contain both pro- and antiangiogenic regulatory proteins and because it has been assumed that the contents of α-granules are homogeneous, it has been unclear how platelets could either stimulate or inhibit angiogenesis. We provide new details about the organization of angiogenesis regulatory proteins in the α-granules of platelets and address the mechanism of how the selective release of these granules leads to the regulation of angiogenesis. Here we report the novel finding that angiogenic and antiangiogenic proteins are segregated into different sets of α-granules in platelets. We provide a mechanism for the differential release of these α-granules, and show that these distinct populations of α-granules may be regulated by differential G-protein-mediated signaling pathways.

Materials and methods

Approval was obtained from the Partners Human Research Committee institutional review board, Boston, MA, for these studies. Informed consent was provided according to the Declaration of Helsinki.

Preparation of Resting Platelets

Human blood from healthy volunteers, drawn into 0.1 volume of Aster-Jandl anticoagulant, was centrifuged at 110 g for 10 min. All volunteers had not ingested

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aspirin or other nonsteroidal anti-inflammatory drugs for at least 10 days prior to blood collection. The platelet-rich plasma was gel-filtered through a Sepharose 2B column equilibrated with a solution containing 145 mM NaCl, 10 mM Hepes, 10 mM glucose, 0.5 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgCl_2$, and 0.3% BSA, pH 7.4. The number of platelets was counted by fluorescence activated cell sorting and adjusted to 2×10^8/ml. The isolated platelet suspension was incubated at 37° C for up to 1 hr. The resting state of the platelets was routinely confirmed by PAC1 antibody and anti-tubulin immunofluorescence staining.

Activation of platelets

Release of α-granules was examined in vitro in response to 10 μm AYPGK-NH2, a selective PAR4-activating peptide, or 8 μm TFLLR-NH2, a PAR1-activating peptide. Peptides were prepared by solid-phase synthesis at the Peptide Synthesis Facility of Synbiocsi (Livermore, CA). Isolated platelets were exposed to PAR activating peptide or vehicle for 10 minutes, fixed with 4% formaldehyde for 20 minutes, attached to polylysine-coated coverslips, and then processed for immunofluorescence microscopy.

Megakaryocyte Cultures

Livers were recovered from mouse fetuses and single cell suspensions were generated using methods described previously[22]. Between the fourth and sixth day of megakaryocyte culture, cells were placed on a 1.5-3% albumin step gradient and sedimented [23] to obtain enriched populations of megakaryocytes.

5

Immunofluorescence Microscopy

Rabbit anti-VEGF antibody (Ab-1) and mouse anti-VEGF (Ab-7) were obtained from Lab Visions (Fremont, CA). Rabbit anti-endostatin antibody (Ab-1) was obtained from Lab Visions. Mouse anti-thrombospondin antibody (Ab-4, 6.1) was obtained from Lab Visions. Rabbit polyclonal anti-fibroblast growth factor basic was obtained from Abcam. Mouse anti-fibrinogen was obtained from BD Biosciences (Franklin Lakes, NJ) and Rabbit anti-fibrinogen was obtained from Santa Cruz Biotechnologies. Rabbit anti-von Willebrand factor was obtained from Chemicon and Dako. Alexa 568 anti-mouse, Alexa 488 anti-rabbit, Alexa 568 anti-rabbit, and Alexa 488 anti-mouse secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA). Actin filament integrity was assayed by fluorescence microscopy of fixed specimens stained with 1 mM phalloidin-Alexa 488 (Molecular Probes, Eugene, OR) for 30 min and washed 4 times with blocking buffer. Resting platelets were fixed for 20 min in suspension by the addition of 1 vol of 8% formaldehyde. Solutions of fixed platelets in suspension were placed in wells of a 24-well microliter plate, each containing a polylysine-coated coverslip, and the plate was centrifuged at 250 g for 5 min to attach the cells to the coverslip. Megakaryocytes were fixed with 4% formaldehyde in Hank's balanced salt solution (GIBCO BRL) for 20 min, centrifuged at 500 g for 4 min onto coverslips previously coated with poly-L-lysine, and permeabilized with 0.5% Triton X-100 in Hanks'. Specimens were blocked overnight in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA), incubated in primary antibody for 2-3 hours, washed, and treated with appropriate secondary antibody for 1 hr, and then washed extensively. Primary antibodies were used at 1 μg/ml in PBS containing 1% BSA and

secondary antibodies at 1:500 dilution in the same buffer. Controls were processed

identically except for omission of the primary antibody. Controls consisted of either

incubating cells with one or both primary antibodies without fluorescently labeled

secondary antibodies, or cells incubated with one or both fluorescently labeled secondary

antibodies in the absence of primary antibodies. Preparations were mounted in Aqua

polymount from Polysciences (Warrington, PA) and analyzed at room temperature on a

Nikon TE 2000 Eclipse microscope equipped with a Nikon100X objective (numerical

aperture, 1.4), and a 100-W mercury lamp. Images were acquired with a Hamamatsu

(Bridgewater, NJ) Orca IIER CCD camera. Electronic shutters and image acquisition

were under the control of Molecular Devices Metamorph software (Downington, PA).

Images were acquired by fluorescence microscopy with an image capture time of 200-

500 milliseconds.

Immunogold-electron microscopy

For preparation of cryosections, isolated human platelets were fixed with 4%

paraformaldehyde in 0.1M Na Phosphate buffer, pH 7.4. After 2 hours of fixation at

room temperature the cell pellets were washed with PBS containing 0.2M glycine to

quench free aldehyde groups from the fixative. Prior to freezing in liquid nitrogen cell

pellets were infiltrated with 2.3M sucrose in PBS for 15 minutes. Frozen samples were

sectioned at -120° C, the sections were transferred to formvar-carbon coated copper grids

and floated on PBS until the immunogold labeling was carried out. The gold labeling

was carried out at room temperature on a piece of parafilm. All antibodies and protein A

gold were diluted with 1% BSA. The diluted antibody solution was centrifuged for 1

minute at 14,000 rpm prior to labeling to avoid possible aggregates. All antibodies were used at a concentration of 1 µg/ml. Grids were floated on drops of 1% BSA for 10 minutes to block for nonspecific labeling, transferred to 5µl drops of primary antibody and incubated for 30 minutes. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5µl drops of Protein-A gold for 20 minutes, washed in 4 drops of PBS for 15 minutes and 6 drops of double distilled water. For double labeling, after the first Protein A gold incubation, grids were washed in 4 drops of PBS for a total of 15 minutes then transferred to a drop of 1% glutaraldehyde in PBS for 5 minutes, and washed in 4 drops of PBS/0.15M glycine. The second primary antibody was then applied, followed by PBS washing and treatment with different size Protein-A gold as above. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetete in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops, leaving a thin coat of methyl cellulose. The grids were examined in a Tecnai G² Spirit BioTWIN transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Preparation of photomicrographs

The digital images produced in Metamorph were assembled into composite images by using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA).

Results

The localization of angiogenesis regulatory proteins within the platelet is important for understanding how platelets contribute to new blood vessel formation. The

capacity of platelets to regulate angiogenesis could result from segregation of pro-angiogenic and antiangiogenic regulators into separate granules. To test this possibility, we compared the localization of the most well-characterized pro-angiogenic protein VEGF, and the established antiangiogenic regulator endostatin, in resting platelets by immunofluorescence microscopy. The majority of α-granules stained for either VEGF (labeled green) or endostatin (labeled red), and little evidence of co-localization as would be indicated by yellow in the merged image was observed (Figure 1a-c). Similarly, double immunofluorescence microscopy comparing the localization of the endogenous angiogenesis inhibitor thrombospondin-1 and basic fibroblast growth factor, another angiogenesis stimulator, also showed segregation of these proteins into separate, distinct granules (Figure 1d-f). To establish whether the segregation of proteins into distinct α-granules was specific to angiogenesis regulatory proteins, we examined the localization of von Willebrand factor (vWf) and fibrinogen. To evaluate the degree of overlap of proteins, we investigated the combination of fibrinogen and vWf. Surprsingly, fibrinogen and vWF also segregated into separate and distinct α-granules (Figure 1g-i). Immunofluorescence microscopy further revealed that vWf co-localized with endostatin (Figure 1j-l) and fibrinogen predominantly with the VEGF-containing α-granules (Figure 1m-o).

The organization of angiogenesis regulators into distinct α-granules is not exclusive to platelets. Megakaryocytes have been shown to generate platelets by remodeling their cytoplasm into long proplatelet extensions that transport individual α-granules on their microtubule tracks.[24] To address whether inhibitors and stimulators of angiogenesis are packaged into distinct populations of α-granules in the precursor cells of

platelets, we analyzed the distribution of angiogenic regulatory proteins in proplatelet-producing mouse megakaryocytes. As observed in platelets, VEGF and endostatin were localized to separate α-granules in the proplatelet extensions (Figure 2a-c). A similar segregated staining pattern was also observed for thrombospondin-1 and basic FGF (Figure 2d-f) as well as fibrinogen and vWf (Figure 2g-i). Most vWf co-localized with the endostatin-containing α-granules (Figure 2j-l). We confirmed the presence of distinct populations of α-granules in human platelets at the ultra-structural level using immuno-electron microscopy (Figure 3). As expected, anti-VEGF (Figure 3a) and anti-endostatin antibodies (Figure 3b) label only a subpopulation of α-granules. Double immunogold microscopy confirmed that the majority of VEGF and endostatin are localized to separate and distinct granules in platelets (Figure 3c). Single immunogold studies revealed that anti-fibrinogen (Figure 3d) and anti-vWf antibodies (Figure 3e) label only a subpopulation of α-granules. Double immunogold microscopy confirmed that the majority of fibrinogen and vWf are localized to separate and distinct granules in resting platelets (Figure 3g). Anti-P-selectin antibodies specifically labeled almost all α-granules and the plasma membrane of resting platelets (Figure 3g). Quantitative analysis of gold labeling in serial sections revealed that antibodies to VEGF, endostatin, vWF and fibrinogen each stain approximately 50% of the granule population (Figure 3h). In contrast, anti-P-selectin antibodies label the membrane of all α-granules as well as the surface of the resting platelet. Less than 10% of granules contained gold labeling for both endostatin and VEGF or vWF and fibrinogen together (Figure 3h).

The packaging of VEGF and endostatin into separate α-granules suggested that distinct granule populations may undergo selective release. We tested this hypothesis by

stimulating platelets with either PAR4-activating peptide or PAR1-activating peptide. Selective granule release was assessed by immunofluorescence microscopy (Figure 4). Phalloidin staining demonstrated that exposure of platelets to either ligand resulted in aggregation and extension of lamellipodia and filopodia, leading to activation (Figure 4b,d,f,h). Immunofluorescence microscopy revealed that PAR4 treatment resulted in loss of the endostatin labeling, suggesting that most of the endostatin-containing granules were released from the platelets ligated with PAR4-activating peptide (Figure 4c). However, numerous VEGF-containing granules were retained in the cytoplasm of PAR4-treated platelets (Figure 4a). In contrast, immunofluorescence microscopy revealed that ligation of PAR1 resulted in the release of VEGF-containing (green) granules, suggesting that release of VEGF-containing granules was elicited by the PAR1 agonist (Figure 4e). However, a large number of endostatin-containing granules were still retained in the cytoplasm of PAR1-treated platelets. (Figure 4g). To confirm the phenomenon of differential granule release, we analyzed the agonist-mediated release of α-granules at higher resolution using immunoelectron microscopy. Stimulation of platelets with PAR4 agonist resulted in the release of almost all endostatin-containing granules; the majority (84%) of granules remaining in the activated platelets were positive for VEGF (Figure 4i). Treatment of platelets with PAR1 agonist induced the release of the majority of VEGF-containing granules; the majority (88%) of granules remaining in the PAR1-activated platelets were positive for endostatin (Figure 4j).

DISCUSSION

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Angiogenesis is a critical element of many physiological processes such as wound healing, as well as pathological processes such as tumor growth. In both situations, new blood vessel development is driven locally, by the release of pro-angiogenic factors such as VEGF, bFGF, and PDGF. However, angiogenesis can also be inhibited by local release of antiangiogenic factors such as endostatin and thrombospondin. The proximity to and interaction with the endothelium allow platelets to strongly influence tumor development and wound healing. Platelets have been presumed to contribute to these angiogenesis-dependent processes by providing many pro- and antiangiogenic proteins, but their regulatory role is incompletely understood. In this study, we have shown that platelets contain distinct populations of α-granules that can undergo differential release in vitro. This study suggests that at least two populations of α-granules containing endogenous angiogenic regulatory proteins are present in platelets and raises the possibility that platelets contain multiple types of α-granules. Platelets contain a large number of angiogenic regulatory proteins, whose localization will need to be thoroughly established to understand the complexity of α-granule organization within resting platelets. Yet, it can be inferred that this subcellular organization has a physiological purpose in facilitating the differential release of these proteins in response to tissue stimuli. Our findings of differential granule release also support and provide a mechanistic explanation for earlier studies examining the secretion reaction of platelets. Two independent groups have documented the differential release of α-granule proteins from platelets[25,26]. In addition, morphometric evaluation of the platelet release reaction during thrombogenesis has demonstrated that platelets do not release all of their granules when they are incorporated into a thrombus [27]. The above results also raise the question

of whether other cell types containing secretory granules segregate angiogenic regulatory proteins to regulate differential release. For example, Weibel Palade bodies, the specific secretory organelles of endothelial cells, contain a number of angiogenesis regulators and have been recently shown to differentially package and release P-selectin and vWf through protease activated receptors[28].

What molecular mechanisms regulate the differential packaging of specific proteins into α-granules? α-granules contain a mixture of proteins synthesized by the megakaryocyte as well as proteins endocytosed from the circulation by both megakaryocytes and the platelets. While the formation of α-granules is poorly understood[29,30], it appears that α-granules develop from budding vesicles in the Golgi complex within megakaryocytes, where they transform into multivesicular bodies, which also fuse with endocytic vesicles. Coated pits and vesicles have been observed in platelets and function to take up proteins, such as fibrinogen, by receptor mediated endocytosis. These endocytic vesicles fuse with the multivesicular bodies. Multivesicular bodies, which are prevalent in early megakaryocytes, are believed to be a common precursor of both α- and dense granules. However, the mechanisms by which α- and dense granules develop into distinct entities are unknown. It is tempting to speculate that a similar process may be employed to segregate proteins into distinct subsets of α-granules, and that genetic defects which affect the α-granule segregation or differential release may provide an explanation of the wide range of angiogenic responses manifested by different individuals[31]. Several angiogenesis-dependent processes may be explained by the sequential release of angiogenesis regulators. For example, in early endothelial injury, an unstable platelet clot is formed and the high-affinity thrombin receptor (PAR-1) signals to

release by majority pro-angiogenic proteins such as VEGF. In the late stage of tissue reconstruction, a high thrombin state occurs when the majority of the clot is crosslinked by factor XIII, and the low affinity protease-activated receptor (PAR-4) is engaged and mainly inhibitors of angiogenesis are released.

Recognizing that activated platelets release growth factors, investigators have begun to enhance tissue regeneration by applying platelets and their derivatives into sites of injuries or surgical intervention. The concept of platelet and tissue interaction and the resulting release of angiogenesis regulators has already been used in the empirical application of platelet preparations to chronic diabetic ulcers[32,33], chronic cutaneous ulcers[34], dehiscent wounds[35-37] and tissue regeneration[38]. Although the majority of evidence indicates that platelets and their derivatives (gels, releasates, and lysates) are promising therapeutic agents for regenerative medicine, little is known about the specific mechanisms underlying platelet-accelerated tissue repair[39]. The ability to generate selective platelet releasates by manipulating protease activated receptors may provide new opportunities for research and applications of tissue engineering and may aid in therapeutic strategies to promote or inhibit angiogenesis.

Our findings of distinct populations of α-granules that can be differentially released suggest implications and potential for a substantial role in anti-angiogenic therapy. It is now well accepted that the growth of a tumor beyond ~1 mm is dependent on the development of a neovasculature. One possibility is that tumors also hijack the angiogenic properties of platelets to promote new blood vessel growth by manipulating the protease activated receptors on platelets and triggering the selective release of predominantly pro-angiogenic factors. The protease-activated receptors on platelets and

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endothelial cells are likely to play an important role in the sequential and highly selective contribution of angiogenesis regulators to tissues. If confirmed, then it may be possible to develop drugs that instruct platelets which interact with tumors to release predominantly anti-angiogenic proteins.

Acknowledgments

Authorship

Contribution: JEI designed and performed experiments and data analysis, interpreted results, provided guidance for the group, and drafted the manuscript. JLR, SPH, EB, AZ, and SS performed experiments and data analysis, interpreted results.
SR and JF designed experiments, interpreted results, formulated discussions and assisted in manuscript preparation and editing. GLK, corresponding author, designed experiments, provided guidance for the group, interpreted results, formulated discussions and assisted in manuscript preparation and editing.

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Figure Legends

Figure 1. Pro- and anti-angiogenic regulators organize into separate, distinct α-granules in resting platelets. a, b, c, Double immunofluorescence microscopy of resting platelets using antibodies against VEGF (a) and endostatin (b) and an overlay (c). d, e, f, Double immunofluorescence microscopy of resting platelets using antibodies against bFGF (d) and TSP-1 (e) and an overlay (f). g, h, i, Double immunofluorescence microscopy of resting platelets using antibodies against fibrinogen (g) and von Willebrand factor (h) and an overlay (i). j, k, l, Double immunofluorescence microscopy of resting platelets using antibodies against von Willebrand factor (j) and endostatin (k) and an overlay (l). m, n, o, Double immunofluorescence microscopy of resting platelets using antibodies against VEGF (m) and fibrinogen (n) and an overlay (o).

Figure 2. Pro- and anti-angiogenic regulatory proteins are segregated into separate, distinct α-granules in megakaryocyte proplatelets. a, b, c, Double immunofluorescence microscopy of proplatelets using antibodies against VEGF (a) and endostatin (b) and an overlay (c). d, e, f, Double immunofluorescence microscopy of proplatelets using antibodies against bFGF (d) and TSP-1 (e) and an overlay (f). g, h, i, Double immunofluorescence microscopy of proplatelets using antibodies against fibrinogen (g) and von Willebrand factor (h) and an overlay (i). j, k, l, Double

immunofluorescence microscopy of proplatelets against VEGF (j) and fibrinogen (k) and an overlay (l).

Figure 3. **Localization of proteins in resting, human platelets using immunoelectron microscopy of ultrathin cryosections.** Single immunogold labeling on ultrathin platelet sections was performed with anti-VEGF (a) and anti-endostatin (b) antibodies. Double immunogold labeling on platelet sections was performed with the use of anti-VEGF antibody and anti-endostatin antibodies. Large gold particles representing anti-VEGF staining (15 nm, arrows) are evident on one population of α-granules and small gold particles (5nm) representing endostatin staining are abundantly present on a different population of α-granules (arrowheads) (c). Single immunogold labeling on ultrathin platelet sections was performed with anti-fibrinogen (d) and anti-vWf (e) antibodies. Double immunogold labeling on platelet sections was performed with the use of anti-fibrinogen antibody, which was revealed with a 15–nm, gold-conjugate (arrows) and then with an antibody to vWf, which was revealed with a 5-nm, gold-conjugate (arrowheads) (f). Single immunogold labeling on ultrathin platelet sections was performed with anti-P-selectin antibody (g). Gold particles representing P-selectin staining are abundantly present on the α-granules as well as the cell-surface membrane. Bar, 300 nm. The bar graph shows the quantitation of the percent of α-granules positive (via immunogold staining) for specific factors. The data represents three separate experiments. Over 100 granules were scored for each study.

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Figure 4. Activation of specific protease activated receptors stimulates the selective release of α-granules containing either endostatin or VEGF. Platelets were treated with platelet buffer in the presence of agonists for 10 minutes with PAR4-activating peptide (a,b,c,d) , and PAR1-activating peptide (e,f,g,h) and then fixed and processed for immunofluorescence microscopy. Cells were stained with either anti-VEGF antibodies (Alexa 488 green labeling; a,e) or anti-endostatin antibodies (Alexa 568 red labeling; c,g,) to assay for granule retention or release. All micrographs were taken at the same exposure time. Corresponding staining with Alexa-phalloidin (b,d,f,h) in the lower panels highlights the morphology of the platelets. Negative controls consisting of incubation with both secondary fluorescently labeled antibodies only or incubation with only primary antibodies failed to show appreciable fluorescence (data not shown). Images are representative of at least 10 high power fields for each experiment, and each experiment was performed 3 times. (i,j) Representative images of immunoelectron microscopy of platelets treated with either PAR4-AP (i) or PAR1-AP (j). Double immunogold labeling on platelet sections was performed with the use of anti-VEGF antibody and anti-endostatin antibodies. In the PAR4-treated samples (i), large gold particles representing anti-endostatin staining (15 nm) are evident on one α-granule (arrow) and small gold particles (5nm) representing VEGF staining are abundantly present on separate population of multiple α-granules. In the PAR1-treated samples (j), large gold particles representing anti-VEGF staining (15 nm, arrow) are evident on one α-granule (arrow) and small gold particles (5nm) representing endostatin staining are abundantly present on separate population of multiple α-granules. (k) A model illustrating the mechanism of differential granule release from platelets. A simplified

summary of the pathway is shown. Resting platelets contain both pro-angiogenic (green) and anti-angiogenic (red) granules. Selective activation of the PAR1 receptor causes release of granules containing pro-angiogenic factors, whereas selective activation of the PAR4 receptor causes release of granules containing anti-angiogenic factors.
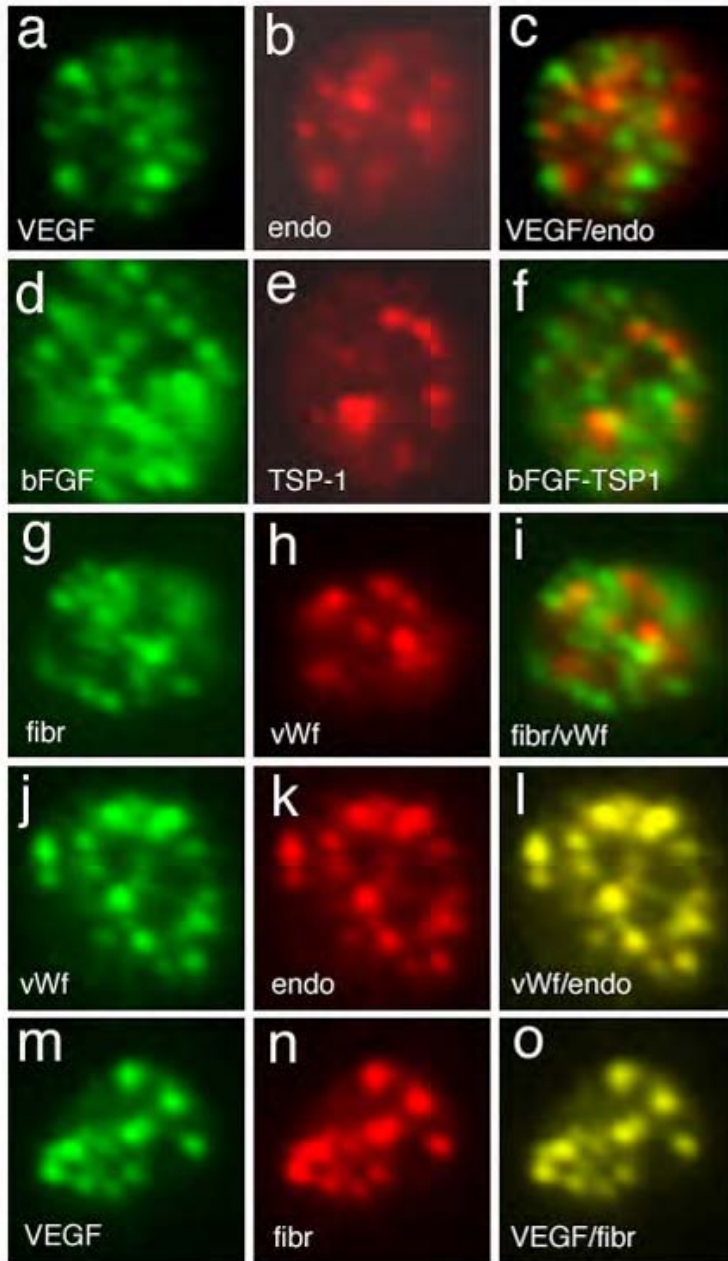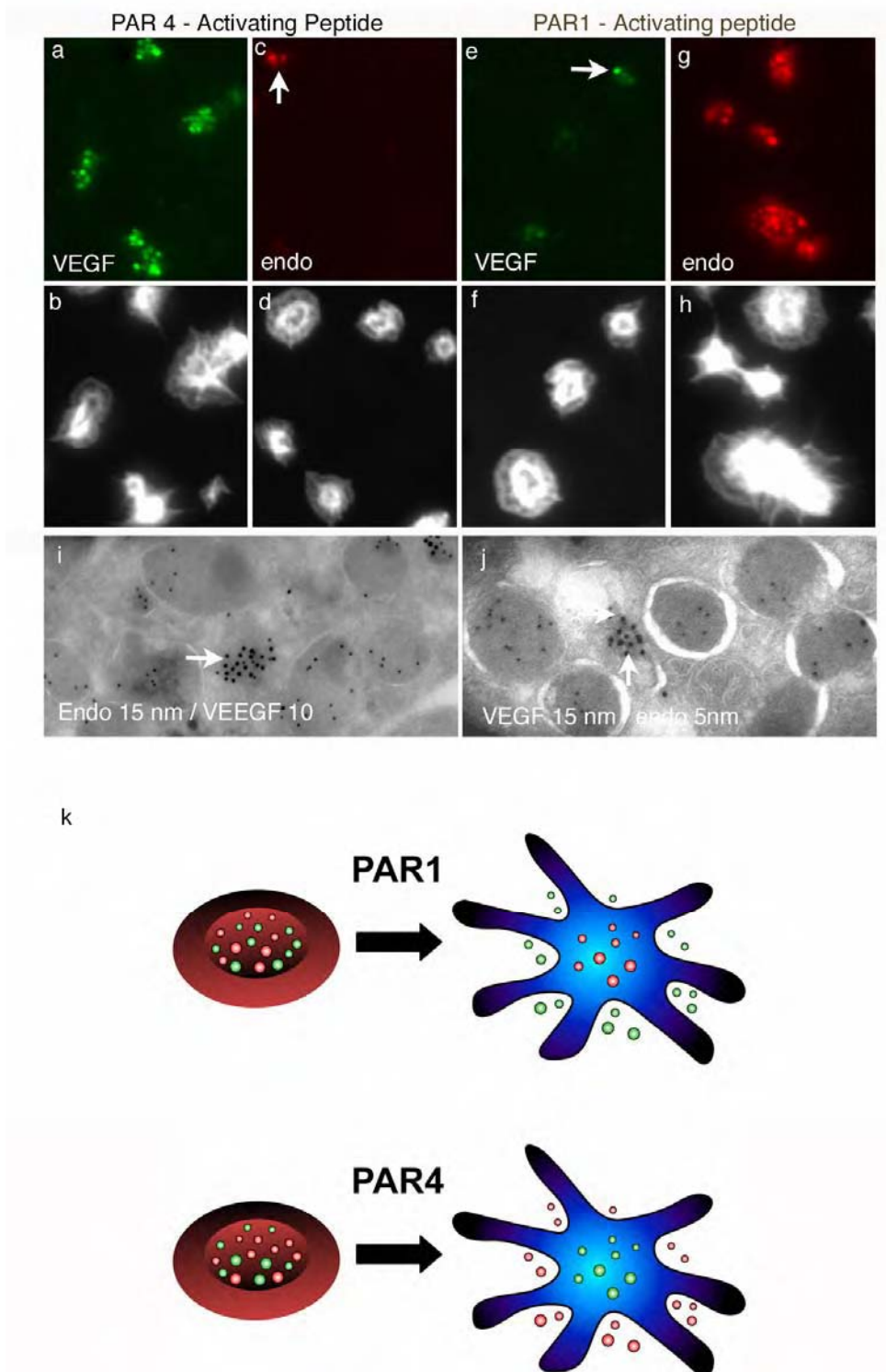
Figure 1

Figure 2

Figure 3

PAR 4 - Activating Peptide   PAR1 - Activating peptide
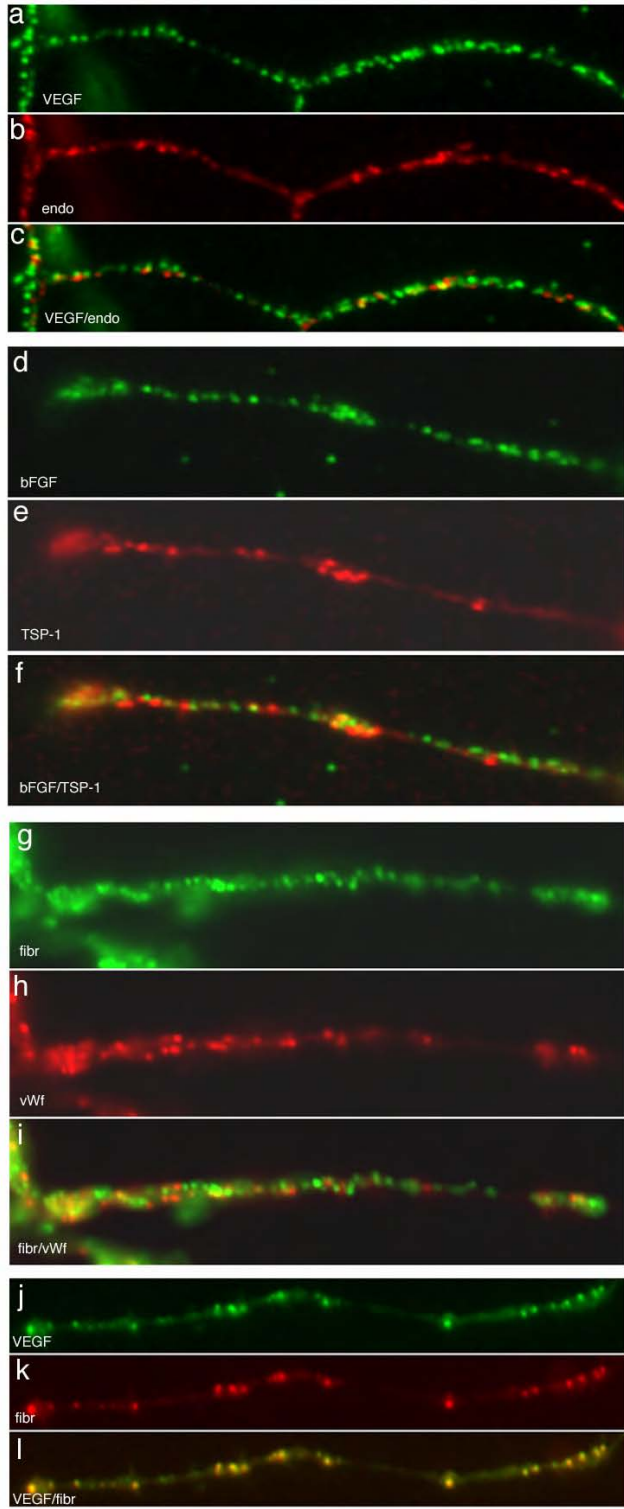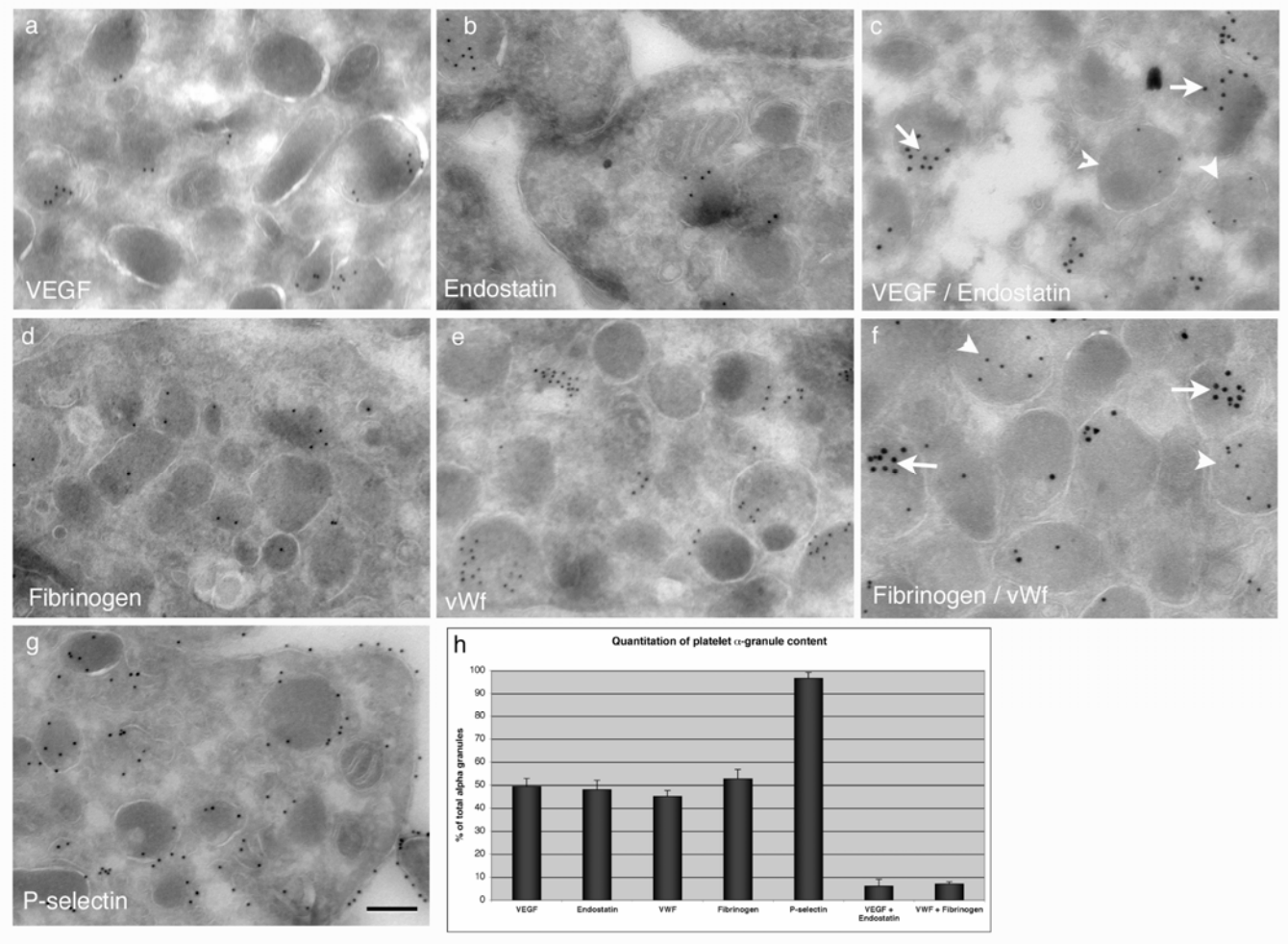
Figure 4

1 Strategies to Prolong the Nonangiogenic Dormant State of Human Cancer

George N. Naumov and Judah Folkman

CONTENTS

1.1 CLINICAL "LATENCY" IN CANCER RECURRENCE FOLLOWING A PRIMARY TUMOR TREATMENT

Cancer recurrence after treatment of the primary tumor is a major cause of mortality among cancer patients. It may take years to decades before local or distant (i.e., metastatic) recurrence becomes clinically detectable as cancer. This "disease-free" period is a time of uncertainty for patients who appear "cured." For example, Demicheli et al.[1] have demonstrated two hazardous peaks of breast cancer recurrence in patients undergoing mastectomy alone without adjuvant therapy. In a group of 1173 patients, the first peak of cancer recurrence occurred at ~18 months after surgery. A second peak in cancer recurrence developed at ~5 years after surgery and was associated with a plateau-like tail extending up to 15 years.[1] Patients experiencing cancer recurrences within 5 years following surgery have a shorter overall survival than those with recurrences occurring at a later time point.[2]

Similar "latency periods" in cancer recurrence have been documented since the beginning of the twentieth century. Rupert A. Willis has summarized the "time elapsing between the excision of a human malignant tumor and the appearance of a clinically recognizable recurrence" for a variety of human cancers. For example, the latency period in breast cancer patients can be from 6 to 20 years; cutaneous and ocular melanoma, 14 to 32 years; kidney carcinoma,

6 to 8 years; and stomach and colon carcinoma, 5 to 6 years.[3] Moreover, Willis was the first to realize that these latency periods do not correspond with the natural progression of cancer, and he introduced the concept of a "dormant cancer cell" as a possible explanation.

Over the past few years, several hypotheses have been proposed in an attempt to explain the phenomenon of human tumor dormancy. Initially, it was proposed that tumor cells enter a prolonged state of mitotic arrest.[4,5] Others hypothesized that tumor size is controlled by the immune system[6–11] or hormonal deprivation in hormone-dependent tumors.[11–13] In 1972, Folkman and Gimbrone demonstrated that dormancy in human tumors could be due to blocked angiogenesis. In the following years, Folkman and colleagues have presented evidence supporting the concept that most human tumors arise without angiogenic activity and exist in a microscopic dormant state for months to years without neovascularization.[14] Such protection may be attributed in part to host-derived factors, which prevent microscopic tumors from switching to the angiogenic phenotype.

1.2 ANGIOGENESIS DEPENDENCE OF TUMOR GROWTH

Cancer progression is a multistep process (Figure 1.1). With each step, the genetic and epigenetic events in the process become increasingly complex and may be more difficult to
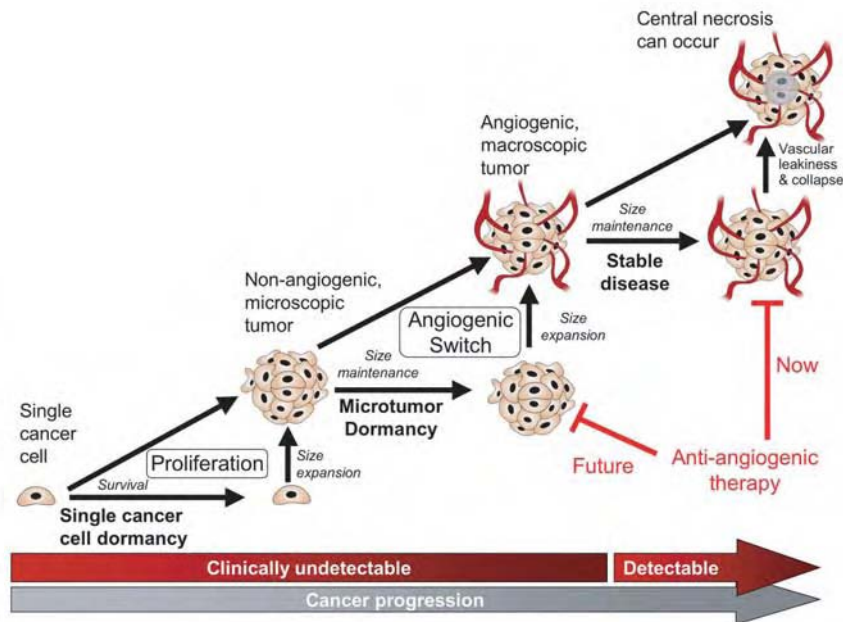
FIGURE 1.1 (See color insert following page 558.) Rate-limiting steps in the tumor progression. Solitary nonproliferating, dormant cancer cells can persist for long periods of time, until they come out of G_0 arrest and start to proliferate. Tumor mass can expand only to a microscopic mass without the recruitment of new blood vessels. Human cancers can remain nonangiogenic and dormant for long periods of time, delaying the tumor progression process. During this microscopic dormant state, nonangiogenic tumors are actively proliferating and undergoing apoptosis. Nonangiogenic tumors can expand in mass after undergoing the angiogenic switch and recruitment of new blood vessels. Angiogenic macroscopic tumors that do not expand in mass are known as "stable disease," although angiogenic tumors can remain at a constant size for prolonged periods of time. Current antiangiogenic therapy targets angiogenic microscopic and macroscopic tumors. However, future antiangiogenic therapy will target nonangiogenic microscopic tumors with the aim of keeping them in a dormant state by preventing the angiogenic switch.

treat. As the cancer progresses from a single neoplastic cell to a large, lethal tumor, it acquires a series of mutations, becoming: (1) self-sufficient in growth signaling, by oncogene activation and loss of tumor suppressor genes, (2) insensitive to antigrowth signaling, (3) unresponsive to apoptotic signaling, (4) capable of limitless cell replications, and (5) tumorigenic and metastatic.[15] Current experimental and clinical evidence indicates that these neoplastic properties may be necessary, but not sufficient, for a cancer cell to progress into a population of tumor cells, which becomes clinically detectable, metastatic, and lethal. For a tumor to develop a highly malignant and deadly phenotype, it must first recruit and sustain its own blood supply, a process known as tumor angiogenesis.[16,17]

For more than a century, it has been observed that surgically removed tumors are hyperemic compared to normal tissues.[18,19] Generally, this phenomenon was explained as simple dilation of existing blood vessels induced by tumor factors. However, Ide et al.[20] demonstrated that tumor-associated hyperemia could be related to new blood vessel growth, and vasodilation may not be the sole explanation for this phenomenon. They showed that when a wound induced in a transparent rabbit ear chamber completely regressed, the implantation of a tumor in the chamber resulted in the growth of new capillary blood vessels.[20] These initial observations were later confirmed by Algire et al.,[21,22] demonstrating that new vessels in the periphery of a tumor implant arose from preexisting host vessels, and not from the tumor implant itself. At the time, this novel concept of tumor-induced neovascularization was generally attributed to an inflammatory reaction, thought to be a side effect of tumor growth, and it was not perceived as a requirement for tumor growth.[23]

In the early 1960s, Folkman and Becker observed that tumor growth in isolated perfused organs was severely restricted in the absence of tumor vascularization.[24-28] In 1971, Folkman proposed the hypothesis that tumor growth is angiogenesis-dependent.[16] This hypothesis suggested that tumor cells and vascular endothelial cells within a neoplasm may constitute a highly integrated, two-compartment system, which dictates tumor growth. This concept indicated that endothelial cells may switch from a resting state to a rapid growth phase because of "diffusible" signals secreted from the tumor cells. Moreover, Folkman proposed that angiogenesis could be a relevant target for tumor therapy (i.e., antiangiogenic therapy).

We now know that angiogenesis plays an important role in numerous physiologic and pathologic processes. The hallmark of pathologic angiogenesis is the persistent growth of blood vessels. Sustained neovascularization can continue for months or years during the progression of many neoplastic and nonneoplastic diseases.[29,30] However, tumor angiogenesis is rarely, if ever, downregulated spontaneously. The fundamental objective of antiangiogenic therapy is to inhibit the progression of pathologic angiogenesis. In contrast, the goal of antivascular therapy is to rapidly occlude new blood vessels so that the blood flow stops. Both therapeutic approaches target the ability of tumors to progress from the nonangiogenic to the angiogenic phenotype, a process termed the "angiogenic switch."[31,40]

Cancer usually becomes clinically detectable only after tumors have become angiogenic and have expanded in mass. Failure of a tumor to recruit new vasculature or to reorganize the existing surrounding vasculature results in a nonangiogenic tumor, which is microscopic in size and unable to increase in mass (Figure 1.1). Without new blood supply, microscopic tumors are usually restricted to a size of $<1-2$ mm in diameter and are highly dependent on surrounding blood vessels for oxygen and nutrient supply. At sea level, the diffusion limit of oxygen is ~ 100 μm.[32] Therefore, all mammalian cells, including neoplastic cells, are required to be within 100–200 μm of a blood vessel. As nonangiogenic tumors attempt to expand in mass, attributed to uncontrolled cancer cell proliferation, some tumor cells fall outside the oxygen diffusion limit and become hypoxic. It is well known that hypoxic conditions induce a set of compensatory responses within cancer cells, such as increased transcription of the

hypoxia-inducible factor (HIF). Subsequently, this hypoxic signaling leads to upregulation of proangiogenic proteins, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and nitric oxide synthase (NOS).[33] The angiogenic switch in tumors is presumed to be closely regulated by the presence of pro- and antiangiogenic proteins in the tumor microenvironment. An increase in the local concentration of proangiogenic proteins allows angiogenesis to occur and ultimately permits a tumor to expand in mass. Antiangiogenic therapy offers a fourth anti-cancer modality, in addition to conventional therapeutic approaches, which target well-established and genetically unstable tumors.

1.3 EXPERIMENTAL MODELS OF HUMAN TUMOR DORMANCY

As early as the 1940s, experimental systems involving the transplantation of tumor pieces in isolated perfused organs and in the anterior chamber of the eyes of various species of animals have demonstrated the effects of neovascularization on tumor growth. Greene et al.,[34] observed that H-31 rabbit carcinoma tumor implanted into the eyes of guinea pigs did not vascularize and failed to grow for 16–26 months. During this period, the transplants measured ~2.5 mm in diameter. However, when the same tumors were reimplanted into their original host (i.e., rabbit eyes), they vascularized and grew to fill the anterior chamber within 50 days. Similarly, Folkman et al.[25] showed that in isolated perfused thyroid and intestinal segment tumors, implants grew and arrested at a small size (2–3 mm diameter). This inability of neoplasms to evoke a new blood supply was later attributed to endothelial cell degeneration in the perfused organs that are perfused with platelet-free hemoglobin solution.[35] In 1972, Gimbrone et al.[36] provided in vivo evidence that the progressive growth of a homologous solid tumor can be deliberately arrested at a microscopic size when neovascularization is prevented. In these experiments, two comparable tumor pieces were implanted in each eye of the same animal: one directly on the iris (i.e., angiogenic milieu) and the other suspended in the anterior chamber (i.e., avascular milieu) in the opposing eye. The vascularized tumor implanted on the iris grew to a size 15,000 times the initial volume and filled the anterior chamber of the rabbit eye within 14 days. In contrast, the tumor implant in the avascular anterior chamber remained avascular, and by day 14 after implantation, had only increased by 4 times its initial volume. These "dormant" tumors remained at a size of ~1 mm in diameter for up to 44 days. During this period, the tumors developed a central necrotic core surrounded by a layer of viable tumor cells, in which mitotic figures were observed. Overall, these microscopic tumors remained avascular, as demonstrated through microscopic and histological analyses and fluorescein tests. The malignant growth potential of these microscopic tumors was demonstrated in vivo by reimplanting the tumors directly on the irises of fresh animals. In the irises, the dormant tumors became vascularized and grew rapidly until the anterior chambers of the eyes were filled with tumor, in a manner similar to the control iris implants. These fundamental observations established the relationship between tumor growth and angiogenesis. Moreover, they provided an in vivo experimental model for further investigations of tumor dormancy.

One of the most pressing questions at that time was whether tumor-induced angiogenesis could be inhibited, preventing dormant tumors from progressing to the angiogenic phenotype. Using a V2 rabbit carcinoma in the corneal implant animal model, Brem et al.,[37] demonstrated that tumor angiogenesis could be blocked by diffusible proteins from the cartilage of newborn rabbits. The coimplants of tumor and cartilage pieces completely prevented vascularization in 28% of tumors and significantly delayed the vascularization in the remaining tumors, which eventually became vascularized. In addition, cartilage pieces inhibited vessel formation around a tumor implant in the chorioallantoic membrane (CAM) of chick embryos. The inhibitory proteins found in cartilage were later identified, isolated, and characterized as tissue inhibitors of metalloproteinases (TIMPs).[37a] Subsequently, other
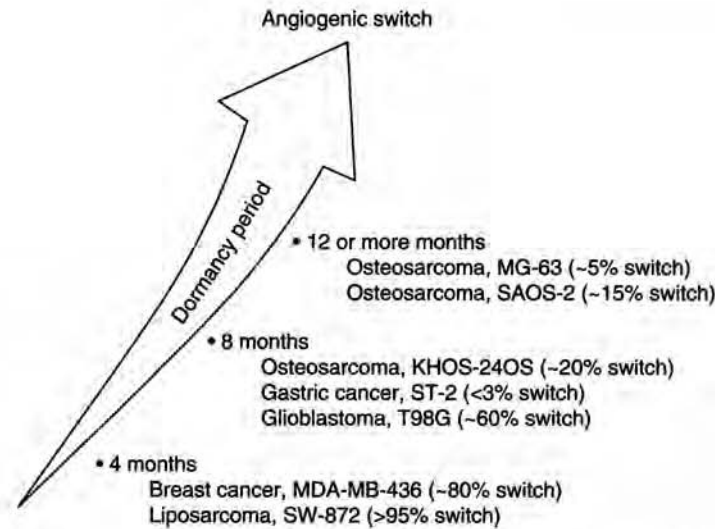
angiogenesis inhibitors were identified. Endostatin and angiostatin were discovered as internal peptide fragments of plasminogen and 20 kDa C-terminal fragments of collagen XVIII, respectively.[38,39]

A spontaneous tumor dormancy model in transgenic mice was described by Hanahan and Folkman,[40] in which autochthonous tumors arise in the pancreatic islets as a result of simian virus 40 T antigen (Tag) oncogene expression. In this experimental model, only 4% of tumors become angiogenic after 13 weeks. In contrast, the remaining 96% of pancreatic islet tumors remain microscopic and nonangiogenic.[40,41] The spontaneous progression of nonangiogenic lesions to the angiogenic phenotype in these transgenic tumor-bearing mice led to the development of the "angiogenic switch" concept.[40]

More recently, Achilles et al.[42] reported that human cancers contain subpopulations that differ in their angiogenic potential. These findings suggested that the angiogenic phenotype of a human tumor cell may be controlled by genetic and epigenetic mechanisms. Therefore, human tumors can contain both angiogenic and nonangiogenic tumor cell populations, characterized by their in vivo ability to recruit new blood vessels to a tumor. However, the factors involved in the proportional regulation of these two tumor cell populations are still unknown. The observed heterogeneity of angiogenic activity among human tumor cells allowed for the isolation of these two populations of cancer cells and the development of new and fruitful human tumor dormancy experimental models.

Single-cell cloning of a human tumor cell line was employed as a strategy for the isolation of angiogenic and nonangiogenic tumor cell populations.[42] Achilles et al. established and selected subclones from a human liposarcoma cell line (SW-872) based on high, intermediate, or low proliferation rates in vitro. These clones were expanded in vitro into a population of tumor cells and were then inoculated into immunodeficient (SCID) mice. Three different growth patterns were observed: (1) highly angiogenic and rapidly growing tumors, (2) weakly angiogenic and slowly growing tumors, and (3) nonangiogenic and dormant tumors. In a subsequent experiment, Almog et al.[43] demonstrated that the nonangiogenic tumors spontaneously switch to the angiogenic phenotype and initiate exponential growth ~130 days after subcutaneous inoculation.[43] During the 130 day dormancy period, microscopic (~1–2 mm in diameter) tumors remain avascular and are virtually undetectable by palpation. Because this animal dormancy model was based on the in vitro tumor cell proliferation differences between the angiogenic and nonangiogenic liposarcoma clones, it raised two fundamental questions: (1) Is there a correlation between tumor cell proliferation and angiogenic potential? (2) Can the observed differences in tumor growth be recapitulated using populations of human tumor cells that have not been cloned?

To address these questions, human tumor cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) based on their "no take" phenotype in immunodeficient mice. These cell lines were assessed for in vivo tumor growth over extended time periods. Following a subcutaneous inoculation of a tumor cell suspension, mice were monitored for palpable tumors at the site of inoculation for more than a year (i.e., about half the normal life span of a mouse) and, sometimes, for the life of the animal. Some of the mice inoculated with the "no take" tumor cells spontaneously formed palpable tumors after a dormancy period, which varied from months to more than a year, depending on cancer type (Figure 1.2). With time, tumors became angiogenic, and palpable, expanded in mass exponentially, and within ~50 days of first detection, killed the host animal. Stable cell lines were established from representative angiogenic tumors. When reinoculated into SCID mice, these angiogenic tumor cells formed large (>1 cm in diameter) tumors within a month following inoculation (i.e., without a dormancy period), in 100% of the inoculated mice. It was found that each cancer type had a characteristic and predictable dormancy period and generated a consistent proportion of tumors that switched to the angiogenic phenotype. However, once nonangiogenic tumors switched to the angiogenic phenotype, they escaped

Angiogenic switch

Dormancy period

• 12 or more months
Osteosarcoma, MG-63 (~5% switch)
Osteosarcoma, SAOS-2 (~15% switch)

• 8 months
Osteosarcoma, KHOS-24OS (~20% switch)
Gastric cancer, ST-2 (<3% switch)
Glioblastoma, T98G (~60% switch)

• 4 months
Breast cancer, MDA-MB-436 (~80% switch)
Liposarcoma, SW-872 (>95% switch)

FIGURE 1.2 Human tumor cell lines that spontaneously switch to the angiogenic phenotype after a prolonged dormancy period in immunodeficient mice. The percent of mice that switch to the angiogenic phenotype is shown in brackets and varies between human tumor types.

from the dormancy state and formed lethal tumors in 100% of the mice, regardless of cancer type. At this time, tumor cell population–based animal dormancy models have been developed and characterized for breast cancer, osteosarcoma, and glioblastoma (Figure 1.2).[44] In contrast to the single-cell-derived human liposarcoma animal model, the angiogenic and the nonangiogenic tumor cell populations of the rest of the animal models were derived by in vivo selection for the angiogenic and nonangiogenic phenotypes. These in vivo models of nonangiogenic human tumors permit analysis of the switch to the angiogenic phenotype, and make it possible to address the question of whether the switch can be bi-directional.

1.4 DORMANT TUMORS HAVE BALANCED PROLIFERATION AND APOPTOSIS

After inoculation in animals, human tumor cells can remain dormant for more than a year. However, this does not mean that the tumor cells are in G_0 arrest. Although some tumor cells might be in mitotic arrest, as demonstrated in some tumor dormancy models,[45–47] we reported that the majority of tumor cells are proliferating or undergoing apoptosis. The tumor cell proliferation index in nonangiogenic tumors can be as high as that of large, vascularized tumors. In a human breast cancer (MDA-MB-436) animal model, more than 50% of tumor cells were proliferating and more than 10% were undergoing apoptosis in all microscopic tumors analyzed at various time points during dormancy, as well as in all macroscopic angiogenic tumors.[44] In a different human osteosarcoma (MG-63 and SAOS-2) and gastric (ST-2) cancer dormancy models, microscopic tumors were unable to grow beyond a threshold size of ~1–2 mm in diameter. Within these nonangiogenic tumors, there appears to be a balance between proliferating cells and cells undergoing apoptosis.[48] Tumor cell proliferation in these tumors was ~12%, and tumor cell apoptosis ranged from 4% to 7.5%.

1.5 DEFINITION OF A HUMAN DORMANT TUMOR BASED ON EXPERIMENTAL ANIMAL MODELS

Based on xenograft models of various human tumors inoculated or surgically implanted into immunodeficient animals, a "dormant" tumor can be defined by its microscopic size and nonexpanding mass. In contrast, a "stable" tumor is macroscopic and expanding in mass. In more detail, we define human nonangiogenic tumors as:

1. Unable to induce angiogenic activity, by repulsion of existing blood vessels in the local stroma and/or relative absence of intratumoral microvessels.
2. Remain harmless to the host until they switch to the angiogenic phenotype (i.e., may remain harmless for 1 year or more, which is half the life span of a mouse).
3. Express equal or more antiangiogenic (i.e., thrombospondin-1) than angiogenic (i.e., VEGF, bFGF) proteins.
4. Grow in vivo to ~1 mm in diameter or less, at which time further expansion ceases.
5. Only visible with a hand lens or a dissecting microscope (5–10 × magnification).
6. White or transparent by gross examination.
7. Unable to spontaneously metastasize from the microscopic dormant state.
8. Show active tumor cell proliferation and apoptosis in mice and remain metabolically active during the dormancy period.
9. Can be cloned from a human angiogenic tumor, because human tumors are heterogeneous and contain a mixture of nonangiogenic and angiogenic tumor cells.

In contrast, angiogenic human tumors (as observed in our animal models) are defined as:

1. Able to induce angiogenic activity, by recruiting blood vessels form the surrounding stroma and/or forming new blood vessels within the tumor tissue.
2. Lethal to the host in only few weeks.
3. Express significantly more angiogenic than antiangiogenic proteins.
4. Grow along an exponential curve until they kill the host.
5. Visible and easily detectable based on their macroscopic size.
6. Red by gross examination.
7. Can spontaneously metastasize to various organs.
8. Can be cloned from a human angiogenic tumor, because human tumors are heterogeneous and contain a mixture of nonangiogenic and angiogenic tumor cells.

1.6 IN VIVO IMAGING OF HUMAN DORMANT TUMORS

Traditionally, various in vivo imaging techniques have been used for the detection and quantification of tumors implanted orthotopically or ectopically (i.e., outside their orthotopic site). However, some of these techniques can be employed for the in vivo detection of microscopic dormant tumors. By definition, nonangiogenic, dormant tumors are microscopic in size. Therefore, they are usually undetectable by palpation (limited to tumor sizes 50 mm^3 and smaller) when located in the subcutaneous space or mammary fat pad. It is an even greater challenge to detect microscopic tumors located in internal organs. In the originally published dormancy model of osteosarcoma (MG-63), the presence of dormant tumors in a fraction of the inoculated mice was revealed through careful examination of the hair growth overlying the original tumor inoculation site.[48] The inner side of the skin in the area associated with hair growth contained a microscopic white lesion, from which a histology section showed a viable tumor. Although this detection method clearly reveals this interesting phenomenon, it is terminal (i.e., the animal has to be euthanized) and does not provide longitudinal quantitative information about the tumor size.

Stable infection of tumor cells with fluorescent proteins (such as green fluorescent protein [GFP] and red fluorescent protein [RFP]) or luciferase allows for in vivo longitudinal detection of tumors even at a microscopic size. GFP-expressing tumor cells can be visualized noninvasively from the skin surface by directed blue light (488 nm) epi-illumination. Submillimeter tumors can be localized using this method. The utility of fluorescence visualization of dormant tumors has been reported by Udagawa et al.,[48] using osteosarcoma (MG-63 and SAOS-2) and gastric cancer (ST-2) dormancy models. Tumor-associated blood vessels appear dark against the background of a fluorescent tumor tissue, allowing for morphological (e.g., vessel diameters, tortuousity, branching) and even functional (e.g., red blood cell velocity) quantification of angiogenesis.[49] More recently, this labeling technique was used to determine the minimum number of human tumor cells necessary to form a nonangiogenic, dormant microscopic tumor in mice (Naumov et al., unpublished). However, detecting fluorescently labeled tumors has its limitations. Microscopic tumors in internal organs can only be visualized ex vivo. Certain procedures, such as in vivo videomicroscopy, can be used for visualization of liver and lung metastases.[50,51] However, in the brain, excitation or emission of fluorescently labeled tumor cells is not only limited by tissue depth, but also by light penetration through the skull.

Infection of tumor cells with the luciferase reporter gene allows for the reliable detection in mice of a signal from tumors that are <1 mm in diameter (as verified by histology) in all internal organs, including the brain. Almog et al.[43] has used the luciferase method for the detection of dormant human liposarcoma tumors in the renal fat pad of mice. The method can also be used to monitor the growth of microscopic human glioblastomas stereotactically after tumor cells are inoculated in the brains of mice (Naumov et al., unpublished work). Following intravenous injection of the luciferine substrate, the enzymatic activity of luciferase is rapid and transient. Only viable and metabolically active tumor cells can be detected by luminescence. The transient effect of the enzymatic reaction allows for real-time detection of tumor cells and for monitoring their viability during the dormancy period, as well as at times throughout the angiogenic switch. The persistent luciferase signal during the dormancy period of microscopic human tumors confirms the previous conclusion (based on histology) that dormancy does not result from tumor cell cycle arrest or eradication. Although the intensity of the luciferase signal directly correlates with the size of tumor, this imaging modality does not provide a clear tumor boundary or an anatomical outline of the tumor. However, small animal magnetic resonance imaging (MRI) provides a clear anatomical definition of a microscopic tumor, and it can be effectively used in combination with luciferase imaging (Naumov et al., unpublished work). Recent reports have demonstrated that single cancer cells can be detected in a mouse brain using MRI.[52] Individual tumor cells trapped within the brain microcirculation were detected using MRI and validated using high-resolution confocal microscopy. Graham et al.[53] demonstrated that three-dimensional, high-frequency ultrasound can quantitatively monitor the growth of liver micrometastases as small as 0.5 mm in diameter.

Collectively, these recent advances in animal imaging modalities enable, in most cases, noninvasive, real-time, longitudinal observations of single cancer cell trafficking and detection of nonangiogenic microscopic tumors in vivo during the dormancy period and as they switch to the angiogenic phenotype. Quantitative imaging of tumors throughout their progression to the angiogenic phenotype can be used for evaluating the efficacy of antiangiogenic therapy in primary and metastatic tumors.

1.7 MOLECULAR MECHANISMS OF THE HUMAN ANGIOGENIC SWITCH

Transfection of human osteosarcoma (MG-63 and SAOS-2) and gastric cancer (ST-2) cells with activated c-*Ha-ras* oncogene induces loss of dormancy in otherwise nonangiogenic human cell lines.[48] When inoculated in immunosuppressed mice, wild-type (or control

vector-transfected) tumor cells did not form palpable tumors for more than 8 months. White tumor foci, which were avascular or contained sparse vessels, were found throughout the dormancy period at the site of inoculation. However, *ras*-transfected human osteosarcoma (MG-63 and SAOS-2) and gastric cancer (ST-2) cells formed vascularized large tumors within 1 month. The in vivo growth of *ras*-transfected tumor cells was associated with significantly increased angiogenic response, increased proliferation, and decreased apoptosis when compared with wild-type tumor cells. Loss of the dormant phenotype induced by activated *ras* correlated with increased levels (1.5 to 2.5-fold) of $VEGF_{165}$, as assessed in the conditioned media relative to the control tumor cells.[48] Overexpression of $VEGF_{165}$ in the tumor cells also resulted in a loss of dormancy and induced a robust angiogenic response in 30% of animals inoculated with gastric cancer and 40% of animals inoculated with osteosarcoma. In contrast to *ras*-transfected tumor cells, loss of dormancy in $VEGF_{165}$-transfected tumor cells was not associated with an increase in tumor cell proliferation, but was associated with reduced apoptosis. Therefore, the angiogenic response induced by $VEGF_{165}$ was found to be sufficient for the induction of a loss of dormancy by reducing apoptosis. Activation of *ras* can directly induce tumor cell proliferation and confer resistance to apoptosis.[54,55] In addition, *ras* activation can indirectly stimulate an angiogenic response in tumors by inducing proangiogenic proteins, such as VEGF,[56] and by downregulating angiogenesis inhibitors, such as thrombospondin.[57–60] Similar to *ras*, other oncogenes and tumor suppressor genes can indirectly affect tumor growth via an angiogenic mechanism. For example, *p53*, *PTEN*, and *Smad 4* have been shown to increase thrombospondin-1 expression by upregulation of *Tsp-1* gene or by increased mRNA expression.[12,61–63] Thrombospondin-1 expression can be decreased by *Myc*, *Ras*, *Id1*, *WT1*, *c-jun*, and *v-stc* via transcriptional repression, myc phosphorylation, or regulation of mRNA turnover and stability.[59,60,64–69] The inherently low toxicity of natural angiogenesis inhibitors, in addition to their selective effect on pathological neovascularization without harm to normal vasculature, makes them attractive therapeutic agents.

In addition to thrombospondin-1 regulation, the *p53* tumor suppressor gene regulates other currently unidentified inhibitors of angiogenesis.[70] Teodoro et al.[71] recently reported that wild-type *p53* mobilizes endostatin through a specific α(II) collagen prolyl-4-hydroxylase (α(II)PH gene product), which binds to *p53*. *p53* is inactivated in over 50% of all human tumors. Reintroduction of wild-type *p53* into mouse fibrosarcoma (T241) cells correlates with increased thrombospondin-1 expression and induces angiogenesis-restricted dormancy.[72] Inoculation of parental T241 fibrosarcoma cells into a mouse ear resulted in vascularized, visible tumors within 2 weeks. In contrast, when wild-type *p53* was introduced in the same cells, only 12% of the tumors became angiogenic 2 months after inoculation. Therefore, expression of wild-type *p53* resulted in the loss of an angiogenic phenotype. Loss of the angiogenic phenotype was also correlated with the upregulation of the mRNA-encoding thrombospondin-1. This experimental model demonstrated that *p53* can act as a tumor suppressor, independent of its direct effects on cell proliferation and survival. Moreover, *p53* had an indirect antitumor effect by inhibiting angiogenesis and increasing the rate of apoptosis.

In a recent report, Naumov et al.[44] compared the tumor cell secretion and intracellular levels of thrombospondin-1 in nonangiogenic and angiogenic tumor cell populations isolated from a human breast cancer cell line (MDA-MB-436). Angiogenic cells contain 2.5-fold higher levels of c-Myc and p-Myc than their nonangiogenic counterparts, as assessed by Western blot. In contrast, angiogenic tumor cells contain significantly lower levels of thrombospondin-1 than nonangiogenic tumor cells. Moreover, nonangiogenic human breast cancer cells secrete at least 20-fold higher levels of thrombospondin-1 than angiogenic cells. Similar findings were reported using a different human breast cancer cell line (MDA-MB-435).[60] Watnick et al.[60] reported that phosphoinositide 3-kinase (PI3K) can induce a signal transduction cascade leading to the phosphorylation of c-Myc and the subsequent repression of

thrombospondin-1. Treatment with LY294002 (a PI3K inhibitor) caused thrombospondin-1 levels within angiogenic cells to increase but had no effect on levels in nonangiogenic cells.[44] Therefore, the PI3K signaling pathway is responsible for the repression of thrombospondin-1, and it is regulated differently in angiogenic and nonangiogenic human tumor cells.

1.8 INDUCTION OF TUMOR DORMANCY USING ANTIANGIOGENIC THERAPY

Tumor progression is highly dependent on the surrounding stroma, including the endothelial cells, fibroblasts, local basement membrane factors, macrophages, platelets, T cells, and other cellular compartments. Antiangiogenic therapy can target the endothelial cell compartment in atleast two distinct ways: directly or indirectly (Figure 1.3).[73]

Direct angiogenesis inhibitors block vascular endothelial cells from proliferating, migrating, or increasing their survival. For example, SU 11248 directly blocks VEGF receptors (among other receptors involved with angiogenic signaling) on endothelial cells (Figure 1.3). Direct angiogenesis inhibitors include: (1) synthetic inhibitors or peptides designed to interfere with specific steps in the angiogenic process (e.g., inhibitors of metalloproteinases, antagonists of the $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrins), (2) low molecular weight molecules (e.g., TNP-470, caplostatin, thalidomide, 2-methoxyestradiol), and (3) endogenous (i.e., natural) angiogenesis inhibitors (e.g., TSP-1, platelet factor 4, interferon-α, IL-12, angiostatin, endostatin, arrestin, canstatin, tumstatin).[5,39,74-92]

Bouck et al.[93] were the first to demonstrate that a tumor can generate angiogenesis inhibitor (i.e., thrombospondin-1). They subsequently suggested that the angiogenic phenotype was a result of a net imbalance of endogenous angiogenesis stimulators and inhibitors.
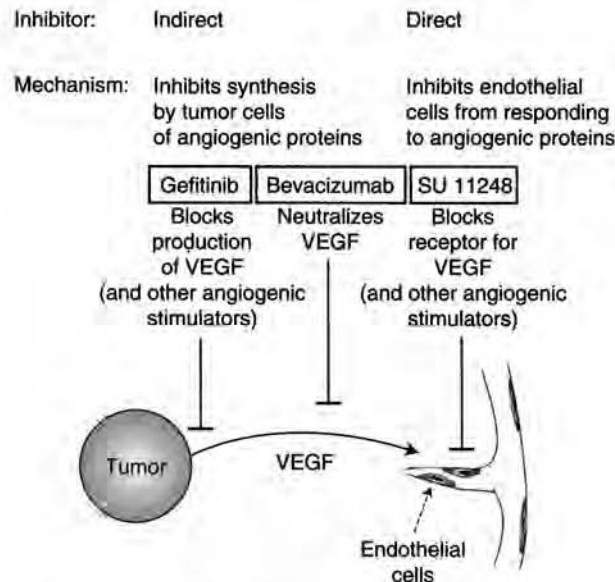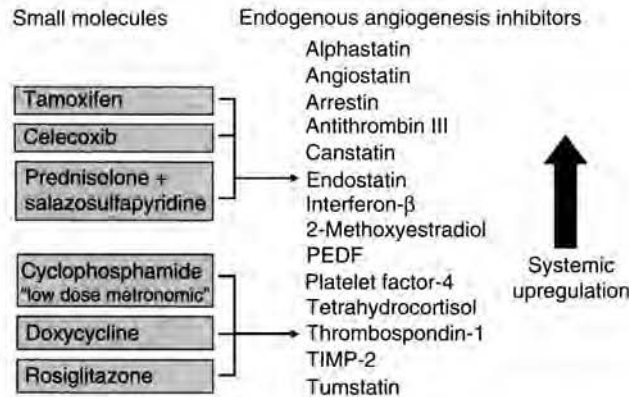

FIGURE 1.3 Examples of direct and indirect angiogenesis inhibitors that can block production of a tumor cell angiogenic protein (Gefitinib), or neutralize a systemic proangiogenic protein (Bevacizumab), or block a receptor for a tumor cell produced angiogenic protein (SU 11248). (Adapted from Folkman, J. et al., *Cancer Medicine*, 7th edn., 2006. With permission.)

In a series of experiments, Folkman and colleagues reported that the surgical removal of a primary Lewis lung carcinoma tumor in mice results in the exponential growth of lung metastases.[94,95] In these experimental animal models, the presence of a primary tumor generated increased circulating angiostatin levels. Angiostatin is a potent antiangiogenic plasminogen fragment,[96] which inhibits the in vivo growth of Lewis lung metastases by preventing neovascularization.[77] However, gene transfer of a cDNA coding for mouse angiostatin into murine T241 fibrosarcoma cells successfully suppressed lung metastatic tumor growth after the removal of the primary tumor.[97] Cao et al.[97] demonstrated that pulmonary micrometastases, expressing angiostatin, remain in a dormant and avascular state for 2–5 months after removal of primary tumors. These dormant micrometastases were characterized as having a high rate of apoptosis counterbalanced by a high proliferation rate.

Holmgren et al.[95] investigated whether treatment with an exogenous angiogenesis inhibitor could replace the endogenous angiogenesis suppressive ability of a primary tumor. In animals with surgically removed primary Lewis lung carcinoma, or T241 mouse sarcomas, treatment with TNP-470 resulted in the suppression of metastases comparable to that observed in the presence of the primary tumor. Therefore, exogenous treatment can be used to replace the endogenous angiogenesis inhibition of a primary tumor. Moreover, it can be used for the systemic suppression of angiogenesis-maintained micrometastases of both Lewis lung cancer and T241 fibrosarcoma in a dormant state. These dormant micrometastases were characterized as having high tumor cell proliferation counterbalanced by high cell death rate (i.e., apoptosis), indicating that inhibition of angiogenesis limits tumor growth by elevating tumor cell apoptosis. Exogenous angiogenesis inhibitors, such as TNP-470, mimic the primary tumor suppression by maintaining high apoptosis in the lung micrometastases, but without having an effect on tumor cell proliferation. A similar mechanism of sustained micrometastatic dormant state has been demonstrated using angiostatin, which maintains a high apoptotic index in lung metastases after the removal of a primary tumor without affecting tumor cell proliferation.[38]

Indirect angiogenesis inhibitors target tumor cell proteins created by oncogenes that drive the angiogenic switch. In general, their mechanism of action is by decreasing or blocking the expression of other tumor cell products, neutralizing the tumor cell product itself, or by blocking receptors on endothelial cells. The impact of oncogenes on tumor angiogenesis has been reviewed by Rak and Kerbel.[59,98,99] For example, gefitinib (Iressa) blocks VEGF production from tumor cells. However, even systemically available VEGF can be neutralized by bevacizumab (Avastin) before it binds to VEGF receptors on endothelial cells (Figure 1.3). There is an emerging group (e.g., thyrosine kinase inhibitors) of anticancer drugs originally developed to target oncogenes, which also have "indirect" antiangiogenic activity. For example, the *ras* farnesyl transferase inhibitors block oncogene signaling pathways, which upregulate tumor cell production of VEGF and downregulate production of Tsp-1.[100] Trastuzumab, an antibody that blocks *HER2/neu* receptor tyrosine kinase signaling, suppresses tumor cell production of angiogenic proteins, such as TGF-α, angiopoietin 1, plasminogen activator inhibitor-1 (PAI-1), and VEGF.[101,102] At the same time, trastuzumab has been shown to upregulate the expression of Tsp-1 (endogenous angiogenesis inhibitor), which may be an important mechanism of its antiangiogenic activity.[102] Upregulation of endogenous antiangiogenic proteins, using direct or indirect angiogenesis inhibitors, can be a useful approach for preventing the angiogenic switch and keeping human tumors in a microscopic dormant state (Figure 1.4).

Acquired drug resistance is a major obstacle in the treatment of cancer. Genetic instability, heterogeneity, and high mutational rates of tumor cells are the major causes of drug resistance.[103] In contrast, antiangiogenic therapy targets endothelial cells, which are genetically stable and have a low mutational rate. In an experimental animal model,

Small molecules   Endogenous angiogenesis inhibitors
                                    Alphastatin
                                    Angiostatin
      Tamoxifen   Arrestin
                                    Antithrombin III
      Celecoxib   Canstatin
      Prednisolone +   →   Endostatin
      salazosulfapyridine   Interferon-β
                                    2-Methoxyestradiol
                                    PEDF   Systemic
      Cyclophosphamide   Platelet factor-4   upregulation
      "low dose metronomic"   Tetrahydrocortisol
      Doxycycline   →   Thrombospondin-1
                                    TIMP-2
      Rosiglitazone   Tumstatin

FIGURE 1.4 Summary of a few small molecules and the corresponding increase in endogenous angiogenesis inhibitors. Examples of small molecules that are orally available and may induce increased systemic levels of endogenous angiogenesis inhibitors. Chronic systemic increase of antiangiogenic proteins can prevent the angiogenic switch and delay the progression of cancer. (Adapted from Folkman, J., *Exp. Cell Res.*, 312(5), 594, 2006. With permission.)

Boehm et al.[104] reported that antiangiogenic therapy targeted against tumor-associated endothelial cells does not result in drug resistance. In this study, Lewis lung cancer, T241 fibrosarcoma, and B16F10 melanoma were repeatedly treated with endostatin. When tumors reached the size of ~350–400 mm³, endostatin treatment was initiated until the tumor became undetectable. Endostatin therapy was then stopped, and the tumor was allowed to regrow. Endostatin therapy was resumed when tumors reached a mean volume of 350–400 mm³. After the second (for melanoma), fourth (for fibrosarcoma), and sixth (for Lewis lung carcinoma) cycles of endostatin treatment, all tumors remained as barely visible subcutaneous nodules (size 5–50 mm³) for up to 360 days. In contrast, endostatin resistance developed rapidly when Lewis lung carcinomas were treated with conventional cytotoxic chemotherapy. These studies demonstrated that repeated cycles of endostatin therapy induced tumor dormancy, which persisted indefinitely after therapy.

1.9 METASTATIC DORMANCY

Metastasis, the spread of cancer from a primary tumor to secondary organs, is the major cause of cancer-related deaths. Hematogenous or lymphatic spread of only a few cancer cells from a primary tumor can successfully form a macroscopic tumor at a secondary site.[50,105,106] However, for a macrometastasis to become lethal, it must successfully complete a number of steps (Figure 1.1).[107] Two different types of tumor dormancy have been identified in the metastatic process: (1) solitary dormant cancer cells, which are in G_0 cell cycle arrest and (2) dormant nonangiogenic tumors, in which tumor cells are actively proliferating and dying, but the tumor fails to recruit blood vessels. Both of these steps can contribute to a latency period associated with metastatic growth of human cancer (Figure 1.1).[108]

Previous studies by Holmgren and colleagues[95,109] have identified nonangiogenic micrometastases as a potential contributor to metastatic dormancy. These studies showed that dormant micrometastases did not grow in size beyond 200 μm, but they remained metabolically active. This size limitation was associated with a steady-state balance between the rates of tumor cell proliferation and apoptosis, with no net growth of the metastases. Changes in the intrinsic properties of these dormant micrometastases, or their microenvironment at a later time, triggered metastatic growth associated with a disturbance of the proliferation and apoptosis balance. Progressive growth in such micrometastases was restricted due to suppression of tumor angiogenesis.

Naumov et al.[110] have identified another possible source of metastatic dormancy: viable, solitary dormant tumor cells that are neither proliferating nor undergoing apoptosis after arriving at a metastatic site. These studies showed that more than 50%–80% of breast cancer cells, distributed to mouse liver via the circulation, can remain in the tissue for extended periods of time (up to 77 days) as solitary nonproliferating dormant cells. This surprising phenomenon was observed in populations of breast cancer cells of high and low metastatic ability. In the case of the highly metastatic cell line (D2A1 cells), lethal macrometastases grew from a very small subset of cells (~0.006%), with the majority (~80% cell loss) of injected cells undergoing apoptosis or destroyed by leukocytes. However, ~20% of the injected cells persisted as nonproliferating dormant cells. In contrast, ~80% of poorly metastatic breast cancer cells remained as nonproliferating dormant solitary cells in the mouse liver. A subset of these cells could be recovered and grown under in vitro culture conditions 11 weeks after injection into mice. The recovered tumor cells retained their ability to form primary tumors in the mammary fat pad of mice. These solitary dormant tumor cells may be a potential source of an occasional nonangiogenic metastasis, and of an even rarer, but lethal, angiogenic metastasis.

Taken together, these studies demonstrated that metastasis is a dynamic process, where solitary, nonproliferating dormant cancer cells, nonangiogenic micrometastases, and angiogenic macrometastases can coexist at each stage of the metastatic process. While nonangiogenic micrometastases could be vulnerable to antiangiogenic and cytotoxic chemotherapeutic agents (administered in a metronomic, low-dose regimen, as described by Browder et al.[111] and Kerbel and colleagues[112,113]), solitary dormant cells could remain unaffected because of their inability to proliferate.

Naumov et al.[114] showed that nonproliferating solitary dormant breast cancer cells remained unaffected by doxorubicin treatment. However, the same treatment successfully inhibited actively growing macrometastases in the same mice. Therefore, doxorubicin chemotherapy, which successfully reduced the metastatic burden, failed to affect the number of solitary dormant cells. These findings have important clinical implications for patients undergoing adjuvant chemotherapy. It is possible that dormant nonproliferating tumor cells can remain unaffected by standard chemotherapy and may retain their potential to initiate growth at a later date. Both solitary cancer cells and nonangiogenic metastases can remain dormant and undetectable for months or years, leading to an uncertainty in the prognosis for patients who have already been treated for the primary cancer.

1.10 ANGIOGENIC SWITCH-RELATED BIOMARKERS FOR DETECTION OF DORMANT TUMORS

Even with recent advances in the clinical detection of human cancer, a tumor that is microscopic in size (~1 mm in diameter) remains undetectable. A panel of angiogenic switch-related biomarkers is under development using the human tumor dormancy models. These biomarkers include circulating endothelial progenitor cells (CEPs) and platelets in the blood, as well as matrix metalloproteinases (MMPs) in the urine. The detection of a single microscopic human tumor in existing animal models can be achieved using each one of these biomarkers alone or in combination.[115–117]

We compared the in vivo ability of angiogenic and nonangiogenic human breast tumors (MDA-MB-436 cells) to mobilize mature circulating endothelial cells (CECs) (CD45−, Flk+, CD31+, CD117−) and CEPs (CD45−, Flk+, CD31+, CD117+).[115] The number of blood-borne CECs and CEPs was quantified using a flow cytometer. There was little difference in the percent of mature CECs in the blood of mice inoculated with angiogenic and nonangiogenic cells. However, mice inoculated with nonangiogenic cells had approximately fourfold decrease in CEPs when compared with control mice. Mice inoculated with angiogenic cells

had levels of CEPs comparable to those in the control mice. Previous reports[44] have shown that these nonangiogenic breast cancer cells (MDA-MB-436 cells) secrete at least 20-fold higher levels of thrombospondin-1 (Tsp-1) than their angiogenic counterparts. Other studies have suggested that endogenous inhibitors of angiogenesis, such as Tsp-1 and endostatin, may inhibit the mobilization of CEPs.[118] These observations suggest that microscopic dormant (nonangiogenic) tumors may suppress the mobilization of CEPs from the bone marrow via systemic thrombospondin-1.

Klement et al.[117] recently reported that blood platelets can sequester both pro- and antiangiogenic proteins. It is estimated that at least 100 billion platelets are produced per day by megakaryocytes in the bone marrow of an average 70 kg person.[119,120] With a life span of 7–8 days in humans, it is estimated that there are approximately a trillion platelets in constant circulation.[119,121] Folkman and colleagues proposed that the platelet compartment of the blood stream can potentially accumulate angiogenesis-related proteins and possibly release them at a later time.[117] Using a novel "platelet angiogenic proteome," as quantitatively assessed by SELDI-ToF technology (Ciphergen, Freemont, CA), the presence of microscopic human tumors in mice can be detected.[117] Using this technology, the accumulation and reduction in angiogenesis-related proteins sequestered in platelets can be quantitatively followed throughout the angiogenic switch. The identification of proteins that are associated with the angiogenic switch and that may be used as angiogenic switch-related biomarkers is currently under investigation.

In summary, the tumor dormancy animal models presented here permit further clarification of the role of CEC/CEPs, platelets, and MMPs as participants in the "angiogenic switch." Moreover, this angiogenic switch-related biomarker panel may prove to be a useful diagnostic method for the presence of microscopic cancers at primary and metastatic sites long before detection by conventional methods. It may be feasible to develop a panel of angiogenesis-associated biomarkers that can identify the presence of a microscopic human tumor, predict its switch to the angiogenic phenotype, and possibly serve as a guide for antiangiogenic therapy. In the future, it may be possible for a patient who is at risk for cancer recurrence to take an oral drug that can elevate endogenous platelet-associated antiangiogenic proteins and delay, if not prevent, the formation of recurrent tumors.

1.11 CONCLUSION

We speculate that the development of more specific and sensitive biomarkers may permit the very early detection of recurrent cancer, possibly years before symptoms or anatomical location. If this concept can be validated, then relatively nontoxic angiogenesis inhibitors may be used to "treat the biomarkers" without ever seeing the recurrent tumor (i.e., cancer without disease).[14]

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blood

Endostatin finds a new partner: nucleolin

Judah Folkman

Comparison of angiotensin-II (Ang-II)–mediated leukocyte recruitment in arterioles and venules. In arterioles, high constitutive expression of IL-4 and Ang-II–induced TNF induces expression of CC chemokines such as MCP-1 and RANTES over a time course of several hours. These chemokines mediate selective arrest of mononuclear leukocytes in arterioles. In contrast, in postcapillary venules, Ang-II induces rolling and arrest within 60 minutes, predominantly recruiting neutrophils to the endothelial surface. This process does not require either IL-4 or TNF.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ∎

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● ● ● **HEMOSTASIS**

Comment on Shi et al, page 2899

Endostatin finds a new partner: nucleolin

Judah Folkman CHILDREN'S HOSPITAL AT HARVARD MEDICAL SCHOOL

In this issue of *Blood*, Shi and colleagues examine the endostatin binding of nucleolin on angiogenic endothelial cells, as well as the transport of nucleolin to the nucleus, where it prevents proliferation—thus revealing a novel mechanism for endostatin's antiangiogenic activity.

During the past 3 years, 8 new drugs with antiangiogenic activity have received approval from the Food and Drug Administration of the United States for the treatment of cancer and age-related macular degeneration, and have also been approved in more than 30 other countries. These are mainly antibodies, aptamers, or synthetic molecules. They block at least 1 to 3 proangiogenic proteins or their receptors.[1] This new class of drugs has been prescribed for more than 1.2 million patients. More than

50 drugs with antiangiogenic activity are in phase 2 or 3 clinical trials.[2] Since 1980, 28 endogenous angiogenesis inhibitors, including platelet factor 4, angiostatin, endostatin, thrombospondin-1, tumstatin, and canstatin, have been discovered in blood or tissues.[3,4] At this writing, more than 1000 reports on endostatin have been published since its discovery in 1997, and reveal that endostatin has the broadest antitumor spectrum of the endogenous angiogenesis inhibitors. It is also the first endogenous inhibitor

to receive approval for anticancer therapy, under the trade name "Endostar" in China.

The integrin α5β1 has been proposed as a receptor for endostatin, and endostatin has been shown to regulate an entire program of antiangiogenic gene expression in human microvascular endothelial cells stimulated by VEGF or bFGF.[5] Nevertheless, certain antiangiogenic actions of endostatin have no molecular explanation and remain as open questions. In this issue of *Blood*, Shi and colleagues address 3 of these questions. Why does endostatin specifically target angiogenic blood vessels, but not quiescent blood vessels? Why does endostatin inhibit tumor angiogenesis with virtually no toxicity in animal studies and clinical trials? Why has the antiangiogenic activity of endostatin appeared to be heparin-dependent in previous studies? These questions are answered by a novel and important finding: that nucleolin is expressed on the surface of proliferating angiogenic human microvascular endothelial cells, but not on the surface of quiescent endothelium. In angiogenic endothelial cells, the cell-surface nucleolin binds endostatin and transports it to the nucleus, where endostatin inhibits phosphorylation of nucleolin. Phosphorylation of nucleolin induced by VEGF or bFGF has been reported to be essential for cell proliferation. Furthermore, endostatin does not inhibit proliferation of many types of tumor cells per se, possibly because while they express nucleolin on their surfaces, they do not internalize it in the presence of endostatin. The heparin binding sites on nucleolin were found to be critical for endostatin. Increasing concentrations of exogenous heparin dissociated the binding of endostatin to nucleolin.

The article by Shi and colleagues is also thought-provoking because the endostatin-nucleolin connection is now fertile soil for future studies. For example, it will be interesting to learn how specific the binding of endostatin is to nucleolin compared with other proteins that also bind to endostatin, as reported by the authors. Furthermore, it will be helpful if the relationship of nucleolin to other cell-surface endostatin-binding proteins can be uncovered, particularly α5β1. Will this endostatin-nucleolin connection lead to the uncovering of a mechanism

for the biphasic, ∪-shaped anticancer dose-response curve recently reported for endostatin,[6] and originally shown for the antiendothelial activity of interferon α?[7] This biphasic dose response is common to other angiogenesis inhibitors. Endostatin increases nucleolin expression in human microvascular endothelial cells in vitro by approximately 20%.[5] Is p53-mediated inhibition of angiogenesis, in part through increased expression of endostatin,[8,9] also regulated by nucleolin? Because the endothelial-cell expression of another endogenous angiogenesis inhibitor, thrombospondin-1, is up-regulated by endostatin, is thrombospondin-1 indirectly nucleolin-dependent? Are any other endogenous angiogenesis inhibitors regulated by binding to nucleolin? Angiogenesis in wound healing and pregnancy is not delayed by high endostatin levels, as for example in individuals with Down syndrome, or in animals receiving endostatin therapy.[10] Is this because proliferating endothelial cells in reproduction and repair do not express nucleolin on their cell surface, or do not internalize it?

The novel role for nucleolin, as a regulator of the antiangiogenic activities of endostatin, has fundamental implications for understanding the biology of endostatin and for its clinical application.

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● ● ● ● **HEMATOPOIESIS** ▬▬▬▬▬

Comment on Chen et al, page 2889

Cited2: master regulator of HSC function?

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Defining intracellular molecules involved in hematopoietic stem cell (HSC) function is important for future efforts to enhance transplantation. Toward this aim, Chen and colleagues report that Cited2, a transcriptional modifier, is necessary for mouse fetal liver hematopoiesis.

Understanding intracellular events that mediate functions of hematopoietic stem cells (HSCs) is important for realizing improved efficacy of HSC transplantation. In this issue of *Blood*, Chen and colleagues have advanced this area by demonstrating a new role for Cited2 (cAMP-responsive element–binding protein [CBP]/p300-interacting transactivators with glutamic acid [E] and aspartic acid [D]–rich tail 2)[1] in steady-state embryogenesis.

Gene-expression profiling linked Cited2 expression with long-term marrow HSC activity.[2] However, the current authors had to study Cited2^{−/−} fetal liver hematopoiesis, since functional deletion of Cited2, a member of a new family of transcriptional modulators, results in embryonic lethality.[3] A role for Cited2 in hematopoiesis was shown by the authors in elegant studies demonstrating that Cited2^{−/−} fetal liver was greatly reduced in phenotyped hematopoietic progenitors

(HPCs); compromised reconstitution of T- and B-lymphocyte and myeloid cells was apparent after both primary and secondary transplantation of Cited2^{−/−} fetal liver cells, and the competitive repopulating capacity of these HSCs was decreased.

Exactly how Cited2 acts, alone or in combination with other mediators, to regulate normal hematopoiesis is not known. A number of receptor and intracellular signals are reported to modulate HSC and HPC functions.[4] These include Notch and Notch ligands, Wnt signaling, HoxB4/PBX1, Bmi-1, C/EBPα, Gfi-1, p21[cip1/waf1], p27[kip1], PTEN, Nov/CLN3, glycogen synthase kinase-3, ERK1/2– and p38–MAP kinase, ME/ELK4, RAR–γ, Stat3, Stat5, and Mad2. Microarray analysis by Chen and colleagues showed decreased expression of Wnt5a and a panel of myeloid markers in Cited2^{−/−} fetal livers, as well as decreased expression of Bmi-1, Notch, LEF-1, Mcl-1, and GATA-2 in Cited2^{−/−} c-kit[+] lineage[−] fetal liver cells. Placing these molecules into correct positions within interacting networks required to properly mediate self-renewal, proliferation, survival, differentiation, and migration of HSCs—and determining where Cited2 fits into this schema—are vital missing pieces of information. For example, which signals are in linear, branching, or completely separate pipelines, and which occupy key regulatory positions in the cascading sequence of events? Do any of these molecules act as a master switch, and is Cited2 a master regulator? What other roles, if any, does Cited2 play in HSC/HPC function and hematopoiesis? The authors propose future studies involving overexpression of genes identified from the current study in Cited2^{−/−} HSCs, and generation of conditional Cited2 knockout mice at specific developmental stages and in specific hematopoietic lineages. This should bring us closer to knowing what Cited2 does and does not do.

How HSCs renew themselves is still essentially unknown. While investigators continue to dig away, little by little, at this crucial HSC function, it may be that the field needs to be more creative and think out of the box to get a true picture of the renewal of HSCs, which may not occur as we currently envision it. Whatever model or models are eventually identified for renewal of HSCs, it may be that Cited2 will be an important player in these events. We look forward to finding out.

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PPARα agonist fenofibrate suppresses tumor growth through direct and indirect angiogenesis inhibition

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Angiogenesis and inflammation are central processes through which the tumor microenvironment influences tumor growth. We have demonstrated recently that peroxisome proliferator-activated receptor (PPAR)α deficiency in the host leads to overt inflammation that suppresses angiogenesis via excess production of thrombospondin (TSP)-1 and prevents tumor growth. Hence, we speculated that pharmacologic activation of PPARα would promote tumor growth. Surprisingly, the PPARα agonist fenofibrate potently suppressed primary tumor growth in mice. This effect was not mediated by cancer-cell-autonomous antiproliferative mechanisms but by the inhibition of angiogenesis and inflammation in the host tissue. Although PPARα-deficient tumors were still susceptible to fenofibrate, absence of PPARα in the host animal abrogated the potent antitumor effect of fenofibrate. In addition, fenofibrate suppressed endothelial cell proliferation and VEGF production, increased TSP-1 and endostatin, and inhibited corneal neovascularization. Thus, both genetic abrogation of PPARα as well as its activation by ligands cause tumor suppression via overlapping antiangiogenic pathways. These findings reveal the potential utility of the well tolerated PPARα agonists beyond their use as lipid-lowering drugs in anticancer therapy. Our results provide a mechanistic rationale for evaluating the clinical benefits of PPARα agonists in cancer treatment, alone and in combination with other therapies.

stroma | inflammation | fibrates | microenvironment

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors comprising three isoforms, PPARα, PPARδ, and PPARγ, which act as ligand-activated transcriptional factors. PPARs play key roles in energy homeostasis by modulating glucose and lipid metabolism and transport (1). PPARα is also critical in inflammation (2) and is the molecular target of the fibrate class of drugs, such as fenofibrate, which act as agonistic ligands of PPARα.

Long-term administration of certain PPARα agonists (clofibrate and WY14643) induces hepatocarcinogenesis in rodents but not in humans (3). Consequently, PPARα has not been established as a molecular target for cancer therapy by its agonistic ligands. In contrast, PPARγ and PPARδ agonists have been extensively studied to evaluate their anticancer effects because of their antiproliferative, proapoptotic, antiapoptotic, and differentiation-promoting activity (4). However, recent studies have revealed the expression of PPARα in tumor cells (5, 6), and PPARα ligands suppress the growth of several cancer lines, including colon, breast, endometrial and skin, in vitro (7–10). PPARα ligands also suppress the metastatic potential of melanoma cells in vitro and in vivo (11, 12). Furthermore, PPARα ligands decrease tumor development in murine colon carcinogenesis (7). Clofibric acid inhibits the growth of human ovarian cancer in mice (13). Most recently, the PPARα agonist WY14643 suppresses tumorigenesis in a PPARα-dependent manner (14).

Together, these data suggest that PPARα ligands may have an important role as antitumor agents, although the mechanism remains elusive. PPARα is expressed not only in tumor cells but also in endothelial and inflammatory cells (15, 16). Also, PPARα ligands can inhibit endothelial cell proliferation and migration and induce endothelial cell apoptosis in vitro (17–19). In addition, fenofibrate reduces adventitial angiogenesis and inflammation in a porcine model (20) and decreases VEGF levels in patients with hyperlipidemia and atherosclerosis (21). However, the relative role of PPARα in tumor angiogenesis and tumor inflammation has not been studied.

Here, we report that PPARα is expressed both in tumor cells and in tumor endothelium. We show that PPARα ligands have potent antitumor and antiangiogenic effects, both in vitro and in vivo. Our data demonstrate that PPARα expression in the host rather than in the tumor cell is critical for the antitumor, antiangiogenic, and antiinflammatory activity of PPARα ligands. This extends the repertoire of potential targets of PPARα ligands beyond cell-autonomous mechanisms of cancer. Our findings may be of clinical relevance because PPARα ligands such as fenofibrate are orally administered, Food and Drug Administration-approved drugs widely used for the treatment of hyperlipidemia with minimal side effects.

Results

PPARα Is Expressed in Tumor Cells in Vitro and in Tumor Endothelium in Vivo. We first screened 19 human tumor cell lines for PPARα expression in vitro. In Western blot analysis of cell cultures, we found that all tumor cell lines examined expressed the PPARα protein, although at varying levels (Fig. 1a). We obtained similar results in murine tumor cell lines, albeit at a lower intensity. The signal could be specifically neutralized with a blocking peptide (Fig. 1b). Expression patterns in tumor tissues were assessed by immunofluorescent double staining for PPARα and the endothelial marker CD31. PPARα staining was examined in s.c. implanted human pancreatic cancer cells (BxPC3) grown in mice (Fig. 1c), as well as in clinical specimens from human prostate carcinoma (Fig. 1d). We found expression of PPARα in the tumor cells as well as in human and murine endothelial cells of microvessels (Fig. 1 c and d).

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Fig. 1. PPARα is expressed in tumor cells and endothelium of neoplastic tissues. (*a*) Western blot analysis of PPARα expression in cultured human tumor cells and hemangioma specimens. Nuclear extract from leukemia cells (HL-60) was used as a control. (*b*) Western blot analysis of PPARα expression in cultured mouse tumor cells. The specificity of protein expression was confirmed by abrogation by a PPARα-blocking peptide. GAPDH and β-actin levels were measured to demonstrate equal loading of protein in each lane. (*c* and *d*) Immunofluorescent double staining for CD31 and PPARα demonstrates PPARα expression in endothelium of human pancreatic cancer (BxPC3) in SCID mice (*c*) and in patient prostate cancer tissue specimens (*d*). CD31-stained endothelial cells are shown in green, PPARα-positive cells are red, and colocalization of the two colors are yellow. Colocalization of red and green fluorescence (yellow) indicates PPARα expression in blood vessels.

PPARα Ligands Have Direct Antitumor and Antiendothelial Effects *in Vitro*. Given the presence of PPARα in multiple cell types, we next compared the effect of PPARα ligands for their ability to inhibit the proliferation of tumor cells, endothelial cells, and fibroblasts. PPARα ligands examined included fenofibrate, gemfibrozil, bezafibrate, WY14643, and 5,8,11,14-eicosatetraynoic acid (ETYA). Fenofibrate was most potent in suppressing the proliferation of all tumor cell lines tested, including melanoma (B16-F10), breast carcinoma (MDA436), and Lewis lung carcinoma (LLC) cells [Fig. 2*a* and supporting information (SI) Fig. 6]. Fenofibrate, WY14643, and ETYA inhibited FGF2-induced proliferation of bovine capillary endothelial cells up to 95% after 3 days (Fig. 2*b*). In addition, fenofibrate inhibited VEGF-stimulated proliferation of human umbilical vein endothelial cells (HUVECs) (data not shown). In contrast to tumor and endothelial cells, PPARα ligands failed to inhibit the proliferation of fibroblasts (foreskin) at doses <50 μM (SI Fig. 7*a*). Moreover, fenofibrate and WY14643 inhibited VEGF-stimulated endothelial cell migration (SI Fig. 7 *b* and *c*). These doses used here are clinically relevant because fibrates in humans readily achieve similar serum levels (22).

To determine whether PPARα ligands could inhibit angiogenesis by down-regulating tumor-secreted growth factors and/or up-regulating endogenous angiogenesis inhibitors, such as thrombospondin (TSP)-1, we measured VEGF, FGF2, and TSP-1 levels in tumor-conditioned media. Fenofibrate and
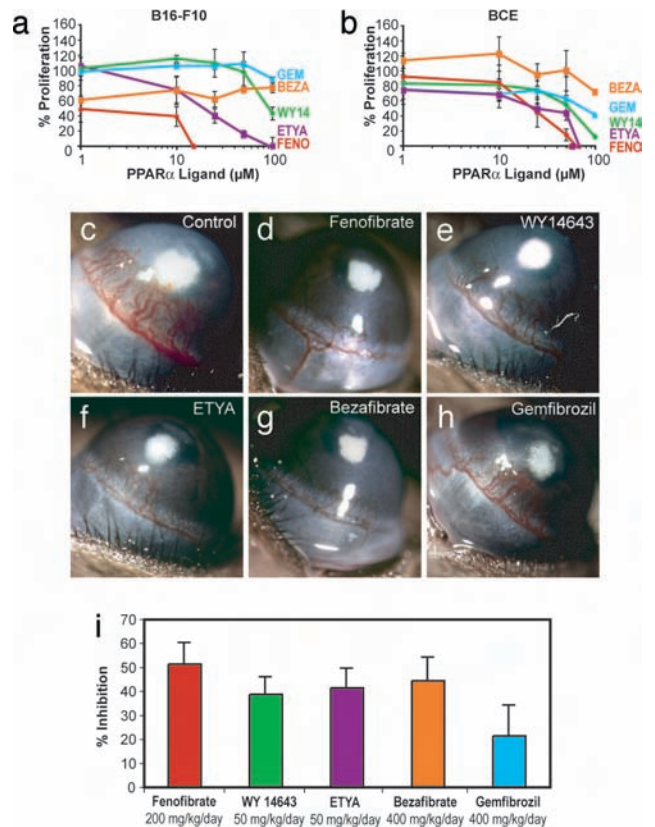
Fig. 2. PPARα ligands have direct antitumor and antiendothelial effects *in vitro* and *in vivo*. (*a*) Percentage of proliferation of tumor cells (B16-F10 melanoma) is determined by comparing cells grown in media plus 10% FBS, and the PPARα ligands, to starved cells. FENO, fenofibrate; WY14, WY14643; BEZA, bezafibrate; GEM, gemfibrozil. (*b*) Percentage of proliferation of BCE cells is determined by comparing cells exposed to an angiogenic stimulus (FGF2) with those exposed to FGF2 and PPARα ligands (fenofibrate, WY14643, gemfibrozil, ETYA, and bezafibrate) relative to unstimulated cells. (*c*) FGF2-induced neovascularization in control cornea on day 6 in a mouse receiving vehicle. (*d–h*) Systemic treatment with fenofibrate at 200 mg/kg per day (*d*), WY14643 at 50 mg/kg per day (*e*), ETYA at 50 mg/kg per day (*f*), bezafibrate at 400 mg/kg per day (*g*), or gemfibrozil at 400 mg/kg per day (*h*). (*i*) Area of inhibition (percentage) by administration of various PPARα ligands: fenofibrate (200 mg/kg per day), 52% inhibition; WY14643 (50 mg/kg per day), 39% inhibition; ETYA (50 mg/kg per day), 42% inhibition; bezafibrate (400 mg/kg per day), 44% inhibition; and gemfibrozil (400 mg/kg per day), 22% inhibition. Inhibition was determined on day 6 by the following formula: pellet distance × 0.2π × neovessel length × clock hours of neovessels. (*n* = 6 eyes per group; the experiment was performed three times.)

WY14643 at 50 μM inhibited VEGF secretion in glioblastoma (U87) cells by 43–55% and in LLC by 51–58% (SI Fig. 8 *a* and *b*). Both PPARα ligands also inhibited FGF2 secretion in K1000 cells (a tumor cell line that expresses and secretes high levels of FGF2) by up to 70% (SI Fig. 8*c*). In addition, fenofibrate also increased the expression of TSP-1 by 3- to 4-fold in HT-1080 fibrosarcoma and in LLC tumor cells (SI Fig. 8*d* and data not shown). Therefore, in addition to their direct antitumor and antiendothelial effects, PPARα ligands may suppress angiogenesis indirectly by inhibiting tumor cell production of VEGF and FGF2 and by increasing TSP-1.

PPARα Ligands Inhibit FGF2-Induced Corneal Neovascularization. To determine the optimal antiangiogenic doses of PPARα ligands for daily administration in mice, we performed the cornea angiogenesis assay in the presence of five different PPARα ligands (Fig. 2 *c–h*). Systemic oral administration of these
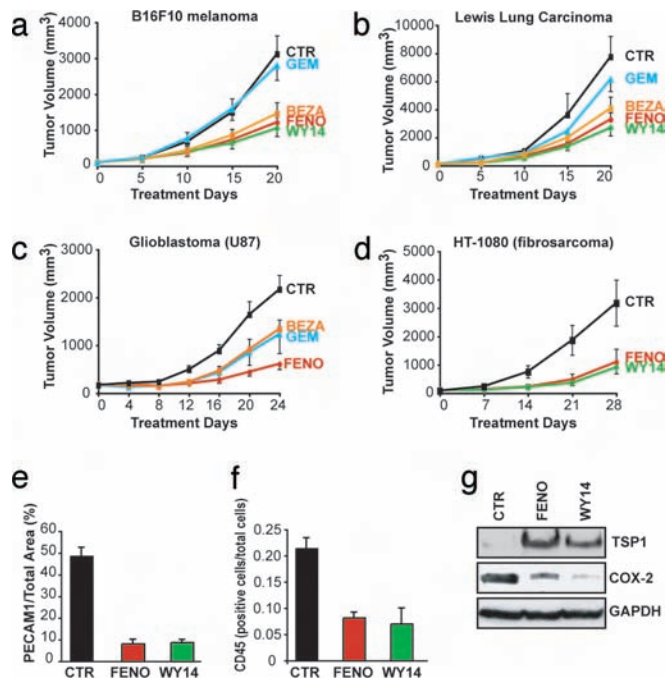
**Fig. 3.** Systemic therapy with PPARα ligands inhibits primary tumor growth. When tumors reached 100 mm³ in size, PPARα ligand treatment was initiated (day 0). On the last day of treatment, the statistical difference between control and treated group was determined by Student's *t* test. The most potent antitumor activity was obtained by fenofibrate and WY14643 at the following doses: fenofibrate, 200 mg/kg per day; WY14643, 50 mg/kg per day; bezafibrate, 200 mg/kg per day; and gemfibrozil, 200 mg/kg per day. (*a*) B16-F10 melanoma (*P* < 0.001). (*b*) LLC (*P* < 0.001). (*c*) Glioblastoma (U87) (*P* < 0.005). (*d*) Fibrosarcoma (HT-1080) (*P* < 0.0001). (*e*) Vessel density in fenofibrate-, WY14643-, and vehicle-treated B16-F10 tumors, as defined by the percentage of vessel area = PECAM1-positive area/tumor area in each field. (*f*) Leukocyte counts per total number of cells per field in fenofibrate-treated and WY14643-treated and vehicle-treated B16-F10 tumors, as determined by CD45 staining. (*g*) Western blot analysis of TSP-1 and COX-2 proteins in tumor lysates of fenofibrate-, WY14643-, and vehicle-treated B16-F10 melanomas on day 20.

PPARα agonists significantly inhibited FGF2-induced corneal angiogenesis by >50% compared with the control (depending on the compound) (Fig. 2*i*).

Systemic Therapy with PPARα Ligands Inhibits Primary Tumor Growth. To determine whether these antiangiogenic effects of PPARα agonists translate to suppression of primary tumors, we treated established s.c. tumors of 100 mm³ with PPARα agonists. Oral fenofibrate (200 mg/kg per day) inhibited B16-F10 melanoma, LLC, glioblastoma (U87), and fibrosarcoma (HT1080) tumor growth by 61%, 58%, 72%, and 66%, respectively, and was more potent than other fibrates, such as bezafibrate and gemfibrozil (Fig. 3 *a–d*). Systemic therapy with WY14643 also inhibited the growth of B16-F10 melanoma, LLC, and fibrosarcoma (HT1080) by 66%, 65%, and 71%, respectively (Fig. 3 *a*, *b*, and *d*). No weight loss or evidence of other drug-related toxicity was observed. Furthermore, no signs of hepatocarcinogenesis were observed in mice treated with PPARα ligands.

Given the *in vitro* evidence for antiangiogenic activity and the known inflammation-modulatory role of PPARα stimulation, we analyzed the tissues of PPARα ligand treated B16-F10 tumors for antiangiogenic and antiinflammatory effects. Fenofibrate and WY14643 treatment reduced vessel density by 83% and 81%, respectively, relative to that in the control tumors (Fig. 3*e* and SI Fig. 9), consistent with the decrease of microvessel density in murine tumor models after treatment with PPARα ligands (13, 14).
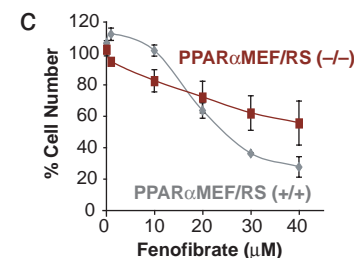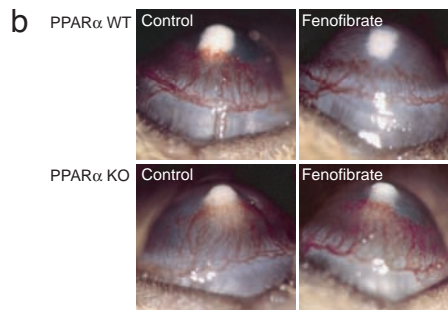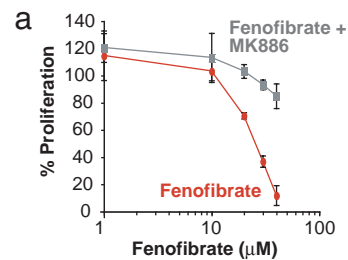
Fig. 4. PPARα ligands have PPARα-dependent and -independent effects. (*a*) Effect of fenofibrate with or without MK886 treatment on the percentage of proliferation on endothelial cells. (*b*) Corneal neovascularization (80 ng of FGF2 pellets) in fenofibrate- and vehicle-treated PPARα WT and PPARα KO mice. (*c*) Effect of fenofibrate treatment on proliferation of PPARα-positive tumor PPARα⁺/⁺MEF/RS and PPARα-negative tumor PPARα⁻/⁻MEF/RS on day 3.

In addition, fenofibrate and WY14643 treated tumors exhibited a dramatic reduction in leukocytes by 62% and 67% (CD45) (Fig. 3*f* and SI Fig. 9). Treatment with PPARα ligands also led to an increase of TSP-1 in B16-F10 tumors (Fig. 3*g*). In contrast, the enzyme COX-2, which is an important mediator of inflammation and also regulates endothelial cell activity (23), was suppressed in both fenofibrate- and WY14643-treated B16-F10 tumors (Fig. 3*g*).

Antiangiogenic and Antitumor Effects of PPARα Ligands Are Specific to the Activation of PPARα. To demonstrate the activation of PPARα in endothelial cells, we measured the kinetics of induction of the medium chain acyl-dehydrogenase (MCAD), a target gene of PPARα, in HUVECs. After 12–24 h of fenofibrate treatment (25 μM), MCAD levels increased in a dose-dependent manner, indicating PPARα activation (data not shown). Furthermore, PPARα ligand-mediated inhibition of FGF2-induced proliferation of bovine capillary endothelial cells was reduced by 90% with the PPARα antagonist MK886 (10 μM; *P* < 0.001) (Fig. 4*a*). In addition, PPARα ligands inhibited corneal neovascularization in PPARα WT (52%) but not in PPARα KO mice (Fig. 4*b*). These findings indicate that the antiangiogenic activity of PPARα ligands specifically depends on activation of PPARα.

To confirm that the suppression of tumor cell proliferation by PPARα agonists was specific to PPARα activation, we examined whether fenofibrate could inhibit the proliferation of PPARα-deficient tumor cells. Therefore, we created a PPARα-negative tumor cell line by transforming mouse embryonic fibroblasts

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(MEFs) derived from PPARα KO mice. Embryonic fibroblasts from PPARα KO and WT mice were transformed with SV40 large T antigen and H-ras, giving rise to two tumorigenic cell lines, PPARα$^{-/-}$MEF/RS and PPARα$^{+/+}$MEF/RS, respectively (SI Fig. 10). Although fenofibrate treatment for 3 days showed 44% dose-dependent inhibition of proliferation of the PPARα$^{-/-}$ cells, suggesting off-target effects, the inhibition in the PPARα$^{+/+}$ was significantly higher ($P < 0.02$), with a maximal proliferation inhibition of 73% (Fig. 4c). Thus, whereas PPARα ligands may have PPARα-independent antiproliferative effects, pronounced inhibition of cell proliferation requires the presence of the nominal PPARα targets.

The Antitumor Activity of PPARα Ligands Depends on Host PPARα Receptors. Our observations suggest a dual effect of PPARα ligands on both endothelial cells and tumor cells. To evaluate the relative importance of host cells versus tumor cells as targets of PPARα ligands, we treated PPARα-positive tumors (PPARα$^{+/+}$MEF/RS) in PPARα WT and KO mice and PPARα-negative tumors (PPARα$^{-/-}$MEF/RS) in PPARα WT mice. The reason we chose the MEF tumor to test the antitumor activity of fenofibrate was that it has shown sufficient growth in the PPARα KO mouse to reveal inhibition by a drug (24). The MEF tumors grew in the PPARα KO mice mainly because they were transfected with two oncogenes. This gave rise to MEF tumors that were capable of inducing angiogenesis over and above the antiangiogenic state imposed by the PPARα KO mice. In contrast, all other tumors remained viable but dormant and did not grow and therefore were not suitable for testing a drug that inhibits tumor growth.

At the stage when the tumors were 100 mm³ (corresponding to 9 days postimplantation in PPARα WT mice and 23 days postimplantation in PPARα KO mice), mice were treated with PPARα ligands or vehicle for 16 days. In PPARα WT mice, fenofibrate and WY14643 inhibited the growth of PPARα-positive tumors by 90–95% (Fig. 5a) and of PPARα negative tumors by 75–90% by day 25 postimplantation (Fig. 5b). The near complete inhibition of tumor growth by PPARα ligands in PPARα-positive and PPARα-negative tumor cells indicates that PPARα in the tumor cell is not the major target of PPARα ligands. Conversely, in PPARα KO mice, fenofibrate and WY14643 failed to significantly inhibit the growth of PPARα$^{+/+}$ tumors (13% and 33%, respectively; Fig. 5c) by day 39 postimplantation. Thus, expression of PPARα in the nontumor host tissue is essential for the antitumor activity of PPARα ligands and is sufficient to mediate the antitumor effects of PPARα agonists even if the tumors lack PPARα (Fig. 5d).

The suppression of tumor growth in the absence of host PPARα has been associated with increased plasma levels of the antiangiogenic protein TSP-1 (24). TSP-1 was not detected in the plasma of WT mice but was present in PPARα KO mice (Fig. 5e). In WT but not PPARα KO mice, fenofibrate induced high levels of TSP-1, consistent with the strong tumor suppression in WT, ligand-treated animals (Fig. 5 a and b). Fenofibrate also induced high levels of endostatin in the plasma of non-tumor-bearing PPARα WT mice. Fenofibrate did not have an effect in PPARα KO mice, which already exhibited elevated basal levels of both TSP-1 and endostatin (Fig. 5 e and f), indicating that these antiangiogenic effects of fenofibrate were PPARα-mediated. In summary, PPARα agonists induced an antiangiogenic state characterized by elevated TSP-1 and endostatin, which is qualitatively similar to the effect of PPARα deficiency.

Discussion

The development of cancer is not simply attributable to the loss of growth control of a single cell clone but rather a developmental disease that involves the tumor cell as well as its interaction with the host tissue. This microenvironment includes
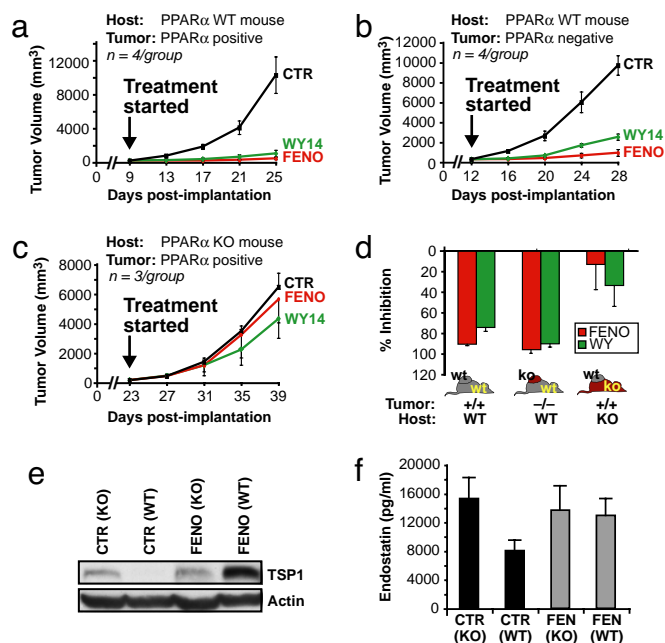
Fig. 5. The antitumor activity of PPARα ligands is host PPARα receptor-dependent. After PPARα-positive (PPARα$^{+/+}$MEF/RS) or PPARα-negative (PPARα$^{-/-}$MEF/RS) tumors were 100 mm³ in size, PPARα ligand treatment was initiated (day 0). The doses were as follows: fenofibrate, 200 mg/kg per day; and WY14643, 50 mg/kg per day. (a) The effect of systemic therapy of PPARα ligands on PPARα-positive tumors (PPARα$^{+/+}$MEF/RS) in PPARα WT mice (90–95% inhibition). (b) The effect of systemic therapy on PPARα-negative tumors (PPARα$^{-/-}$MEF/RS) in PPARα WT mice (75–90% inhibition). (c) The effect of systemic therapy of PPARα-positive tumors (PPARα$^{+/+}$MEF/RS) in PPARα (KO) mice (13–33% inhibition). (d) The columns summarize the effects of fenofibrate and WY14643 in host or tumor cells. (e) Western blot analysis of TSP-1 in plasma from fenofibrate and vehicle treated PPARα WT and PPARα KO mice. (f) Endostatin levels in plasma from fenofibrate- and vehicle-treated PPARα WT and PPARα KO mice.

endothelial cells, inflammatory cells, and other stromal elements. Therefore, targeting the noncancerous host tissue, mainly by antiangiogenesis mechanisms, has emerged as an important opportunity for tumor therapy (25). More recently, modulation of tumor-promoting inflammation in the tumor bed has been proposed as a target for cancer treatment (26).

Here, we report that expression of PPARα in the host tissue is required for PPARα agonists to exert their tumor-suppressing effect. The *in vivo* antitumor effect was not likely mediated by the *in vitro* observed direct antitumor cell activity of PPARα agonists, because in PPARα WT animals, the presence of PPARα in the tumor was not necessary to confer responsiveness to PPARα agonists. In summary, animal studies indicate that expression of PPARα in the nontumor host tissue is necessary and sufficient for the tumor-suppressive effect of PPARα agonists. The host tissue contribution may be local (tumor bed) or systemic. Our analysis suggests that this host-mediated effect of PPARα ligands may be attributable to the inhibition of angiogenesis.

Importantly, the doses of the pharmacological PPARα agonists required for tumor inhibition are in the same range as those used clinically to treat hyperlipidemia (22). PPARα ligands administered at continuous low doses in the diet can suppress tumor and metastatic growth in various experimental tumor models including melanoma, colon, and breast carcinogenesis (11, 27, 28). However, fenofibrate at daily low doses (25 mg/kg or 0.1–0.25%) lacked antitumor activity in primary hamster melanoma (11) and murine endometrial cancer (9). This finding is consistent with our observation that 25 mg/kg of fenofibrate had minimal antitumor and

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antiangiogenic effects (data not shown), whereas 200 mg/kg inhibited angiogenesis and tumor growth.

The antitumor activity of PPARα ligands is primarily PPARα-dependent but may also be mediated by PPARα-independent ("off-target") pathways (22). Here we demonstrated specific PPARα-dependent effects of the nominal PPARα ligands: (*i*) direct activation of the target gene, MCAD, in endothelial cells; (*ii*) inhibition of tumor and endothelial cell proliferation at doses that selectively activate PPARα *in vitro* and that were reversed by a PPARα antagonist; and (*iii*) inhibition of corneal neovascularization in WT but not in PPARα KO mice. Conversely, the presence of PPARα-independent activity was evidenced in the moderate inhibition of proliferation of PPARα-negative cells. However, this effect may not contribute to *in vivo* tumor suppression because the antitumor effect of PPARα agonists was mediated by PPARα expressed in the nontumor host tissue.

In addition to the antiproliferative activity, PPARα ligands such as fenofibrate induce a dose-dependent increase in endothelial cell apoptosis and causes arrest in the cell cycle in the G_0/G_1 phase at higher doses (18). However, even higher PPARα concentrations also can activate PPARγ and/or PPARδ (22). Our studies also show that bezafibrate minimally suppressed the proliferation of endothelial cells yet strongly inhibited corneal neovascularization *in vivo*. Bezafibrate is a pan-PPAR agonist that activates all three nuclear receptors, PPARα, PPARγ, and PPARδ (22). We and others have found that ligand-induced activation of PPARγ inhibits endothelial proliferation and corneal angiogenesis (29). In contrast, activation of PPARδ promotes endothelial proliferation (30). Thus, one possibility to explain the weak antiendothelial activity of bezafibrate *in vitro* compared with its robust antiangiogenic activity *in vivo* is by the relative contribution of each activated PPAR to the overall angiogenic response.

Although our *in vitro* data revealed a potent role of PPARα ligands in the inhibition of tumor cells, endothelial cell proliferation, and angiogenesis, PPARα ligands also have antiinflammatory effects. The antiinflammatory effect of PPARα ligands is mediated notably through inhibition of inducible NOS, COX-2, and TNFα (31). Inflammatory cells present in the tumor play an important tumor-promoting role (26) by secretion of trophic cytokines for tumor cells as well as proangiogenic factors. Suppression of inflammation in tumors may correlate with improved prognosis and growth inhibition. In agreement with the role of inflammatory cells in tumors and the antiinflammatory activity of PPARα agonists, we show that tumor growth suppression caused by PPARα ligands significantly decreased leukocyte expression within the tumor. Moreover, expression of an inflammatory mediator, COX-2, was decreased in the PPARα ligand-treated tumors.

Although the role of PPARα in inflammation has been well characterized, the modulating effect of PPARα on inflammatory processes in the context of tumor growth remains unclear. We recently reported that PPARα KO mice exhibited a significant increase in inflammatory infiltrates in tumors (24), consistent with the role of PPARα in the negative modulation of inflammation (2). However, contrary to the recent notion of inflammation as a tumor promoter (26), this overt inflammation in PPARα KO mice not only failed to support tumor growth but also appeared to actively suppress it. Specifically, the lack of PPARα resulted in an increase of TSP-1 and endostatin levels in plasma and/or tumors, which may explain the observed tumor suppression.

It is counterintuitive that PPARα activation by agonists and genetic abrogation of PPARα in the host would both lead to tumor inhibition. Treatment of PPARα WT mice with a PPARα agonist led to an increase in TSP-1 and endostatin in tumors and/or plasma, producing an antiangiogenic state similar to the PPARα KO mice (24). Two perturbations of PPARα activity in opposite directions both inhibit tumor growth and increase TSP-1 and endostatin levels. This underscores the central role of these angiogenesis inhibitors in the control of tumor growth.

Endostatin induces TSP-1 expression (32). This raises the possibility that these two angiogenesis inhibitors are coordinated.

An analogous paradox of PPARα effect has been described in atherosclerosis. Plaque growth depends on angiogenesis (33). Atherosclerosis is suppressed not only in PPARα KO mice (34) but also in mice treated with fenofibrate (35). In more general terms, these counterintuitive results suggest a biphasic (U-shaped) dose–response curve of host tissue to PPARα activity, as is also observed with PPARγ agonists (29). In other words, very high concentrations or "very low" concentrations of PPARα in the host yield the same outcome: maximal suppression of tumor angiogenesis.

Of interest, clinical evidence suggests that long-term administration of fibrates may reduce melanoma progression. Gemfibrozil-treated patients had a 9-fold decrease in melanoma compared with placebo-treated controls, whereas statin-treated patients had a 1.9-fold reduced incidence of melanoma compared with placebo treated controls (36). Fenofibrate also increased the response rate to retinoids in a human clinical trial for cutaneous T cell lymphoma (37), suggesting that PPARα ligands may potentiate the effect of other anticancer agents.

In conclusion, we provide a mechanistic rationale for extending the clinical use of the well tolerated PPARα agonists to anticancer therapy, and we show their efficacy in tumor treatment in animal models. The antitumor properties of PPARα ligands appear to be mediated primarily by their direct and indirect antiangiogenic effects and their antiinflammatory activity but also by direct antitumor effects. This provides another example for the paradigm of achieving antitumor efficacy through synergistic attack on multiple targets that encompass cell autonomous and non-cell-autonomous mechanisms of cancer growth. Because of their multifaceted effects and excellent safety and tolerability profile after chronic and prolonged exposure, PPARα ligands may be potential tumor-preventative agents. They may be used for maintenance of long-term angiogenesis suppression. Furthermore, our findings support recent studies (11–14) that suggest that PPARα ligands may be ideally suited to complement conventional modalities for cancer treatment. Specifically, because fenofilerate is commercially available, it could be evaluated as an extension of existing multidrug regimens, notably in metronomic (antiangiogenic) chemotherapy schemes (38, 39). However, further research into the pathophysiological role of PPARα and their pharmacological regulators will be paramount to unravel all mechanisms for the antitumor effects of PPARα agonists.

Materials and Methods

Cells and Reagents. Endothelial cells, fibroblasts, and tumor cells were maintained as described (29) in *SI Methods*. Fenofibrate, bezafibrate, and gemfibrozil were obtained from Sigma; WY14643 and ETYA were from Chem-Syn.

Western Blot Analysis. Western blots were performed by using tumor cell lysates collected from plated cells that were 60% confluent. Total protein extracts (30 μg) were analyzed on PVDF membrane blots incubated overnight with rabbit anti-mouse PPARα (Affinity Bioreagents) or rabbit anti-human PPARα (Active Motif). All blots were incubated for 1 h with their corresponding HRP-conjugated secondary antibodies (Amersham Biosciences) and developed with ECL (Pierce). For immunoblotting of TSP and COX-2 (Labvision), the primary antibody was incubated at room temperature for 2 h.

Immunohistochemistry. Sections of tumors were treated with 40 μg/ml proteinase K (Roche Diagnostics) for 25 min at 37°C for PECAM1. PECAM1 was amplified by using tyramide signal amplification direct and indirect kits (NEN Life Science Products). CD45 (BD Biosicences) was detected by using a rat-on-mouse kit (InnoGenex).

Proliferation Assays. Endothelial, fibroblast, and tumor cell proliferation were assayed as described (29). For PPARα antagonist studies, MK886 (Alexis Biochemicals) was used. For proliferation of PPARα-negative and PPARα-positive cells, percentage cell number = $100 \times (cells_{ligand})/(cells_{stimluated})$.

Angiogenesis Assays. Corneal neovascularization assays were performed as described (29). After implantation of 80 ng of FGF-2 into C57Bl6, PPARα WT, and PPARα KO mice, PPARα ligands were administered over 6 days by gavage in an aqueous solution of 10% DMSO in 0.5% methylcellulose, whereas control mice received vehicle. Tumor cells were injected s.c. (1×10^6 cells in 0.1 ml of PBS) into C57BL/6, SCID, PPARα WT, or PPARα KO mice (The Jackson Laboratory). Once tumors reached 100 mm³, PPARα ligands were administered by daily gavage for 20–28 days. Tumor volume was calculated as width squared × length × 0.52.

Statistical Analysis. The Student's paired t test was used to analyze the difference between the two groups. Values were considered significant at $P < 0.05$.

Note. During the finalization of this article, Pozzi *et al.* (14) published a report in which they showed PPARα-mediated inhibition of angiogenesis and tumor growth. Their findings, although by using a different tumor model and focusing on a single agonist, WY14643, are consistent with ours and confirm the important role of PPARα in tumor suppression.

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