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DIETER MARMÉ
EDITOR

Tumor Angiogenesis

A Key Target for Cancer Therapy

Springer

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A Key Target for Cancer Therapy

With 63 Figures and 42 Tables

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This work is dedicated to my grandchildren Ann-Sophie, Aurélie, and Mathis

Foreword

It is now almost 50 years since Judah Folkman published in 1971 the legendary hypothesis paper in the *New England Journal of Medicine* that started the field of modern angiogenesis research. Folkman predicted the existence of a *tumor angiogenesis factor* (TAF) that would induce the growth of blood vessels in tumors and whose blockade would lead to the regression of growing tumors to maintain them in dormant state at microscopic size. Many angiogenic growth factors have been identified since then, but if any one factor comes close to being TAF, it is the vascular endothelial growth factor (VEGF) family of molecules, most notably VEGF-A. VEGF-A stands high up in the hierarchy of events that induce the sprouting of capillaries from existing microvessels. Discovered by Napoleone Ferrara in 1989, it took 15 years from target discovery to clinical approval of the first VEGF blocking drug, the neutralizing antibody Bevacizumab. Bevacizumab and several other VEGF blocking drugs as well as a panel of VEGF receptor neutralizing or blocking drugs are now in clinical use in the fields of oncology and ophthalmology. With several block-buster drugs generating revenues of more than 1 billion USD per year, the total market of anti-angiogenic drugs is well exceeding 10 billion USD. Anti-angiogenic tumor therapy has thereby become the first clinically effective anti-stroma therapy part of standard tumor therapy. Yet, it clearly did not live up to the high public expectations that anti-angiogenic tumor therapies were expected to exert around the turn of the century. Instead, the overall therapeutic benefit is modest resulting in an increase of patients' overall survival in the range of week to months (e.g., from 15.6 to 20.3 months for patient with metastatic colorectal cancers). Still, in terms of percentage, this reflects a highly significant increase in overall survival of 25%.

Many textbooks on tumor angiogenesis have been published during the last decades probably raising the question why another textbook is needed when the major questions in the field of angiogenesis research have apparently all been answered. Well, have they? Probably not, because key questions remain unanswered. The mechanisms of action of anti-angiogenic tumor therapy (i.e., regression vs. normalization) are to this day not well understood, particularly in human tumors. No stratifying procedures have been developed that would be capable of predicting the response to anti-angiogenic therapy. The advent of immuno-oncology (IO) drugs raises a whole plethora of questions on the rationale combination of anti-angiogenic drugs and IO drugs (stromal reprogramming to improve the efficacy of IO drugs). Most importantly, has

the full potential of anti-angiogenic therapy already been exploited? Combinations of VEGF-targeting and angiopoietin-targeting drugs, in recent years, did not yield the anticipated synergistic effects. Still, there may be room for the development of novel combinatorial drugs targeting vascular sprouting and vascular maturation.

Given all these enigmatic questions, the book *Tumor Angiogenesis: A Key Target for Cancer Therapy*, edited by Dieter Marmé, serves as a timely update on a research field that may still hold many surprises in terms of biological mechanisms and phenomena as well as translational window of opportunity. More than 30 chapters cover, on the one hand, all relevant molecular and mechanistic aspects and zoom in, on the other hand, on the specific organ attributes of angiogenesis and anti-angiogenic therapy in 15 different tumor entities. It is this combination that will make the book a useful resource for the experienced and newcomer basic scientist in the field as well as for clinical scientists interested in specific tumor entities. The future will unravel the potential of vascular targeted therapies in oncology and beyond. But in order to get there, much needs to be learned about organ-specific functions of organotypically differentiated blood vessels in health and diseases. *Tumor Angiogenesis* may serve as a most welcome alert that indeed not all questions have been answered and likewise that the full potential of anti-angiogenic therapy has not yet been exploited.

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Hellmut G. Augustin

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Contents

Part I Mechanisms of Tumor Angiogenesis **1**

Mechanisms of Tumor Angiogenesis 3
Iratxe Zuazo-Gaztelu and Oriol Casanovas

The Role of VEGF in Controlling Vascular Permeability 33
Lakshmi Venkatraman and Lena Claesson-Welsh

Benefits and Pitfalls of Tumor Vessel Normalization 51
Jin-Sung Park, Intae Park, and Gou Young Koh

The Impact of Endothelial Transcription Factors in Sprouting Angiogenesis 73
Melanie Hofmann and Joerg Heineke

Endothelial Cell-Cell Junctions in Tumor Angiogenesis 91
Quentin Roux and Julie Gavard

Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis 121
Inga Bekes and Christine Wulff

Part II Mechanisms of Tumor Lymphangiogenesis **133**

Angiopoietins and TIE Receptors in Lymphangiogenesis and Tumor Metastasis 135
Yulong He

Significance and Molecular Regulation of Lymphangiogenesis in Cancer 157
Mihaela Skobe and Bronislaw Pytowski

Part III Mechanisms of Anti-angiogenic Therapy **181**

Mechanisms of Anti-angiogenic Therapy 183
Roser Pons-Cursach and Oriol Casanovas

Part IV Anti-angiogenic Targets **209**

The Role of the VEGF Signaling Pathway in Tumor Angiogenesis 211
Napoleone Ferrara

Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors 227
Elina A. Kiss and Pipsa Saharinen

Part V Pathology of Tumor Angiogenesis **251**

Pathology of Tumor Angiogenesis 253
Peter Bronsert and Martin Werner

Part VI Imaging of Tumor Angiogenesis **275**

Imaging Tumor Angiogenesis 277
Gordon Jayson and James O'Connor

Part VII Biomarkers for Anti-angiogenic Therapy **291**

Biomarkers for Anti-angiogenic Therapy 293
Weibin Hou and Stefan Duensing

Part VIII Mechanisms of Resistance in Anti-angiogenic Therapy **311**

Anti-angiogenic Cancer Therapy: Development of Resistance 313
Domenico Ribatti

Part IX Mechanisms of Synergy in Combinations of Anti-angiogenics and Other Targeted Therapies **325**

Cytotoxics and Anti-angiogenics: Metronomic Therapies 327
Andreas Pircher, Normann Steiner, and Eberhard Gunsilius

Anti-angiogenics and Radiation Therapy 349
Daniel H. Schanne, Anca-L. Grosu, and Dan G. Duda

Combination of Anti-angiogenics and Other Targeted Therapies 359
Katja Zirlik and Justus Duyster

Part X Anti-angiogenics in Gastrointestinal Cancers **377**

Anti-angiogenic Targeting in Metastatic Colorectal Cancer Therapy 379
Thomas Zander, Valentin Goede, and Michael Hallek

Anti-angiogenics in Gastroesophageal Cancer 395
Ulrich Hacker and Florian Lordick

Anti-angiogenics in Pancreatic Cancer Therapy 415
Thilo Hackert, Laura Wüsten, and Markus W. Büchler

Anti-angiogenics in Hepatocellular Cancer Therapy 435
Martha M. Kirstein and Arndt Vogel

Part XI Anti-angiogenics in GIST **447**

Inhibition of Tumor Angiogenesis in GIST Therapy 449
Charlotte Benson and Michela Libertini

Part XII Anti-angiogenics in Other Soft Tissue Sarcomas **463**

The Value of Anti-angiogenics in Soft Tissue Sarcoma Therapy 465
Bernd Kasper and Charlotte Benson

Part XIII Anti-angiogenics in Head and Neck Cancer **475**

The Value of Anti-angiogenics in Head and Neck Cancer Therapy 477
Valérie Cristina and Sandrine Faivre

Part XIV Anti-angiogenics in Lung Cancer **495**

Inhibition of Tumor Angiogenesis in the Treatment of Lung Cancer 497
Massimo Di Maio, Silvia Novello, Enrica Capelletto, and Giorgio Vittorio Scagliotti

Part XV Anti-angiogenics in Gynecological Tumors **513**

The Value of Anti-angiogenics in Breast Cancer Therapy 515
Malgorzata Banys-Paluchowski, Tanja Fehm, and Volkmar Müller

The Value of Anti-angiogenics in Ovarian Cancer Therapy 529
Sven Mahner and Fabian Trillsch

The Value of Anti-angiogenics in Cervical Cancer Therapy 545
Thalia Erbes, Juliane Farthmann, and Kai Berner

Part XVI Anti-angiogenics in Urological Tumor Therapy **567**

Anti-angiogenics in Kidney Cancer Therapy 569
Herbert Rübben and Andrej Panic

The Value of Anti-angiogenics in Prostate Cancer Therapy 579
Krisztian Homicsko and Dominik Berthold

The Value of Anti-angiogenics in Bladder Cancer Therapy 593
Gerald Bastian Schulz and Alexander Karl

Part XVII Anti-angiogenics in Brain Tumors **607**

The Value of Anti-angiogenics in Primary Brain Tumor Therapy 609
E. Schorb and C. F. Waller

Anti-angiogenics in Brain Metastases: Perspectives and Experiences 627
Frank Winkler

Part XVIII Anti-angiogenics in Multiple Myeloma **637**

The Value of Anti-angiogenics in Multiple Myeloma Therapy 639
Thomas Moehler, Dirk Hose, Mindaugas Andrulis, Anja Seckinger, and Hartmut Goldschmidt

Part XIX Anti-angiogenics in Human Metabolism **659**

The Implication of Anti-angiogenic Treatment of Malignancies on Human Metabolism 661
Nina Obad and Rolf Bjerkvig

Index 673

About the Editor

Dieter Marmé is renowned for his scientific work on molecular biology and tumor biology, and in particular for research into the roles of growth factors in the mechanisms by which tumor angiogenesis is initiated and propagated. At the time of his retirement in 2005, he had spent more than two decades at the Tumor Biology Center in Freiburg, Germany. He initially joined the Center as Director of the Institute of Molecular Medicine in 1993 and later served as its Director of Research and Chairman of the Board of Directors. Prior to joining the Tumor Biology Center, Dr. Marmé had been an Extraordinary Professor in the Department of Genetics and Molecular Biology at the University of Freiburg and had then spent a decade working for Gödecke, a division of Parke-Davis (now Pfizer), initially as Head of Pharmacological Biochemistry and later as Head of Biological Research. Based on the understanding of the mechanisms by which tumors progress and tumor angiogenesis is initiated, he focused on the investigation of therapeutic concepts to interfere with the development of malignancies. After retiring from the Tumor Biology Center in 2005, he continued to work for the institution as an Adviser in Oncology and at the same time became a member of the editorial board for various journals in the field of oncology and a consultant for pharmaceutical companies. Dr. Marmé is the coeditor of the previous Springer book *Tumor Angiogenesis: Basic Mechanisms and Cancer Therapy* (2008).

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Part I

Mechanisms of Tumor Angiogenesis

Mechanisms of Tumor Angiogenesis

Iratxe Zuazo-Gaztelu and Oriol Casanovas

Contents

Introduction 4

Molecular Mechanisms of Angiogenesis 4
Mechanisms Involved in Blood Vessel Formation 4
Biological Processes Involved in Angiogenesis 8

The Angiogenic Switch in Tumorigenesis 20
Hypoxia and Tumor Angiogenesis 21
Differences between Physiological and Tumor Neovascularization 23

Conclusion 25

Cross-References 26

References 26

Abstract

Tumor angiogenesis, the process by which blood vessels penetrate and grow in the tumor microenvironment, is essential for oxygen and nutrient supply and hence constitutes a key player for the survival of solid neoplasms. Different mechanisms of angiogenesis are developed during tumor progression such as vasculogenesis, sprouting angiogenesis, intussusception, and vasculogenic mimicry. The transition from a quiescent vasculature to an actively growing one follows a series of synchronic events and is finely tuned by a wide array of molecules and positive and negative regulators of angiogenesis. Beginning with blood vessel sprouting and endothelial cell proliferation, followed by vessel navigation, remodeling, stabilization, and maturation, and finishing with blood vessel regression, the main molecular factors involved in the progression of each step are profoundly detailed. When the balance between positive and negative regulators of angiogenesis is shifted toward proangiogenic molecules, the quiescent vasculature becomes activated and initiates the angiogenic state of tumor development. The role of intratumoral hypoxia as a potent activator of the angiogenic switch, its regulation, and a detailed description of normal and aberrant tumor vessels are also provided. The understanding of the foundations of these mechanisms is crucial for an effective therapeutic targeting of the angiogenic process.

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Introduction

Tumor angiogenesis, the process by which blood vessels penetrate and grow in the tumor microenvironment, is essential for oxygen and nutrient supply and, therefore, for survival of solid neoplasms. Taking into account its role in tumor progression and metastasis, angiogenesis is one of the widely acknowledged hallmarks of cancer (Hanahan and Weinberg 2011). Since the pioneering studies by Folkman established the initial foundations of tumor angiogenesis nearly 40 years ago, research in the field has reached a significant level of maturity, allowing detailed description of the intricate processes of pathological vessel proliferation (Folkman et al. 1971). In this chapter, we compiled the basic mechanisms of blood vessel formation and the biological roles of the main molecular regulators involved in tumor angiogenesis. Furthermore, we describe in detail the "angiogenic switch" occurring in malignant neoplasms, focusing on the role of the complex tumor microenvironment, the differences between physiological and pathological angiogenesis, and the abnormalities found in tumor vasculature.

Molecular Mechanisms of Angiogenesis

Mechanisms Involved in Blood Vessel Formation

Vessels can be described as highly branched and ordered tubular networks that allow transport of gases, nutrients, signaling molecules, and cells. Beyond their nutritive function, blood vessels provide instructive trophic signals essential for organ morphogenesis and the development of every complex organism (Carmeliet and Jain 2011). While the luminal side of all types of blood vessels, including arteries, veins, and capillaries, is formed by a lined monolayer of endothelial cells, vessels are covered in the outside by a basement membrane followed by a layer of mural accessory cells (pericytes and vascular smooth muscle cells (SMCs)).

Prototypically, vasculogenesis and sprouting angiogenesis are the two main mechanisms responsible for neovascularization (Fig. 1a, b). Sprouting angiogenesis is defined as the formation of new vascular structures from a preexisting vessel, while vasculogenesis refers to de novo blood vessel formation due to vascular progenitor cell differentiation. Both mechanisms contribute to the formation and remodeling of the vessel network during development, remain nearly inactive in the adult body, and are only reactivated to allow tissue repair or in the event of a disease.

Beyond vasculogenesis and sprouting angiogenesis, other less frequent mechanisms have recently been reported in neoplasms, including vessel co-option, intussusception, and vasculogenic mimicry (Fig. 1c, d). In most cases, mutual exclusivity between different mechanisms does not exist; indeed, they simultaneously participate both in physiological and pathological angiogenesis.

Vasculogenesis

Vasculogenesis (Fig. 1a) has been extensively described in the early stages of vascular development. It was not until 1997 that the growth of new blood vessels in postembryonic tissues was considered to occur also through vasculogenesis (Asahara et al. 1997). Moreover, compelling evidence suggests that bone marrow-derived circulating endothelial progenitor cells (EPCs) contribute to the induction and progression of postnatal neovascularization (Kopp et al. 2006). EPCs, also known as angioblasts, are circulating cells that express several endothelial lineage-specific markers such as CD34, CD31, VEGFR-2, and Tie-2.

Together with EPCs, mature circulating endothelial cells derived from blood vessel renewal

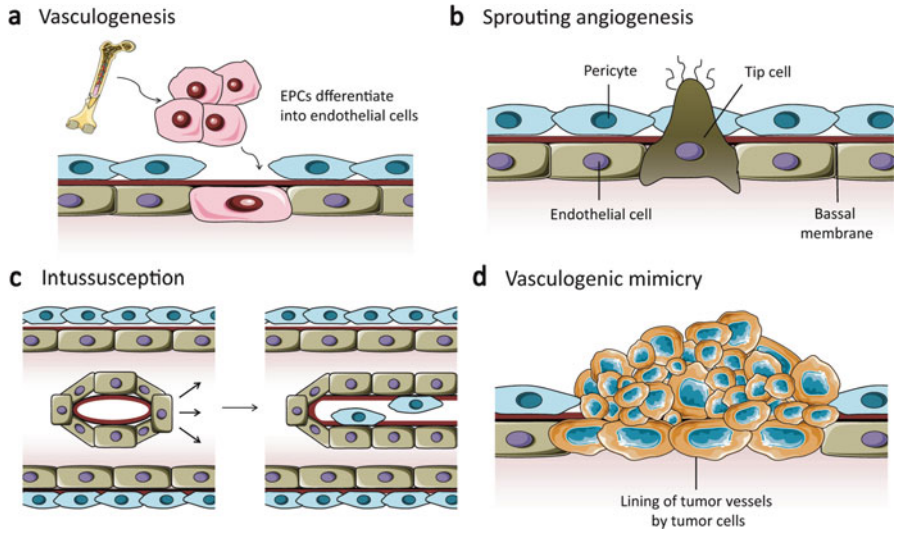

Fig. 1 Mechanisms involved in blood vessel formation. In normal tissues vessels can grow by the recruitment of bone marrow-derived endothelial progenitor cells (EPCs) that differentiate into endothelial cells (**a**), by sprouting angiogenesis (**b**), or by intussusceptive microvascular growth, a process that involves vessel splitting (**c**). Additionally, tumor cells use other mechanisms such as vasculogenic mimicry, during which vessel-like structures are lined by tumor cells (**d**)

also take part in adult vasculogenesis. Moreover, in order to facilitate incorporation of those circulating endothelial adult and progenitor cells and to sustain the stability of the nascent vasculature, hematopoietic stem and progenitor cells are recruited (Kopp et al. 2005). Several chemokines, cytokines, and growth factors that are produced in response to tissue ischemia and tumor growth promote mobilization and recruitment of EPCs. For instance, tumor cells produce proangiogenic factors, such as VEGF, and cytokines (i.e., stromal-derived factor-1) that recruit bone marrow-derived dendritic cells and promote their proliferation and differentiation. The precise mechanisms governing mobilization of precursor cells from the bone marrow and their posterior homing to neo-angiogenic spots are yet not fully understood.

Mobilization of EPCs starts following the activation of the matrix metalloprotease 9 (MMP9) in the osteoblastic zone by the tumor-derived proangiogenic factors. The activation of MMP9 triggers the proteolytic processing of membrane-bound Kit ligand to its soluble active form. The soluble Kit ligand is a stem cell-active cytokine that promotes migration of hematopoietic and endothelial progenitor cells to the vascular zone of the bone marrow and their posterior release in the circulation (Heissig et al. 2002).

When homed, endothelial progenitor cells can be incorporated into the endothelial monolayer of a vessel or recruited to angiogenic sprouts. At this step, P-selectin, E-selectin, and integrins are critical for the correct adhesion of EPCs to the vessel walls (Deb et al. 2004). Differentiation into mature endothelial cells is mainly mediated by VEGF and physically contributes to vessel size since it increases the diameter of the vessel. In addition to the physical contribution of the EPCs to the newly formed vessels, EPCs support angiogenesis by a paracrine mechanism that includes the release of proangiogenic factors in neovascularization sites of the tumor stroma or ischemic tissues (Urbich and Dimmeler 2004).

Formal demonstration of the contribution of vasculogenesis to tumor angiogenesis has been achieved through the use of knockout mice for inhibitors of differentiation factors. These factors initiate the mobilization of bone marrow-derived dendritic cells to angiogenic sites in the tumor. Genetic ablation of inhibitors of differentiation factors disrupted tumor vascularization and

blocked tumor growing as a consequence of an impaired angiogenesis (Benezra et al. 2001). The restoration of the mobilization capability of bone marrow-derived cells by transplantation of wild-type bone marrow rescued tumor neovascularization and growth in these knockout mice.

The contribution of vasculogenesis to tumor vessel formation ranges from 0.1 to 50% depending on the experimental cancer model and tumor type. Lymphomas and hematological tumors are more dependent on bone marrow-derived dendritic cells in comparison to other tumors. The knowledge obtained from the study of tumor vasculogenesis has sparkled the development of new applications at the clinical setting. The potential existing correlation between the levels of endothelial progenitor and circulating cells in blood and the outcome in patients undergoing an anti-angiogenic treatment could be used as a cellular biomarker for monitoring the response to antitumor therapy (Bertolini et al. 2003).

Recently, the study of tumor vasculogenesis has shifted from its role in primary tumor growth toward the study of its implication in dissemination and metastasis. In addition to angiogenesis activation, EPCs are able to promote metastatic growth by homing into metastatic sites prior to tumor cell arrival (Kopp et al. 2005). The spontaneous secretion of SDF-1 by EPCs generates a gradient that could promote the extravasation and development of the pre-metastatic niche (Jin et al. 2012).

Sprouting Angiogenesis

Sprouting angiogenesis is the best described mechanism used by tumors to promote their own vascularization by inducing new capillary sprouts from preexisting host capillaries (Fig. 1b). The mechanism involves several well-defined sequential steps and an extensive interplay between soluble factors, extracellular matrix (ECM) components, and cells (Paku and Paweletz 1991). At the onset of sprouting angiogenesis, there is a destabilization of the endothelial-pericyte contacts which are essential for vessel integrity and quiescence maintenance. Endothelial and mural cells share a complex basement membrane that forms a protective coat around endothelial tubules, preventing resident endothelial cells from leaving their location. Once destabilized, endothelial cells undergo an endothelial-mesenchymal transition that enhances their migratory, invasive, and proliferative properties. These activated cells are then able to degrade the surrounding ECM and the basement membrane by activated proteases (such as MMPs), opening the path for guided migration and proliferation. Vessel lumen is then formed by polarization of the migrating endothelial cells (Ferrara et al. 2003). At this step, an immature blood vessel is formed, and the opposite mesenchymal-endothelial transition directs the reversal of the proliferative state of endothelial cells to the previous resting state. In detail, the return to quiescence is retrieved by synthesis of new basement membrane and pericyte and mural cell recruitment (Jain 2003). This latter step is known as vessel maturation and is characterized by a lack of tumor angiogenesis.

Recent studies have remarked the specialization that endothelial cells undergo in order to enroll the angiogenic process. To achieve locally needed vascular patterns, the multistep process of sprouting angiogenesis requires functional specialization of endothelial cells in the angiogenic sprout together with vascular guidance cues that allow regulation of the topological extension of the forming vessel. The main group of signaling pathways essential for the initial morphogenetic events includes VEGF and Notch (Iruela-Arispe and Dvorak 1997). Attracted by proangiogenic signals, the phenotype of the sprouting endothelial cells gains an invasive and motile behavior, protease activation, cell-cell contact remodeling, and apical-basal polarity reversal. The endothelial cells that are selected to guide the sprouting are located at the tip of the angiogenic sprout and are, therefore, commonly known as “tip cells.” These leading cells respond to VEGF signaling by dynamically extending large filopodia in order to sense and guide the forming vessel along the forthcoming vascular bed. Recent studies show similarities between the molecular regulation of guidance cues of neural and endothelial cells.

These specialized nonproliferative endothelial cells also release different molecular signals that promote the recruitment of mural cells like pericytes, SMCs, and fibroblasts, guaranteeing the stabilization of emerging vessels.

Apart from tip cells, endothelial cells can specialize to highly proliferating cells located in the stalk of the angiogenic sprout. The proliferative potential of the "stalk cells," capable of forming tubes and branches, assures the expansion of the structure. In contrast to tip cells, stalk cells extend fewer filopodia but proliferate to support vessel elongation in response to VEGF-A (Gerhardt et al. 2003). Tip cells are migratory and polarized, whereas stalk cells proliferate during the extension of the sprout and form the new vascular lumen cell population. Stalk cells also produce components of the basement membrane and establish adherens and tight junctions with neighboring cells, thus strengthening the integrity of the new sprout and luminal-abluminal polarity (Dejana et al. 2009).

In order to form a new vascular connection, the tip cell phenotype must be switched off after connecting with the tips of other sprouts or existing vessels. Tip cells build vessel loops by anastomosing with cells from neighboring sprouts. The sprouting process is rehearsed until proangiogenic signals decrease, a new basement membrane is formed, quiescence is reestablished, and VEGF levels dampen (Leslie et al. 2007). In the transition from active sprouting to quiescence, endothelial tip cells adopt a "phalanx"-like phenotype, with features of lumenized, nonproliferative, and immobile cells (Bautch 2009). Eventually, maturity and stabilization are achieved through the generation of a lumen and the migration of pericytes along the basement membrane until vessels are covered, initiating blood flow and allowing perfusion.

The correct extension and morphology of the nascent vessels is regulated by the precarious balance between tip cell navigation and stalk cell proliferation. The phenotypic specialization to tip or stalk cell depends on the balance between proangiogenic factors and endothelial cell proliferation suppressors (Geudens and Gerhardt 2011). The abnormal vascular structures generally found in cancer are a consequence of the imbalance between these two processes. The biological nature of the molecules and signals that initiate the angiogenic cascade from the initial destabilization to the formation of mature and functional vasculature has been profoundly studied and characterized and is further described in the following sections of this chapter.

Alternative Ways of Blood Supply in Tumors

Although sprouting angiogenesis is regarded as the most important contributing mechanism to tumor angiogenesis, there are alternative processes such as intussusceptive microvascular growth (IMG; Fig. 1c) and vasculogenic mimicry (VM; Fig. 1d). These nonconventional pathways introduce an additional level of complexity to the understanding of tumor vascularization mechanisms.

Intussusception

Intussusception (IMG) is a variant of angiogenesis that was first observed in postnatal remodeling of lung capillaries (Caduff et al. 1986; Fig. 1c). This developmental intravascular growth mechanism is based on the splitting of preexisting vessels into two new vessels after the formation of a transvascular connective tissue column, called tissue pillar, into the lumen of the vessel.

In contrast to sprouting angiogenesis, IMG is a fast process that can occur within hours, or even minutes, since it does not require proliferation of endothelial cells. Even though sprouting has the advantage of being invasive and permits joining vascular gaps, it is a slow process that highly relies on endothelial cell proliferation and basal membrane degradation. In IMG, the remodeling of endothelial cells is a consequence of their volume increase and narrowing. It is believed that IMG happens after vasculogenesis or sprouting angiogenesis in order to expand the capillary plexus without a high metabolic demand (Burri et al. 2004).

The onset of IMG is the "touching contact" of endothelial cells from opposite walls. Following the transendothelial cell bridge created from the touching spot, interendothelial junctions are

reorganized, and the endothelial bilayer is performed. Then, the interstitial pillar forms to reinforce the bridges, and mural cells are recruited to cover this new interstitial wall. Due to their contractile features, pericytes are believed to be the main triggers of this phase. Finally, the interstitial pillars widen, endothelial cells retract, and two independent vessels are created (Burri et al. 2004). By IMG, a large vessel can split into smaller vessels.

Even though the precise mechanism of intussusception is poorly understood, there are some key mediators that influence pillar formation. Alterations in blood flow dynamics, changes in shear stress on endothelial cells sensed and transduced by molecules such as CD31, modifications of wall stress on the pericytes, and absences of VEGF are some among the possible factors driving biochemical cascades that result in cytoskeletal rearrangements and intussusception initiation (Djonov and Makanya 2005). For instance, it was observed that human melanomas bare a high number of intraluminal tissue folds together and that a correlation between VEGF and intussusceptive angiogenesis exists in these tumors (Ribatti et al. 2005). In this context, sprouting angiogenesis inhibition might stimulate the process of intussusceptive angiogenesis. Since IMG can only occur on existing vessel networks, its most important contribution is its ability to increase the density and complexity of tumor microvessel networks already established by sprouting angiogenesis. Moreover, IMG also provides additional surface for further sprouting angiogenesis. IMG has been observed in colorectal, melanoma, and mammary tumors (Dome et al. 2007).

Vasculogenic Mimicry

Vasculogenic mimicry (VM; Fig. 1d) describes the ability of some tumor cells to dedifferentiate into multiple cellular phenotypes, obtaining endothelial-like properties (Maniotis et al. 1999). This process leads to the creation of de novo vasculogenic-like matrix embedded networks. The new perfusable vascular-like structures are composed of red blood cells and plasma and contribute to blood circulation (Frenkel et al. 2008). Endothelial cells undergoing VM mimic the pattern of embryonic vascular network, possibly providing tumor cells with a secondary circulation system, independent from angiogenesis.

Molecular analysis comparing highly invasive and noninvasive melanoma cells derived from the same patient suggests a genetic reversion of the aggressive cells to an embryonic-like cell fate and increased cell plasticity. The undifferentiated phenotype includes the expression of endothelium-associated genes such as VE-cadherin and Ephrin-A2, among others (Hendrix et al. 2003). The activation of transmembrane metalloproteinases, release of ECM components, and low levels of oxygen are known to promote VM (Seftor et al. 2005). Although the exact mechanism remains to be unraveled, it involves deregulation of the lineage-specific phenotype and the concomitant transdifferentiation to endothelial-like cells.

VM occurs mainly in aggressive tumors such as melanomas, and even though their occurrence is relatively rare within tumors, the presence of VM-associated patterned networks in tumor tissue correlates with an increased risk of metastasis and poor clinical outcome (Sun et al. 2004). Until now, VM has been described in melanomas, breast carcinoma, prostatic carcinoma, hepatocellular carcinoma, bladder carcinoma, and other aggressive tumors. Increasing evidence demonstrates that tumor cell-dominant VM has a key role in tumor progression and metastasis.

Biological Processes Involved in Angiogenesis

As stated above, in order to build new and fully functional vascular structures, several biological processes must be accurately regulated. Different pro- and anti-angiogenic regulators are needed to perform and control each specific step of the angiogenic cascade. Multifunctionality among those factors is one of their relatively common features, empowering some molecules with the outstanding ability to either activate or inhibit vascularization. On account of simplification, we will describe the molecular regulation of the main biological processes involved in vessel formation:

sprouting and proliferation (Fig. 2a), guidance and navigation (Fig. 2b), stabilization, maturation, and remodeling (Fig. 2c), and regression.

Sprouting of Blood Vessels and Endothelial Cell Proliferation

Endothelial cells in the adult organism remain quiescent and are protected against external insults by autocrine maintenance signals. These cells form a monolayer of phalanx cells and are interconnected by junctional molecules like VE-cadherin and claudins. The surface of the endothelium monolayer is covered by pericytes, which suppress endothelial cell proliferation, and releases pro-survival signals such as VEGF and Ang-1. When quiescent vessels sense an angiogenic signal such as VEGF, fibroblast growth factors (FGFs), or chemokines, released by hypoxic, inflammatory, or tumor cells, sprouting angiogenesis is triggered (Fig. 2a).

VEGF-VEGFR signaling pathway is established as the master regulator of the formation and remodeling of vasculature. VEGF ligands are the prototypical, multifunctional pro-angiogenic factors that control endothelial cell proliferation and migration and regulate cardiovascular system homeostasis (Carmeliet and Jain 2011). Until now, VEGF molecules are allegedly the most potent vascular permeability factors and vasodilatation inductors. Besides, endothelial precursor cell differentiation and vascular guidance of tip cells are also controlled by VEGF family.

The VEGF family is composed of six different members: VEGF-A (referred herein as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). Contrary to other angiogenic superfamilies, the VEGF family distinguishes itself by the nonredundant role of its members. At least five different isoforms of VEGF are generated by alternative splicing of a single gene: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. These isoforms differ in their binding affinity for heparin, which might affect their diffusion rates in the extracellular space. The predominant splicing variant of VEGF both in normal and tumor cells is the $VEGF_{165}$ isoform. All the members of the VEGF family and their corresponding receptors usually work as homodimers, although heterodimers between different members have also been reported (DiSalvo et al. 1995).

During tumor progression, VEGF levels are controlled through diverse mechanisms such as hypoxia, oncogene activation, loss of tumor suppressors, cytokines, and growth factors levels. There is also an autocrine production of VEGF by endothelial cells which is critical for vascular homeostasis and early stages of vascular development (Lee et al. 2007). In general terms, paracrine VEGF, released by tumor or stromal cells, increases vessel branching and promotes tumor vessel abnormalities (Stockmann et al. 2008), whereas autocrine VEGF, released by endothelial cells, empowers vascular homeostasis. The deletion of a single allele of VEGF-A causes embryonic lethality, reinforcing the believed key role of this family in developmental vascular physiology.

There are three different tyrosine kinase-type receptors for the VEGF family: VEGFR-1 (or Flt1 in mouse), VEGFR-2 (also known as kinase insert domain-containing receptor in humans or Flk1), and VEGFR-3 (or Flt4). These receptors are principally expressed in endothelial cells (Roskoski 2008). Furthermore, certain VEGF isoforms are also able to bind to non-tyrosine kinase coreceptors such as neuropilins 1 and 2 to enhance VEGFR-2 activity. Neuropilins are best known for their interaction with semaphorin and their angiogenesis-independent function in axonal guidance (Gluzman-Poltorak et al. 2000).

VEGFR-1 has been identified as the high affinity receptor, whereas VEGFR-2 is the low-affinity receptor. While placental growth factor and VEGF-B bind to VEGFR-1, VEGF-A can bind both VEGFR-1 and VEGFR-2, VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3, and VEGF-E binds to VEGFR-2. Binding of the ligand leads to the dimerization of the receptors; this in turn initiates the autophosphorylation of several intracellularly located tyrosine residues. The activated dimers now expose new docking sites for the recruitment of different types of intermediary signaling molecules by protein-protein interactions through specific SH2 and SH3 domains. These large signaling complexes are known as signalosomes and can be different

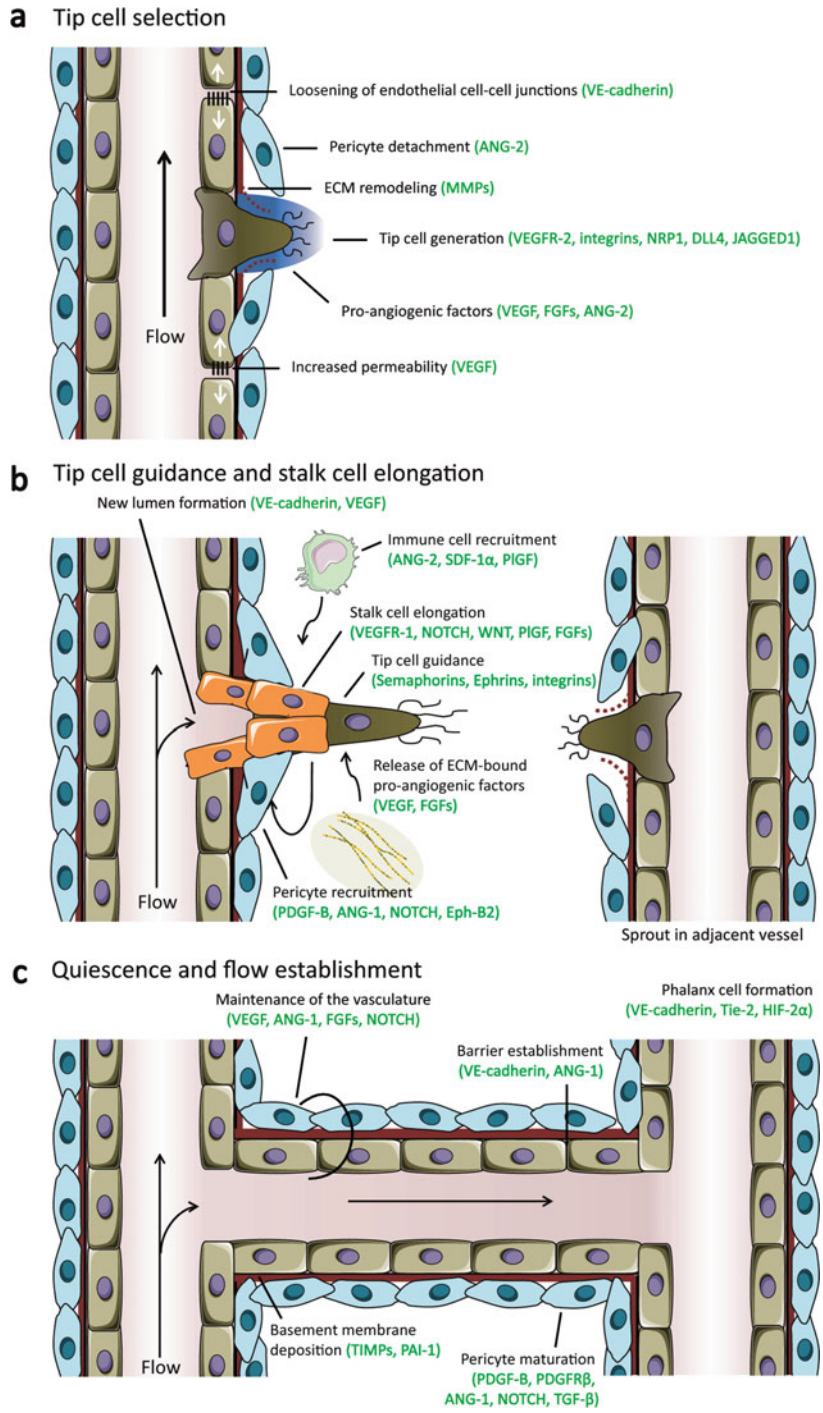

Fig. 2 Molecular basis of angiogenesis. Sequential steps of blood vessel formation and their features are depicted. The most important molecular players involved in each process are denoted in parentheses. (**a**) Upon angiogenic stimulation by proangiogenic factors, the quiescent vessel dilates and an endothelial tip cell is selected. Tip cell generation requires basement membrane degradation, loosening of endothelial cell-cell junctions, and pericyte detachment. A provisional matrix layer is deposited by extravasation of plasma proteins (e.g., fibrinogen) due to increased permeability. Cell migration is favored by protease-mediated matrix remodeling. (**b**) Tip

depending on the combination of VEGF ligand and receptor. In fact, differences between signalosomes allow a broad range of biological effects to VEGF stimulation that include increased endothelial cell proliferation, migration, survival, permeability, and ECM degradation.

Several studies indicate that VEGF stimulates both physiological and tumor angiogenesis by signaling through VEGFR-2 in a dose-dependent manner (Carmeliet and Jain 2011). In fact, VEGFR-2 null mice die during embryonic development by defects in the vascular system, reinforcing its role in proliferation, survival, and migration of vascular endothelial cells. The activation of VEGFR-2 promotes differentiation of progenitors, mitogenesis, chemotaxis, survival, and vascular permeability. It also increases the expression of matrix metalloproteinases and plasminogen activators for ECM degradation and further endothelial cell migration. In detail, VEGF release causes plasma proteins extravasation and the deposition of a provisional ECM scaffold toward which endothelial cells migrate in response to integrin signaling. The activated proteases liberate the angiogenic molecules stored in the ECM such as VEGF and FGF, and the ECM is remodeled into an angio-competent milieu. Once VEGF is released, it binds to the VEGFR-2 receptors of the endothelial cells. Tip cell migration is regulated by VEGF gradient, whereas stalk cell proliferation depends on VEGF concentration (Gerhardt et al. 2003). VEGF/VEGFR-2 signaling axis induces the formation and extension of filopodia and the expression of delta-like ligand 4 (Dll4) protein in tip cells, which activates Notch in stalk cells. Notch, in turn, downregulates VEGFR-2 expression in stalk cells, rendering them less responsive to VEGF and ensuring tip cell leading. The blockade of VEGFR2 signaling is associated with sprouting defects (Bentley et al. 2009).

On the other hand, VEGFR-1 is only slightly activated by proangiogenic factors, and its precise role in angiogenesis is poorly understood (Schwartz et al. 2010). Nevertheless, it has been shown that VEGFR-1 acts as a decoy receptor, since it is able to sequester VEGF, implying a negative role for this receptor in angiogenesis. In addition, mice lacking VEGFR-1 present a higher number of endothelial cells than wild-type mice, whereas the decreased expression of VEGFR-1 increases VEGF availability and VEGFR-2 activity. In fact, a soluble isoform of VEGFR-1 (sVEGFR-1), which encodes the extracellular ligand-binding domain, can be produced by surrounding cells. While soluble VEGF isoforms promote vessel enlargement, matrix-bound isoforms stimulate the branching pattern. sVEGFR-1 inhibits angiogenesis by acting as a molecular trap for VEGF ligand, assisting the guidance of emerging branches or inhibiting the sprouting. The imbalance between the functions of VEGFR-1 and VEGFR-2 causes hemangiomas and benign tumors with increased aberrant angiogenesis (Jinnin et al. 2008).

In pathological conditions, PlGF contributes to the angiogenic switch by affecting multiple cell type directly and indirectly and also activates bone marrow-derived endothelial progenitor cells. PlGF can induce its own signaling and amplify VEGF-driven angiogenesis through direct effects on endothelial cells (Autiero et al. 2003). The synergism between VEGF and PlGF is also stated by the fact that PlGF upregulates the expression of VEGF. PlGF might also indirectly influence SMC proliferation and migration through activated endothelial cell cytokine release (Luttun et al. 2002).

Fig. 2 (continued) cells sense the environment and navigate in response to guidance cues (e.g., semaphorins and ephrins) while adhering to the extracellular matrix (ECM) in order to migrate. Stalk cells behind the leading tip cell proliferate and elongate, attracting pericytes in the process. Stalk cells are further stabilized by the deposition of the new basement membrane. Immune myeloid cell recruitment of tumor-associated macrophages (TAMs) and TIE-2-expressing monocytes (TEMs) produces additional proangiogenic factors and triggers the release of ECM-bound factors. (**c**) Fusion of adjacent branches and posterior lumen formation drive neovessel perfusion, which concludes with quiescence by a phalanx phenotype promotion, deposition of the new basement membrane, maturation of pericytes, reestablishment of cell-cell junctions, and release of vascular maintenance molecules

Other prototypical proangiogenic signaling pathway involves the *fibroblast growth factor* (*FGF*) family, comprised by 23 different ligands and four tyrosine kinase-type receptors (FGFR-1–4) expressed widely in the organism (Presta et al. 2005). FGFR-1 and FGFR-2 are expressed by endothelial cells, and their binding to ligands FGF-1, FGF-2, FGF-4, and FGF-5 leads to the induction of critical stages of angiogenesis in vivo. Among the four pleiotropic proangiogenic ligands, at least FGF-1 and FGF-2 directly stimulate endothelial cell proliferation, detachment, migration, and ultimate differentiation into a functional capillary vessel. For the maintenance of vascular integrity, endothelial quiescent cells require low levels of FGF, since vessel disintegration has been observed as a consequence of FGFR signaling inhibition (Murakami et al. 2008). FGF ligands exert their functions in endothelial cells after paracrine release by stromal or tumor cells, or by endogenous FGF in an autocrine fashion.

Even though VEGF has a pivotal role during angiogenesis, an important cross talk takes place between FGF and VEGF. For instance, VEGF system activation is required for later FGF induction and in vivo angiogenesis promotion. The opposite cross regulation has also been demonstrated, and FGF also seems able to stimulate tumor angiogenesis under certain experimental conditions. The effects of FGF are due to its dual action including a direct effect over endothelial cells and an indirect effect concerning the regulation of the production of other proangiogenic molecules like VEGF, angiopoietin-2 (Ang-2), or interleukin-8 (IL-8) by tumor or stromal cells (Beenken and Mohammadi 2009). Both in mouse and human tumors, the role of FGF in tumor growth and neovascularization has been described (Presta et al. 2005).

The third proangiogenic pathway includes the *epidermal growth factor (EGF)-like family* of growth factors and their receptors (ErbB). The integrants of this family play various functions in different tissues, but they are basically involved in cell proliferation and survival stimulation. Some of the ligands included are EGF and transforming growth factor alpha (TGF-α), which bind to the ErbB family of tyrosine kinase receptors. The ErbB family of receptors is composed of ErbB1/HER1/EGFR, ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER418.

Both in tumor and physiological settings, EGF family members display proangiogenic activity. However, whether the effects of EGF are direct or indirect remains unclear. EGF stimulation produces the release of proangiogenic factor such as VEGF, IL-8, and FGF by tumor and stromal cells. The ErbB family receptors in endothelial cell membranes enable these cells to respond to EGF-like factors by increased proliferation and survival. Furthermore, other angiogenic factors like TGF-β induce the autocrine production of EGF-like molecules such as TGF-α, thus promoting endothelial cell survival through PI3K-Akt signaling (Viñals and Pouyssegur 2001).

The ECM itself provides a link between vascular cells and their surrounding environment. Proteolytic degradation of the basement membrane and the surrounding ECM is an integral part of angiogenesis. In this step, several *proteinase families* are involved, including matrix metalloproteinases (MMP and their tissue-type inhibitors or TIMP), plasminogen activators (uPA and its inhibitor PAI-1), heparanases, tryptases, chymases, cathepsins, etc. Besides breaking down ECM components and clearing a path for endothelial cell migration, proteinases are able to switch on angiogenesis by the liberation of matrix-bound angiogenic activators (bFGF, VEGF, TGF-β, HGH, etc.) and proteolytically activating angiogenic chemokines such as IL-1β. Whereas VEGF isoforms cleaved by MMPs preferentially enlarge vessels, MMP-resistant matrix-bound VEGF is involved in vessel branching (Iruela-Arispe and Davis 2009). Moreover, proteases such as MMP9 participate in the mobilization of bone marrow progenitors by the liberation of cytokines such as the Kit ligand (Heissig et al. 2002) and by the establishment of a pre-metastatic niche (Kaplan et al. 2005). The proteolytic remodeling of the ECM occurs in a sharply controlled mode, and the pleiotropic activities of proteinases are context and concentration dependent. In fact, excessive breakdown removes guidance cues for endothelial cell migration, thus inhibiting

angiogenesis, while insufficient degradation prevents vascular cell mobility (Carmeliet and Jain 2011).

Moreover, proteinases can switch off angiogenesis, as they liberate matrix-bound angiogenic inhibitors such as arrestin, angiostatin, TSP-1, and inactivate angiogenic cytokines like SDF-1. The basement membrane of quiescent vessels is composed mainly of collagen IV and laminin, whereas the interstitial matrix of collagen I and elastin between vascular cells provides further viscoelasticity and strength to the endothelial cell wall. Proteinases expose novel epitopes of these ECM proteins, which ultimately induce endothelial cell and perivascular cell migration and generate the angiogenic scaffold for neovascularization.

Specific ECM molecules have cell surface receptors such as the heterodimeric *integrins*, which transmit bidirectional information between vascular cell cytoplasm and their surrounding environment. Integrin signaling assists vascular cells at new vessel building by favoring association of endothelial cells with ECM proteins such as vitronectin, fibrinogen, and fibronectin. In addition to their contribution to the ligation of ECM components, integrins interact with several extracellular molecules, functioning as "hubs" that modulate endothelial cell and perivascular cell behavior during angiogenesis. Hence, the binding of integrins to VEGF, FGFs, and Ang-1 or their receptors stimulates vessel growth. Among the integrin family, αvβ3 and αvβ5 integrins are reported to positively regulate the angiogenic switch (Desgrosellier and Cheresh 2009).

In this same context, *VE-cadherin* is an endothelium-specific protein that mediates in homotypic cell-cell interactions (Cavallaro et al. 2006). This protein is coexpressed with VEGFR-2 in developing blood vessels, suggesting a potential interaction between both receptors. VE-cadherin is critical for the final steps of capillary development and lumen formation and is associated with the inhibition of endothelial cell migration. During neovessel sprouting, the adhesive function of VE-cadherin is reduced by endocytosis in response to VEGF and angiogenic factors (Dejana et al. 2009). Meantime, the localization of VE-cadherin at filopodia allows the establishment of new contacts by tip cells with cells on the outreaching vessel sprouts.

Chemokines are a large group of molecules secreted by stromal cells that promote inflammation and pathological angiogenesis by the recruitment of immune cells and EPCs to malignant, inflammatory, and ischemic tissues. Besides, chemokines directly activate the G protein-coupled chemokine receptors (GPCRs) signaling in endothelial cells. Endothelial cells express chemokine CXCR receptor such as the angiogenic CXCR2 and CXCR4 receptors, which are bound by GROa, IL-8 and SDF-1, and angiostatic CXCR3, whose ligands are PF-4, MIG, etc. Hence, SDF-1 binds to the CXCR4 receptor on tip cells (Duda et al. 2011) and is upregulated in hypoxia, supporting mobilization and retention of proangiogenic bone marrow-derived cells to promote revascularization. Depending on the temporospatial balance of these modulators, chemokine release has an overall role in initiating or terminating angiogenesis.

Cues that Guide Vessel Navigation

There are many similarities between tip cell environment sensing and axonal cone growing, and therefore it is not surprising that many molecular, mechanistic, and morphological aspects of vascular guidance are shared with the axon guidance process during nervous system development (Adams and Eichmann 2010; Fig. 2b). In the last decade, a bundle of attractive (VEGF, Slit) and repulsive (semaphorin, ephrin, netrin) vascular guidance cues have been described. However, some cues such as semaphorins, netrins, and Slits are able to function as either attractive or repulsive depending on the molecular context. Specific receptors for each vascular signal are expressed by endothelial cells. Some attractive cues such as *VEGF* are displayed as gradients of soluble factors, whereas others establish communication networks through transmembrane proteins like ephrins and their EPH receptors.

One of the main families governing vessel guidance comprises *Ephrin/EPH*. This family is involved in the regulation of arterial-venous plexus formation, remodeling and maturation,

axonal outgrowth, fasciculation and pathfinding, and lymphatic plexus formation (Mosch et al. 2010). Ephrin and EPH are membrane-bound proteins that function as a bidirectional receptor-ligand pairs whose signaling regulates cell-cell contact-dependent patterning. Whereas EPH becomes autophosphorylated and activates intracellular signalosomes (known as forward signaling), the phosphorylation of ephrin cytoplasmic tail prompts the binding of PDZ domain-containing proteins (known as reverse signaling).

Among the different family members, ephrin-B2 and its receptor EPHB4 have been found to play a key role in angiogenesis by vessel morphogenesis regulation. During vasculogenesis, the vascular plexus is characterized by ephrin-B2-positive arteries and EPHB4-positive venous regions. These two populations of cells prevent intermingling and segregate from each other by avoiding repulsive actions, suggesting an "artery-to-vein push and pull" model of angiogenesis. Besides, ephrin-B2 reverse signaling in tip cells induces VEGFR-2 internalization, which is necessary for downstream signaling of the receptor to cause tip-cell filopodial extension (Sawamiphak et al. 2010). Ephrin-B2 also drives mural cell and EPC recruitment. Moreover, EPHB4 upregulation stimulates tumor angiogenesis and induces malignant transformation, classifying the receptor as a proangiogenic and tumorigenic molecule. Other ephrin ligands and EPH receptors, like ephrin-A1 and EPHA2, have also a role in vessel growth and maturation. Since the discovery of the ephrin/EPH family, several members have been described to be deregulated in different tumor types (Mosch et al. 2010).

Semaphorins belong to a large family of membrane-bound and secreted proteins involved in both attractive and repulsive activities in the vascular and nervous system formation. Semaphorin family ligands are characterized by the presence of a highly conserved extracellular sema domain that mediates the binding to multimeric receptor complexes, mainly formed by plexins and neuropilins (NRPs) (Suchting et al. 2006). Regarding the dual role in promoting or inhibiting the angiogenic response, the signaling cascade initiated after plexin and neuropilin coreceptors (NRPs) activation remains to be completely elucidated.

SEMA3A, SEMA3B, SEMA3D, SEMA3F, and SEMA4A are regarded as negative regulators of tumor angiogenesis, whereas SEMA3C and SEMA4D promote tumor angiogenesis. For instance, SEMA3A expression by endothelial cells of developing vessels inhibits endothelial cell migration as it interferes with integrin function (Serini et al. 2003). The loss of Plexin D1 receptor produces aberrant sprouting into SEMA3E expressing tissues, as seen in zebrafish embryos (Adams and Eichmann 2010). In mice, Plexin D1 removal induces erroneous navigation, since endothelial cells are not able to recognize the repulsive SEMA3E signals in the surrounding environment. Moreover, SEMA3E signaling regulates the balance of tip and stalk cells necessary for growing sprouts by coordinating the activity of VEGF in a negative feedback loop (Kim et al. 2011). SEMA3F has recently been described as a metastasis suppressor in different animal models due to its ability to block peritumoral vessel sprouting, tumor cell adhesion, and migration (Bielenberg et al. 2006). Indeed, tumors have been reported to produce a soluble form of neuropilin 1 (sNpn-1), which might function as a VEGF trap, and therefore inhibit tumor angiogenesis and growth (Guttmann-Raviv et al. 2006). On the contrary, SEMA4D induces endothelial cell migration and tubulogenesis apart from stimulating blood vessel formation in vivo through interaction with its Plexin B1 receptor in endothelial cells (Basile et al. 2004).

Similar dual attractive and repulsive roles are found in two other families of secreted factors: *Slits* and their roundabouts (Robo) receptors and by *netrins* and their receptors uncoordinated 5 (UNC5) and deleted in colorectal cancer (DCC). Slits and Robo are proteins with multiple binding domains that are involved in physiological vasculogenesis (Park et al. 2003). In detail, Robo-4 receptor for Slits is reported to be expressed in sites of active angiogenesis in the adult, which include tumor vessels. Robo-4 expression in endothelial cells maintains vessel integrity, and its deficiency induces leakiness

and hypervascularization (London et al. 2009). The permeability-promoting actions of VEGF are counteracted by Robo-4 activity, which impedes VEGFR-2 activation of Src. In addition, in vitro studies showed that the exposure of endothelial cells expressing Robo-1 to a Slit-2 source promoted their chemotaxis (Wang et al. 2003). Indeed, Slit-2 has been found to be expressed in many tumor cell lines and biopsies.

Netrins are a protein family that contains a laminin VI domain and a carboxy-terminal domain that binds heparin, several proteoglycans, and membrane glucolipids, thus allowing interaction with the cell surface or with ECM components (Barallobre et al. 2005). UNC5B is a netrin receptor whose expression is enriched in tip cells. Netrin-1 binding to UNC5B receptor has shown to act as a repellent in blood vessel guidance (Lu et al. 2004). Other studies have reported stimulation of endothelial cell and perivascular cell proliferation and migration in vitro by Netrin-1 and Netrin-4 (Wilson et al. 2006). In fact, UNC5B inactivation results in enhanced sprouting, whereas netrin-1 promotes filopodia retraction of endothelial cells, consistent with a suppressive function of UNC5B and netrins on vessel growth (Adams and Eichmann 2010).

Vessel Remodeling, Stabilization, and Maturation

In order to achieve functionality, vessels must mature both at the level of the endothelium and vessel wall and as a vascular network. This maturation involves the remodeling of the network into a hierarchically branched structure, which implies the formation of large and small vessels, the association with perivascular cells, the establishment of directional flow, and the adjustment of density to meet the nutritional requirements of the surrounding tissue (Fig. 2c). Preceding the phenomenon of vascular remodeling, capillary retraction takes place. This occurs during the rapid growth phase of capillaries and is generally associated with the elongation of other capillaries in the vicinity (Clark and Clark 1939).

Late events in the angiogenic process include the stabilization of the newly formed vessels and the maintenance of the vasculature. Vessel integrity in different vascular beds is maintained by a coordinated regulation between several cellular (including endothelial cells, pericytes, fibroblasts, SMC, inflammatory cells, etc.) and noncellular components such as the ECM (Stratman and Davis 2011). In short, pericyte recruitment, adhesion, and wrapping around endothelial cells are fundamental events during blood vessel stabilization and maturation. Whereas pericytes establish direct cell-cell contacts with endothelial cells in capillaries and immature vessels, vascular SMC are separated from endothelial cells by a matrix and function as covers of veins and arteries (Gaengel et al. 2009).

Vascular remodeling is a complex process that requires an extensive array of molecular signaling. During vessel maturation, while endothelial proliferation is detained, endothelial cells express survival signals in order to maintain integrity of the vessel lining. One such survival factor is the canonical *VEGF*, produced by endothelial cells themselves. The "intracrine" VEGF activates the PI3K/AKT survival pathway, thus preventing apoptosis in nonpathological conditions (Warren and Iruela-Arispe 2010). This pro-survival activity of VEGF differs from its paracrine function described in the previous section, where the loss of endothelial VEGF does not cause developmental vascular defects. Moreover, *FGFs* have also been involved in the maintenance of vascular integrity for their ability to strengthen adherens junctions (Beenken and Mohammadi 2009). The inhibition of FGF causes dissociation of tight junctions and further endothelial cell loss and vessel disintegration (Murakami et al. 2008). Another important survival cue is *blood flow*, as shear stress inhibits endothelial cell apoptosis by KLF2 activation. Active KLF2 evokes quiescence by downregulating VEGFR-2 and upregulating nitric oxide synthase and thrombomodulin, favoring vessel dilation, perfusion, and absence of clots.

Nevertheless, the prototypical vascular remodeling factor family par excellence is the *angiopoietin/Tie* family, formed by angiopoietins (Ang) and their receptors, known as Tie (Augustin et al. 2009). The angiopoietin family comprises

the ligands Ang-1, Ang-2, and Ang-4 and the Tie tyrosine kinase receptors Tie-1 and Tie-2. Since all known angiopoietins bind only to Tie-2 receptor, Tie-1 persists as an orphan receptor that might act as a negative regulator of Tie-2. The main function of angiopoietins is the control of the switch between endothelial cell quiescence and activation. At the molecular level, the activation of the angiopoietin/Tie signaling cascade modulates, in a positive or negative fashion depending on the molecular context, pro-survival pathways (such as PI3K-Akt) and endothelial cell permeability (by Src kinase regulation).

The binding of Ang-1 produced by cells in the vicinity of developing vessels (mural cells, fibroblasts, and tumor cells) to the Tie-2 receptor expressed in endothelial cells promotes vessel maturation through endothelial cell quiescence and pericyte recruitment. Ang-1 or Tie-2 deficiency causes premature death in mice due to severe defects in the vascular system characterized by a poorly organized and immature capillary network. As a result, the Ang-1 functions to induce vasculature stabilization by a mature and nonproliferative state maintenance of endothelial cells. Moreover, Ang-1 tightens vessels via effects on PECAM, VE-cadherin and occludin and favors endothelial cell-pericyte interactions by serving as a sticky ECM-associated and α5-binding protein (Saharinen et al. 2008). Roles for Ang-1 in endothelial cell growth and capillary tube formation by its synergistic activity with VEGF and in circulating EPC mobilization have also been described (Hattori et al. 2001).

Opposed to Ang-1, Ang-2 is produced by endothelial tip cells in angiogenic and vascular remodeling sites and acts as an Ang-1 antagonist, contributing to the detachment of perivascular cells. Ang-2 binds specifically to Tie-2, hence competing with Ang-1 for the binding to the same receptor, and its action depends on the endothelial cell state. Intriguingly, whereas Ang-2 inhibits Tie-2 signaling in the resting vasculature, it stimulates Tie-2 signaling on stressed endothelium (Augustin et al. 2009). Even though deficiency of Ang-2 does not impair normal development, adult mice lacking Ang-2 present vascular defects in angiogenically active organs. This suggests that the dual role of Ang-2 is related to vascular remodeling activation, being its final effect dependent on the presence or absence of other proangiogenic factors (Gale et al. 2004). For instance, in the absence of VEGF, Ang-2 promotes vascular regression by endothelial cell apoptosis. On the contrary, VEGF presence stimulates Ang-2 activation of pericyte detachment, enabling endothelial cell exit, proliferation, and migration, thus contributing to new vessel formation.

The overall effects of angiopoietin/Tie signaling on tumors depend on the context (Augustin et al. 2009). Moderate Ang-1 or strong Ang-2 overexpression have been observed in tumor cells. Induced overexpression of Ang-1 in tumor cells stabilizes the vasculature and diminishes angiogenesis, thus promoting antitumor effects. Contrarily, Ang-2 overexpression activates angiogenesis and enhances tumor growth, while its systemic delivery results in tumor vessel regression. Tumor-derived Ang-2 also stimulates angiogenesis by recruiting proangiogenic monocytes (de Palma et al. 2005). On the whole, these studies highlight the significance of maintaining an accurate balance of angiopoietin/Tie signaling for normal vascular homeostasis. Ang-1/Ang-2 balance shifting in favor of Ang-2 makes the vasculature more plastic and susceptible to sprouting.

Other of the main molecular families involved in vessel maturation contains *platelet-derived growth factor* (*PDGF*) and its receptors. The PDGF family comprises four different isoforms (PDGF-A, PDGF-B, PDGF-C, PDGF-D) closely related to the VEGF family and are expressed and impact different types of cells including fibroblasts, SMC, neurons, and endothelium (Andrae et al. 2008). Although PDGF ligands act as homodimers, functional heterodimers (such as PDGF-AB) are also found. Two tyrosine kinase receptors have been described: PDGFR-α and PDGFR-β. Consequent to ligand binding, the receptor dimerizes forming homo- or heterodimeric receptor complexes. Some of the typical interactions are PDGF-AA and PDGF-CC interacting with PDGFR-α and PDGF-BB and PDGF-DD binding to PDGFR-β. In contrast to isoforms, PDGF-A

and PDGF-B, PDGF-C and PDGF-D are secreted as zymogens and require a previous activation by proteolytic cleavage. Similar to VEGF, PDGFs also contain "retention matrix" structural motifs that allow their interaction with ECM proteins and the regulation of their biological availability (LaRochelle et al. 1991).

The proangiogenic PDGF/PDGFR family works in a paracrine fashion. Endothelial and stromal cells produce PDGF factors that bind to their receptors in mural cells (pericytes and SMC). In order to stabilize endothelial cell channel, angiogenic cells release PDGF-B to chemoattract PDGFR-β expressing pericytes (Gaengel et al. 2009). Hence, PDGF-B functions as an attractant, stimulating cell migration, proliferation, and cell fate. Consequently, genetic ablation of either ligands or receptors of the PDGF family in mice provokes pericyte deficiency, which in turn causes vessel leakage, microaneurysm formation, tortuosity, and bleeding, leading to defects in the blood-brain barrier and premature death (Quaegebeur et al. 2010). Among the signaling pathways stimulated upon ligand binding and receptor dimerization are the Ras-ERK, PI3K-Akt, and phopholipase C-γ that induce proliferation, migration, and survival of mural cells.

During tumor development, paracrine PDGF-B produced by tumor cells recruits pericytes and elicits angiogenesis. In addition, PDGF stimulates also tumor cells directly in an autocrine manner, as reported in gliomas. For instance, PDGF-D has been described as a potent stimulator of tumor neovascularization (Li et al. 2003). Besides, tumor-derived PDGF-B also recruits pericytes in an indirect manner by upregulating SDF-1α. In metastasis, PDGFR-β expressing pericytes have a dual role. In primary tumors, pericytes are a physical barrier for tumor cell intravasation, so the absence of pericyte correlates with metastasis (Gerhardt and Semb 2008). Nevertheless, other studies have reported that pericytes at micrometastatic sites support tumor colonization by proangiogenic factor release.

The third major group of remodeling factors involves the *transforming growth factor-beta* (*TGF-β*) family of cytokines which includes, among others, TGF-β, bone morphogenetic proteins (BMPs), and activins. The TGF-β family is involved in the control of many biological responses and cellular functions such as proliferation, apoptosis, or differentiation and is produced by nearly every cell type. Three members of the TGF-β family with partially overlapping expression patterns but distinct functions have been identified (TGF-β1–3). These growth factors are secreted as latent forms and later activated proteolytic processing or binding to thrombospondin-1 (TSP-1). The different effects of TGF-β signaling depend on the molecular, temporal, and spatial context. Genetically modified mice lacking various TGF-β signaling components harbor extensive vascular defects, remarking their key role in angiogenesis (Pardali et al. 2010). In the adult organisms, TGF-β exerts a proangiogenic role on activated sprouting endothelial cells by the stimulation of their proliferation and migration, whereas it induces quiescence and maturation in the resting endothelium.

Signal transduction by TGF-β requires a series of receptors that have a serine/threonine kinase intracellular domain, accessory receptors, Smad proteins and Smad transcription factors (Akhurst 2006). There are two different TGF-β receptor families: type I receptors (TGFβRI, also known as activin-like kinase (ALK)) and type II receptors (TGFβRII), which are constitutively active serine/threonine kinases. Ligand binding of TGF-β to its TGFβRII receptor induces heterodimerization with TGFβRI, which is then phosphorylated at the serine and threonine residues by TGFβRII kinase. Once activated, TGFβRII phosphorylates intracellular proteins such as the Smad family of transcription factors. In turn, the active Smad proteins are translocated into the nucleus where they activate the transcription of target genes.

Two different TGFβRI receptors are expressed in endothelial cells: ALK1 and ALK5. ALK1 stimulates endothelial cell proliferation and migration, whereas ALK5 inhibits these processes, maintains endothelium quiescence, and induces ECM deposition. Furthermore, endoglin (CD105) is a TGF-β coreceptor (or type III TGF-β receptor), which is highly expressed in

proliferative endothelial cells, and is required for ALK1 signaling. The ratio of ALK5/ALK1 expression explains the dual role exerted by TGβ in angiogenesis, since their net balance dictates the outcome of TGF-β response (Lamouille et al. 2002). While proliferation is stimulated through ALK1 signaling in endoglin-positive sprouting endothelial cells, resting endothelium-lacking endoglin is subjected to TGF-β/ALK5-induced quiescence and inhibition of cell proliferation and migration. Besides, differential TGF-β concentration also triggers different responses. At low doses, it contributes to the angiogenic switch, through the upregulation of angiogenic factors such as VEGF and several proteinases. At high doses, TGF-β inhibits endothelial cell proliferation and migration by stimulating the reformation of the basement membrane and the recruitment and differentiation of mesenchymal cells via PDGF-B and SM22a upregulation (Taylor and Khachigian 2000). Endothelial cell proliferation inhibition occurs as a result of the impeded pRb phosphorylation that provokes endothelial cell cycle arrest at G1 phase (Gupta and Qin 2003).

The *Notch* pathway includes another central superfamily of molecules with important roles in vascular biology, controlling not only remodeling but also endothelial cell fate during vascular development and vascular guidance in sprouting angiogenesis (Roca and Adams 2007). There are four transmembrane Notch receptors (Notch-1, Notch-2, Notch-3, Notch-4) which have large extracellular domains named NECD. Unlike the VEGF signaling pathway, their five ligands (Jagged-1, Jagged-2, Delta-like 1, Delta-like 3, and Delta-like 4) are also transmembrane proteins which are exposed by neighboring cells. Thus, interaction requires cell-cell contact. These ligands stimulate Notch-presenting cells in a juxtacrine manner. Upon binding, Notch is subjected to two proteolytic cleavages: extracellular and intracellular (catalyzed by γ-secretase). The intracellular cleavage liberates a portion of Notch known as NICD that translocates to the nucleus and regulates transcription of Notch target genes.

The Notch superfamily has been shown to take part in cell fate decisions, either by initiating differentiation of these cells or by maintaining their undifferentiated state. Notch signaling is critical for the control of endothelial cell fate during arteriovenous differentiation (Gridley 2010). Whereas the inactivation of Notch determines venous identity, its active form determines arterial one. Precisely, Notch-1, Delta-like 1, and Delta-like 4 are expressed in endothelial cell arteries and control arteriogenesis both in the embryo and in the adult.

Besides its roles in vascular development, Notch contributes to sprouting angiogenesis regulation. A deletion of a single copy of Delta-like 4 (Dll4) or Notch-1 provokes vascular defects and embryonic lethality (Gale et al. 2004). Tip or stalk cell specification of endothelial cells is controlled by the Notch pathway (Eilken and Adams 2010). High levels of Notch where noted in stalk cells, whereas Notch signaling was shown to be low in tip cells. During physiological angiogenesis or tumor progression, blockade of Notch or Dll4 augments filopodia and sprouting following excessive tip cell formation (Thurston et al. 2007). Notch-1 expression is critical for tip cell behavior suppression in stalk cells. Both the hypersprouting phenotype and the aberrant number of tip cells after Notch inhibition suggest that tip cell phenotype is the default endothelial response to proangiogenic stimuli. Contrarily to Dll4, the Notch ligand Jagged-1 (JAG1) is primarily expressed by stalk cells. Nevertheless, a modification of Notch by Fringe glycosyltransferase favors the activation of this receptor by Dll4, leaving JAG1 as a poor antagonist that favors phenotype maintenance (Eilken and Adams 2010).

Tip and stalk cell fate are transient phenotypes of endothelial cells. In order to expand the vascular plexus, endothelial cells undergo repetitive cycles of sprouting, branching, and tubulogenesis, requiring active transitions between tip and stalk cells. The coordinated function of VEGFR-2 and Notch pathways controls branching. Indeed, this integrated intercellular feedback works as a "branching pattern generator" and involves the regulation of all VEGFRs by Notch. Dll4 expression is activated through VEGFR-2 signaling in endothelial tip cells. This Dll4 activates Notch signaling in adjacent neighboring endothelial

cells, thus dictating a stalk fate. The inhibition of tip cell behavior occurs as a consequence of Notch-mediated downregulation of VEGFR-2, VEGFR-3, and NRP-1 and VEGFR-1 upregulation (Jakobsson et al. 2010). Through Notch/Dll4 signaling, endothelial cells located at the angiogenic sprout dynamically compete for tip position. Upon VEGF signaling, although all cells upregulate Dll4, the ones that express it quicker or at higher levels have a competitive advantage to become tip cells. Regarding the dynamic shifting of tip-stalk position during sprouting angiogenesis, Dll4 expression is highly regulated at various levels. For example, a TEL/CtBP repressor complex at the Dll4 promoter is transiently disassembled following VEGF stimulation, permitting a restricted pulse of Dll4 transcription (Roukens et al. 2010). Several other pathways such as the Wnt/β-catenin, one converges on the transcriptional control of Dll4 (Corada et al. 2010). Other examples include stalk cell JAG-1 expression, which by antagonizing Dll4 activity, reduces Notch signaling induction in the adjacent tip cells, thus maintaining their responsiveness to VEGF stimulation (Benedito et al. 2009).

Dll4a is highly expressed in tumor blood vessels, implying a role for this protein in the control of tumor angiogenesis. Intriguingly, the inhibition of Notch/Dll4 signaling pathway aberrantly increases tip cell count, leading to augmented vessel density, defective perfusion, and consequently tumor hypoxia and growth inhibition (Thurston et al. 2007). Nevertheless, chronic Dll4 blockade results in vascular neoplasms (Yan et al. 2010).

Regression of Blood Vessels and Endogenous Inhibitors of Angiogenesis

Some endogenous proteins or fragments of proteins formed in the body act as physiological inhibitors of angiogenesis (Ribatti 2009). Apart from inhibiting blood vessel formation, endogenous anti-angiogenic factors block cell cycle progression, migration, and induce apoptosis.

Many of these inhibitors are fragments of larger ECM molecules that are released following proteolysis by enzymes like metalloproteinases, cathepsins, and elastases. For instance, *arresten*, *tumstatin*, and *canstatin* are parts of type IV collagen; *endostatin* is a fragment of type XVIII collagen, and *endorepellin* is part of the proteoglycan perlecan. All fragments bind integrins expressed by endothelial cells. Integrin-dependent signaling pathways are crucial for the anti-angiogenic effects of these molecules. Recombinant tumstatin was reported to specifically induce apoptosis of proliferating endothelial cells and promote a potent anti-angiogenic activity in several in vitro and in vivo angiogenesis models (Maeshima et al. 2002). Similarly, arresten has also been described to inhibit endothelial cell proliferation, migration, tube formation, and growth of primary tumors and metastases in mouse xenograft tumor models (Sudhakar et al. 2005).

Two other molecules that are critical for the negative regulation of angiogenesis are thrombospondin-1 (*TSP-1*) and thrombospondin-2 (*TSP-2*). TSP-1 and TSP-2 are potent anti-angiogenic heparin-binding proteins that although constituents of the ECM can also be secreted and found in blood circulation (Armstrong and Bornstein 2003). Through primary binding to CD36 endothelial cell membrane receptor, TSP-1 is thought to activate anti-proliferative and proapoptotic effects. Furthermore, TSP-1 directly affects the ECM by activation of TGF-β. TSP-2 also inhibits endothelial cell migration and tube formation, as well as specifically increasing apoptosis of these cells (Noh et al. 2003).

Apart from the matrix-derived molecules, the heterogeneous group of other endogenous anti-angiogenic molecules contains several growth factors, cytokines, metabolites of hormones, and clotting factors (Folkman 2004). For example, interferon α (*IFN-α*) and β (*IFN-β*), *interleukin-1β* (*IL-1β*), *IL-4*, *IL-12*, *IL-18*, and *pigment epithelium-derived factor* are all cytokines with the strong capacity of blocking angiogenesis. Both IFNα and IFNβ inhibit angiogenesis in mouse models by modulating the pro-angiogenic signals generated by tumor cells. Moreover, they also modify the activity and expression of several proteases such as MMP-9

(Ma et al. 2001) and downregulate bFGF expression (Dinney et al. 1998). Regarding interleukins, IL-1β inhibits by an autocrine pathway angiogenesis stimulated by FGF. Fragments derived from or related to blood coagulation factors such as *angiostatin* (cleaved from plasminogen produced by tumor cells), anti-angiogenic antithrombin III (derived from antithrombin III) and *platelet factor 4* play a role in angiogenesis inhibition and endothelial cell apoptosis.

The Angiogenic Switch in Tumorigenesis

Without new vessels, tumor outgrowth is usually restricted to no more than 1–2 mm^3. During this phase, known as avascular phase, the tumor is nourished by the diffusion of nutrients and oxygen obtained from nearby blood vessels, and tumor-related new blood vessel formation is not observed. These avascular tumors reach a steady state, where proliferation and apoptosis are balanced, and there is no net increase of tumor volume. In order to sustain unlimited proliferation and to grow beyond the restricted size, tumors demand an extension of the local vessel network, thereby ensuring adequate delivery of oxygen and nutrients to meet their metabolic needs. The transition from the avascular phase to the angiogenic state of tumor development is known as the "angiogenic switch." Since tumor neovascularization is critical for tumor growth, the ability of forming a dense microvascular plexus is a prerequisite acquired early during tumor progression (Folkman 1990). To achieve this end, tumor cells are subjected to numerous genetic and epigenetic changes that endow them with angiogenic potential. The angiogenic phenotype serves the development of malignant neoplasm at multiple stages, since it plays an important role both in the growth and blood supply of the primary tumor and in the tumor metastasis. Several experiments have demonstrated that in the absence of a functional vasculature, tumors become necrotic or apoptotic, reinforcing the dependence of tumors on access to vasculature in order to thrive (Holmgren et al. 1995). The mechanism through which the tumor manages to reactivate the quiescent vasculature from its dormant state to an angiogenic trait and the therapeutic exploitation of its inhibition for cancer treatment has been broadly studied in the past years.

A dynamic balance between positive (pro-angiogenic) and negative (anti-angiogenic) factors controls vascular homeostasis (Hanahan and Folkman 1996). In normal tissues, under physiological conditions, the balance is shifted toward negative regulators of angiogenesis, which maintain the resting state of the vasculature. During tumor progression, several mechanisms contribute to the reversion of this balance. For instance, the loss of tumor suppressor genes and upregulation of oncogenes provoke the loss of the inhibitory phenotype and the gain of inducers that trigger the formation of an excessive and aberrant vascular bed. In early stages of tumorigenesis, tumor cells release high levels of strong angiogenic inducers such as VEGF and FGF. Several studies have appointed VEGF as a key angiogenic player in tumor progression. VEGF is expressed in most types of cancer. Its expression is induced by oncogenes, hypoxia, hypoglycemia, and growth factors and correlates with tumor progression. For example, Myc overexpression leads to a ten-fold increase of VEGF in a B-cell line (Mezquita et al. 2005). Besides, other positive regulators of angiogenesis such as PDGF, FGF, EGF, TGF-β, MMPs, TNF, and angiopoietins (described in the previous sections of this chapter) are also deregulated during the angiogenic switch.

Intriguingly, it has been shown that cancer cells may escape from ECM-associated endogenous inhibitors by further upregulation of pro-angiogenic factors (Fernando et al. 2008). Nevertheless, an increase of an inducer does not suffice to switch on tumor angiogenesis, since inhibitors like TSP-1 are continuously produced at significantly high levels. The loss of endogenous angiostatic factors by subsequent additional genetic alterations in tumor suppressor genes such as p53 is a necessary step to switch on the angiogenic program (Volpert and Alani 2003). Emerging data also demonstrate that tumor cells play an active role in the vascular stem cell and metastatic niche development in order to ensure

cancer stem and progenitor cell expansion. Additionally, tumor cell metabolism creates an acidic tumor microenvironment that promotes EMT and increased tumor cell stemness.

Hypoxia and Tumor Angiogenesis

Strong evidence supports a role for hypoxia in the activation of tumor angiogenesis. Generally, neoplasms have been described to harbor-extensive regions of hypoxia if compared to the corresponding non-tumoral tissue (Vaupel 2004). Hypoxia occurs as a consequence of the rapid proliferation of the tumor mass and the formation of a distorted and abnormal vasculature, which is inefficient in oxygen transport. Low oxygen levels upregulate inducers and downregulate inhibitors, contribution to switching on angiogenesis. Besides, hypoxia drives upregulation of the expression of endothelial-pericyte destabilizing molecules such as Ang-2, which further contributes to the start of sprouting angiogenesis. The mobilization of multiple types of precursor cells from the bone marrow to the tumor mass, and the recruitment of immune cells are also positively modulated by tumor hypoxia (Blouw et al. 2003). Furthermore, low oxygen concentration downregulates DNA repair mechanisms, promoting genomic instability in cancer cells (Bristow and Hill 2008). Changes in gene expression elicited by hypoxia trigger a switch to anaerobic metabolism, inhibition of apoptosis, increased invasiveness, EMT, and metastasis (Cairns et al. 2011).

Moreover, oxygen-sensing enzymes (prolyl hydroxylases) in endothelial cells have been reported to play a fundamental role in tumor vessel morphology and functionality control (Mazzone et al. 2009). All these findings suggest that a number of key initiating events of tumor angiogenesis are subjected to the control of the hypoxia response program (Fig. 3a). When sufficient oxygen is available, the prolyl hydroxylase domain (PHD) proteins PHD1–3 hydroxylate the hypoxia-inducible factor (HIF) proteins HIF-1α and HIF-2α, regarded as master regulators of the hypoxia driven response. When hydroxylated in a region referred to as oxygen-dependent degradation domain (ODD domain), HIFs are attracted to the von Hippel-Lindau (VHL) protein, member of the E3 multiprotein ubiquitin ligase complex, which marks HIF with a multi-ubiquitin chain that directs it toward proteasomal degradation (Majmundar et al. 2010).

Under hypoxic conditions, PHDs remain inactive, and HIFs initiate their transcriptional activity in order to increase the oxygen supply by angiogenesis, through the upregulation of angiogenic factors (Fraisl et al. 2009). In detail, active HIFs translocate to the nucleus, where they form heterodimers with HIF-1β, which is oxygen independent and constitutively expressed, and bind to hypoxia-response elements (HRE), of the sequence 5′-RCGTG-3′. The binding of HIF-1α/HIF-1β heterodimers to HRE activates the transcription of more than 100 different genes (Semenza 2003; Fig. 3b). Many of these genes, such as VE-cadherin, nodal, VEGF, VEGFR-2, Ephrin-B, CD31, sema4D, plexinB1, integrins, MMPs, and other ECM components, have vasculogenic and prometastatic properties. Other genes include those involved in cell survival, apoptosis, cell motility and cytoskeletal structures, adhesion, transcriptional regulation, and drug resistance.

As a rule, HIF-1α promotes vessel sprouting, while HIF-2α intervenes in vascular maintenance. They have both overlapping and unique target genes and may also trigger specific roles. For instance, whereas HIF-1 has been predominantly regarded as a driver of the initial response to hypoxia (<24 h), HIF-2 seems to be responsible for chronic response (>24 h). Both transcription factors are highly expressed in a wide range of aggressive and metastatic tumors (Yang et al. 2014). Moreover, recent evidence relates HIF-1α overexpression with tumor cell-dominant VM in several types of cancers (Mazzone et al. 2009). HIF-1α depletion in mice impairs embryonic vascular development and revascularization and angiogenesis of injured tissues and tumors. There is also an indirect regulation of tumor angiogenesis by HIF-1α, since it promotes the release of chemoattractants such as SDF-1α to recruit bone marrow-derived progenitors

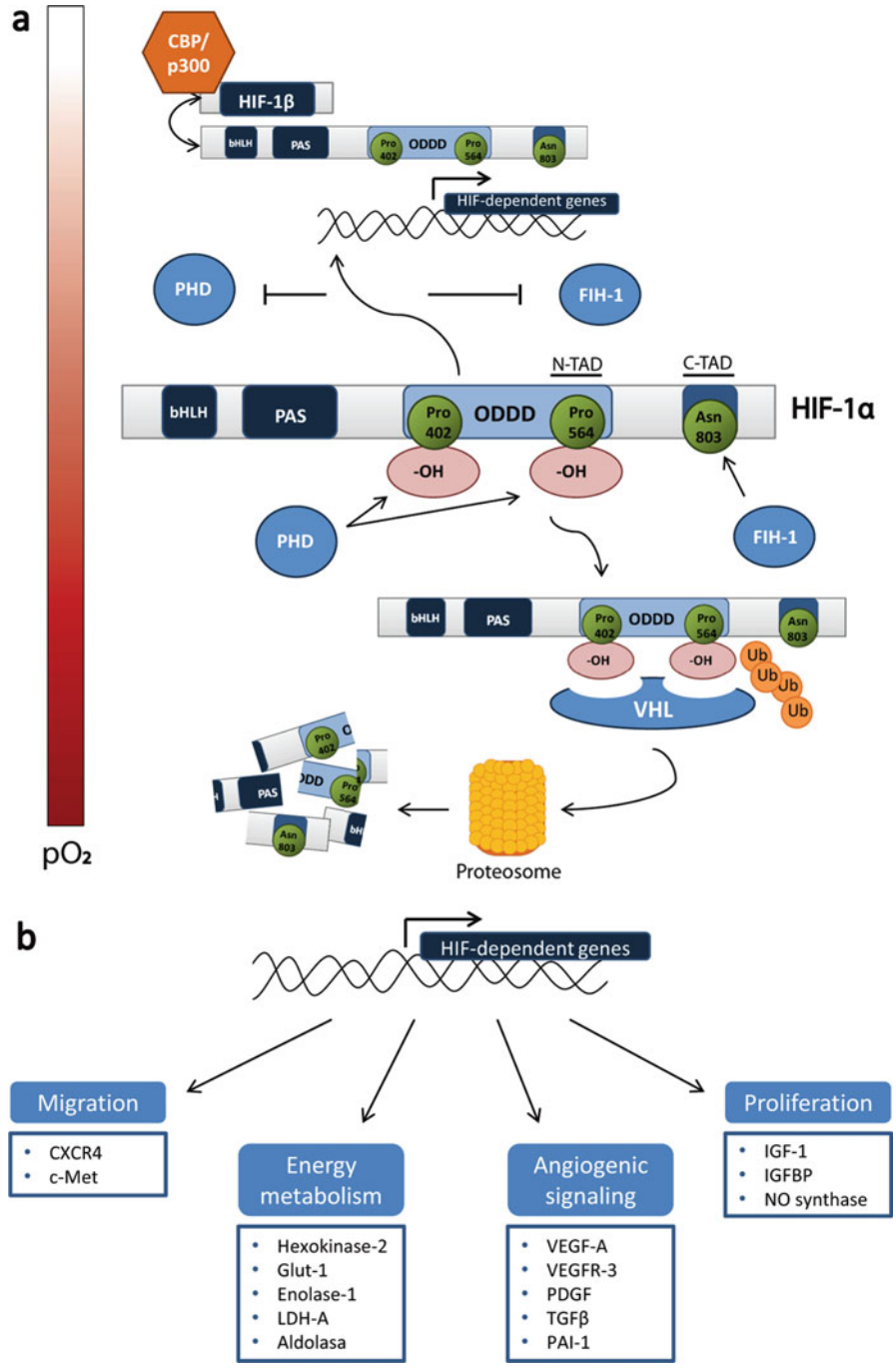

Fig. 3 Stability and transcriptional activity regulation of HIF. (**a**) Under normoxic conditions, prolyl-hydroxylase domain (PHD) proteins hydroxylate two proline residues (402 and 564) located in the oxygen-dependent degradation domain (ODDD) of HIF-1α. Another oxygen sensor, factor-inhibiting HIF-1 (FIH-1), also hydroxylates HIF-1α on an asparagine (803) residue located at its C-terminal transcription activation domain (C-TAF). The hydroxylated protein interacts with the von Hippel-Lindau (VHL) protein that adds a multi-ubiquitin chain to HIF-1α, addressing it for degradation by the proteasome. Under hypoxic conditions, oxygen sensors PHDs and FIH-1 are inhibited, and HIF-1α is no longer degraded. Interaction of HIF-1α with its co-activators such as CBP/p300 and the

(Du et al. 2008). HIF-1α and HIF-2α also regulate TAM polarization and proangiogenic activity with different effects. The relationship between hypoxia and inflammation is illustrated by the interlink between HIF-1α signaling and nuclear factor-kB, which are mutually cross activated. Moreover, HIF activation leads to pro-malignant reprogramming of tumor gene expression and selection of hypoxia-resistant genotypes, like p53 tumor suppressor mutations. Under certain conditions, hypoxic upregulation of VEGF is HIF-1α independent and is mediated by the metabolic regulator PGC-1α, which prepares the ischemic tissue for oxidative metabolism after its revascularization (Arany et al. 2008).

Regarding the regulation of HIFs activation, abundant research shows that it involves reactive oxygen species (ROS) production by mitochondria under limited oxygenation. These ROS have been reported to inhibit PHDs through oxidation to ferric iron or ascorbate depletion (Page et al. 2008). Furthermore, the transcriptional activity of HIFs is regulated at the C-terminal region via two transcriptional activation domains (TAD) known as N-TAD and C-TAD. Precisely, the C-TAD activity is subjected to regulation by the factor inhibition HIF-1 protein (FIH-1) that hydroxylates HIF in an asparagine residue. This hydroxylation abrogates the transcriptional activity by impairing the interaction with other co-activators such as p300 and CBP. In non-hypoxic conditions, HIFs can also be activated by oncogenes and growth factors so as to allow tumor cells to enhance angiogenesis before oxygen deprivation.

Differences between Physiological and Tumor Neovascularization

In physiological conditions, most adult blood vessels remain quiescent, with a minimum rate of endothelial cell proliferation for the purpose of maintaining cell turnover and vascular integrity. Angiogenesis is limited to the high metabolic demands of growing tissues or wound healing and tissue repair. During adulthood, three different locations of the female reproductive organs comprise the few adult tissues requiring ongoing angiogenesis: (i) monthly, during the reproductive cycle, so as to rebuild the uterus lining; (ii) in the ovaries during egg maturation in ovulation; and (iii) during pregnancy in order to synthesize the placenta (Jaffe 2000). Particularly, follicular growth and corpus luteum development depend entirely on angiogenesis, which allows initial rapid corpus luteum growth and later the regression of the follicular blood vessels. A coordinated and time-regulated action of inducers and inhibitors of angiogenesis regulates the course of the ovarian cycle (Goede et al. 1998). VEGF is regarded as the master player during vascular growth in ovarian function, with its expression temporally and spatially associated with blood vessel proliferation in the ovary and occurring first in perivascular cells. Besides, nitric oxide, a potent vasodilator and stimulator of VEGF production, is released by endothelial cells of luteal arterioles and capillaries. Therefore, a paracrine signaling loop is established between perivascular cells, which produce VEGF, and endothelial cells, which produce nitric oxide, ensuring a coordinated regulation of angiogenesis and vasodilation (Reynolds et al. 2000).

Even though most of the vascular plexus remains quiescent in the adult tissues, endothelial cells retain the ability of dividing rapidly in response to a physiological stimulus such as inflammation or hypoxia. For instance, during wound healing, angiogenesis is reactivated for the regeneration of damaged tissues. In this context, several proangiogenic factors such as VEGF and Ang-2 are rapidly overexpressed, whereas

Fig. 3 (continued) HIF-1β subunit activates the binding to hypoxia response elements (HRE). (**b**) Main biological processes and genes regulated by HIF mediated transcription. HIF-dependent processes include migration, energy metabolism, angiogenic signaling, and proliferation among others

Ang-1 is downregulated with similar kinetics, allowing the destabilization of preexisting vessels and the formation of new capillaries. Finally, VEGF and Ang-2 decrease to baseline levels to allow immediate maturation and stabilization of the new vessels. The whole process is finely controlled in a specific temporal and spatial sequence that results in a tightly regulated balance of pro- and anti-angiogenic molecules (Bloch et al. 2000). As a result of the tuned balance, these physiological processes lead to the formation of a stable and functional vascular tree.

Contrarily, an alteration of the equilibrium between negative and positive regulators of angiogenesis promotes abnormal vessel growth, as seen in many pathological conditions. The shift in the balance can lead to either excessive or defective angiogenesis that can exacerbate or worsen the pathological symptoms. As explained in the previous sections, the best-known condition in which angiogenesis is switched on is cancer disease. However, other examples of excessive angiogenesis occurring when the diseased cells produce abnormal amounts of growth factors include, among others, ocular and inflammatory disorders, obesity, diabetes, cirrhosis, or asthma. By contrast, insufficient angiogenesis is related to vessel dysfunction and to diseases like osteoporosis, ischemic heart disease, or preeclampsia (Carmeliet and Jain 2011).

Although physiological and pathological angiogenesis share most molecular mechanisms, they differ in many features. Several lines of evidence indicate that molecules like cyclooxigenase-2, proteases, TSP-2, and placental growth factor are specifically involved in pathological neovascularization. Moreover, pathological angiogenesis is normally determined by inflammation or hypoxia and is, therefore, characterized by macrophage and leukocyte infiltration in the diseased tissues. For instance, tumors where described by Dvorak in 1986 as "wounds that never heal."

Differences between Normal and Tumor Vessels

Both at the morphological and functional levels, tumor vessels display unique characteristics that make them different to the normal vasculature (Fig. 4). The tumor microenvironment is characterized by uncontrolled and continuous overproduction of angiogenic factors. Such an extreme stimulation of the endothelium leads to the development of immature and structurally and functionally abnormal vasculature (Goel et al. 2011). In fact, the tumor vascular tree is chaotic, populated by dead-end vascular branches and areas of intermittent and inverted blood flow that impairs the vascular function and punctually leads to regions of lowered perfusion and subsequent hypoxia (Baluk et al. 2005). The resulting irregular perfusion impedes nutrient, oxygen, and drug delivery. Vessel-poor regions are followed by highly dense areas, and, when looked at the microscope, tumor vessels vary from irregular, abnormally wide, dilated, and tortuous serpentine-like shapes, with uneven diameter and excessive branching, to thin capillaries with small lumens. Furthermore, every layer of the tumor vessel wall is also abnormal. Endothelial cells of the tumor vasculature are poorly interconnected, lacking a cobblestone appearance and forming occasional multilayers. Moreover, tumor vessels are characterized by an irregular basement membrane and a lack of functional perivascular cells, which renders them leaky and with numerous openings, widened interendothelial junctions and discontinuous basal membrane. This leads to increased permeability to circulating molecules and even to entire cells, promoting tumor cell intravasation, dissemination, and metastasis. Another frequent outcome of hyperpermeability is an increase in interstitial pressure in the tumors, thereby impeding nutrient and drug distribution, and extravasation of erythrocytes, a process known as microhemorrhaging. At the molecular level, endothelial cells from tumors have been reported to upregulate different genes if compared to normal endothelial cells, termed tumor endothelial markers or TEMs (St Croix et al. 2000).

The structural abnormalities of tumor vessels are consequences of the pathological imbalance of activators and inhibitors of angiogenesis. Molecular studies have shown a marked upregulation of VEGF mRNA in the majority of human tumors (Ferrara et al. 2003). VEGF released by tumor and

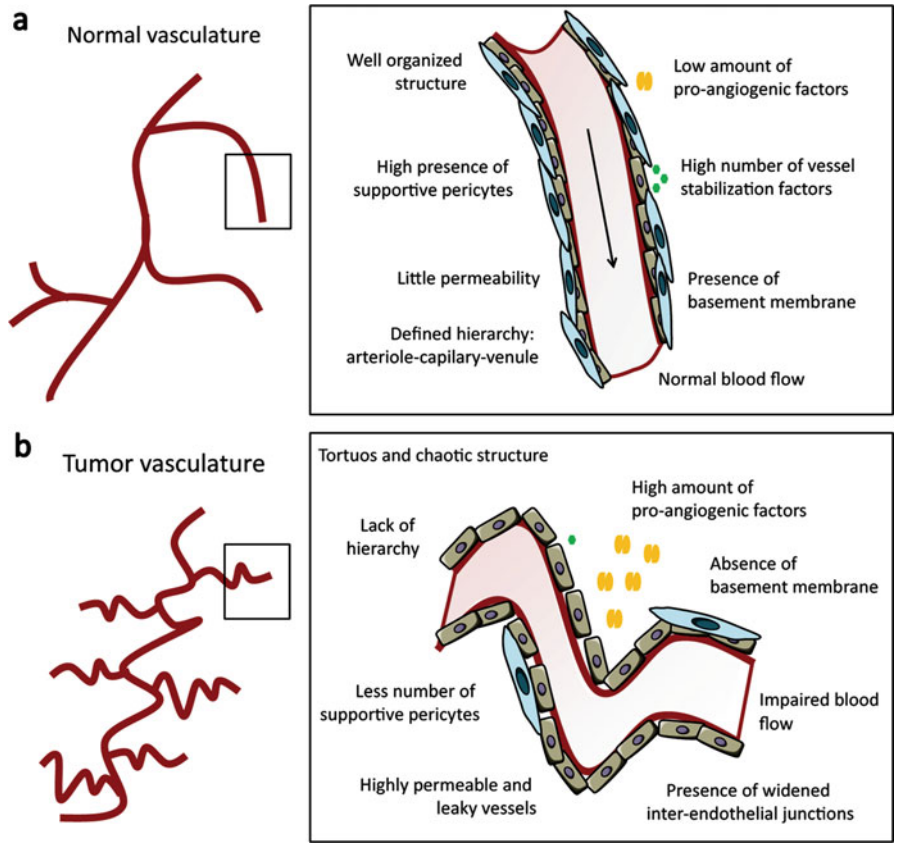

Fig. 4 Schematic differences between (**a**) normal and (**b**) pathological angiogenesis. The uncontrolled overproduction of angiogenic factors in (**b**) pathological conditions leads to an immature and structurally and functionally altered vasculature which is characterized by chaotic and tortuous vascular branches, regions of hypoxia, irregularities in the basement membrane, and lack of coverage of perivascular cells, among others. By contrast, (**a**) the normal vasculature has a well-organized structure, normal blood flow, low amount of proangiogenic factors, and a high presence of supportive pericytes

stromal cells not only triggers proliferation of endothelial cells and sprouting angiogenesis but also promotes increased vascular permeability, which renders tumor vessels highly leaky. Furthermore, tumor-produced Ang-2 mediates in the dissolution of endothelial junctions, while proteases digest the basement membrane and the ECM, thus allowing endothelial cell migration and sprouting. The sustained imbalance in the production of proangiogenic factors and the persistent lack of vessel-stabilizing factors produces an immature and dysfunctional vascular network that resembles a vessel structure that is not able to cope with the rapid growth rate of the expanding tumor mass (Baluk et al. 2005).

Conclusion

Tumor angiogenesis is a well-established hallmark of cancer. Starting with the archetypal sprouting angiogenesis and ending with the less familiar vasculogenic mimicry, the understanding of the different mechanisms that drive the angiogenic process is essential for successful therapeutic targeting. The main biological processes involved in angiogenesis progression are endothelial cell proliferation, vessel guidance, maturation, stabilization and quiescence, and, finally, regression. The interplay between the main molecular families composing each step of the vessel-branching

process is crucial to understand the chemical and physical changes that endothelial cells and their surroundings undergo both in normal and pathological conditions. Moreover, aberrant regulation of some of the molecules involved in normal angiogenesis, such as VEGF, FGF, or MMPs, is critical for the engagement of the angiogenic switch and the subsequent tumor progression. In this context, the role of intratumoral hypoxia as a catalyst of the overproduction of proangiogenic molecules both by tumor and stromal cells is emphasized. However, even though the molecular pathways followed both in normal and pathological angiogenesis are shared, the morphology of the newly formed vascular tree in the tumor stroma is completely different due to an aberrant imbalance of proangiogenic and anti-angiogenic molecules. In conclusion, a profound knowledge of the mechanisms, mediators, and main players of the angiogenic process, together with the focus on the main differences between physiological and tumor angiogenesis, is decisive for an effective development of therapeutic strategies.

Cross-References

- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Mechanisms of Anti-angiogenic Therapy
- Pathology of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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The Role of VEGF in Controlling Vascular Permeability

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Contents

Introduction 34

Vascular Endothelial Growth Factors (VEGFs) and Their Receptors 35

Features Regulating Vessel Integrity 38
Basal Permeability 38
Endothelial Fenestrations 39
The Glycocalyx 39
The Blood-Brain Barrier (BBB) 39
The Vesiculo-Vacuolar Organelle (VVO) 40
Endothelial Junctions in Lymphatic and Blood Vessels 40

Blood Flow Regulation: Implication for Basal Sieving 42

VEGF-Induced Transient Vessel Leakage 42
Leakage of Molecules 43
Leakage of Cells 43

Vascular Leakage in Disease 43
Vascular Leakage and Cancer 43
Vascular Leakage and Myocardial Pathology 44
Vascular Leakage in Ocular Diseases 44
Lymphatic Neoangiogenesis and Cancer 44

Imaging Vascular Flow and Integrity 45

Perspectives 46

Acknowledgments 46

Cross-References 46

References 46

Abstract

Blood vessels in different organs have vastly different morphologies and functions. One important aspect of vessel heterogeneity is its exchange with the surrounding tissue. While vessels in the CNS are highly restricted in their

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exchange, vessels in peripheral organs may be quite permeable and allow solvent and small molecules to pass across the vessel wall. A more extensive permeability, or leakage, can be induced in an acute, transient manner by specific factors, with the purpose to deliver blood constituents to the interstitial space. The interstitial fluid is drained by the lymphatic vasculature and eventually delivered back to the blood circulation via the subclavian veins. Larger volumes of accumulated interstitial fluid, edema, are a sign of extensive leakage and/or poor uptake of fluid by the lymphatics. Through the continuous blood and lymphatic circulation, the maintenance of tissue homeostasis is ensured through the delivery of oxygen and nutrients to the tissues. In pathologies, the vasculature is often affected by, and engaged in, the disease process. This may result in excessive formation of new, unstable, and leaky vessels with poor blood flow and tissue swelling potentially exacerbated by poorly functioning lymphatics. Elevated interstitial pressure, hypoxia, and a chaotic tissue microenvironment promote the disease. This review is focused on the role of vascular endothelial growth factors (VEGFs) and their receptors in the control of vessel integrity.

Keywords
VEGF · Permeability · Edema · Flow · Pore · Junction

Introduction

Endothelial cells, key constituents of blood and lymphatic vessels, rest with their basolateral side on a specialized basement membrane, while their apical side faces the blood and the lymph, respectively. The main function of the blood vasculature is to serve as a conduit for the blood to ensure efficient oxygenation and nutrition of tissues. The lymphatic vasculature is pivotal for a range of homeostatic functions such as maintenance of a physiological interstitial pressure, lipid transport, and immune surveillance (Lanitis et al. 2015).

The blood vasculature is stable and its endothelial cells turn over very slowly in the healthy individual (Lee et al. 2007). During particular physiological responses such as embryo development, ovulation, and regrowth of the endometrium, or in conjunction with injury or disease, there is a need for new vessels to form, to nurture the growing or regenerating tissue. In fact, the growth of all new tissues, whether healthy or not, is accompanied by blood vessel formation, neoangiogenesis. A main underlying mechanism in neoangiogenesis is the relative hypoxia in the growing tissue (Liao and Johnson 2007), which drives expression of a wide range of growth factors including vascular endothelial growth factor (VEGFA), described below. VEGFA is essential in stimulating formation of new vessels and in survival of existing ones (Simons et al. 2016). During embryogenesis, vessels form de novo in a process denoted vasculogenesis, while angiogenesis implies vessel formation from the preexisting vasculature. The newly formed vasculature undergoes remodeling, also denoted "pruning," to form a hierarchical order consisting of arteries, capillaries, and veins. Pruning may involve apoptosis, cell death, of endothelial cells in vessels that lack flow; alternatively, it depends on the local motility of endothelial cells to reshape the vessels to new dimensions and densities to meet the needs of the tissue (Korn and Augustin 2015).

Blood vascular endothelial cells in different blood vessels and in different organs have distinct functions, and display in part, unique gene expression patterns (Augustin and Koh 2017). Thus, certain molecules such as neuropilins and members of the Eph family of receptor tyrosine kinases are preferentially expressed in arteries and not in veins, while expression of other molecules is restricted to veins. Other distinguishing hallmarks of different vessel categories are their different perivascular mural cell supports, their typical dimension, and their particular blood flow velocities. While arteries are surrounded by a multilayered coat of α-smooth muscle actin expressing mural cells (smooth muscle cells), veins are sparsely covered by smooth muscle cells as well as a distinct type of mural cell, the pericyte.

Pericytes also support capillaries (Bergers and Song 2005). Blood flow is about ten times faster in arteries than in veins and capillaries (Wayland and Johnson 1967; Ma et al. 1974). Moreover, veins, but not capillaries or arteries, are equipped with valves to prevent backflow of the blood. Endothelial cell junctions are essential in regulating the exchange between the blood and the surrounding tissue. Endothelial cell-specific adherens junctions can dissolve to permit extravasation of blood components, while tight junctions provide a persistent barrier in a vessel- and organ-type-specific manner (Dejana et al. 2001).

The lymphatics are organized in capillaries which drain unidirectionally into larger, collecting vessels. The collecting vessels bring the lymph to a sentinel lymph node from which it is carried further to eventually be drained into the subclavian vein. Lymphatic capillaries are blind-ended tubes that open up with increased interstitial pressure. The increased pressure acts to open up the lymphatic capillary by pulling on filaments anchoring the capillary to the surrounding connective tissue (Stacker et al. 2014). Collecting vessels are surrounded by an incomplete basement membrane and a thin layer of smooth muscle cells that contract and relax to propagate the lymph. Lymphatic valves in the collecting vessels prevent backflow of the lymph. Although blood and lymphatic endothelial cells are morphologically similar and share several specialized functions, they also have distinct features such as unique molecular expression patterns. This is particularly noticeable when studying endothelial cells of blood and lymphatic origin in vivo, compared to cultured cells (Wick et al. 2007). Lymphatic endothelial cells in different tissues have distinct developmental origin, but whether this reflects unique functions is not known (Potente and Makinen 2017).

Vascular Endothelial Growth Factors (VEGFs) and Their Receptors

The polypeptide growth factor VEGF was initially denoted vascular permeability factor (VPF) implying its essential role in regulation of the vascular barrier (Senger et al. 1983). The originally discovered VEGF, now denoted VEGFA, was subsequently found to be a member of a larger family of related factors. The VEGF family consists of five mammalian factors, VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF). Of these, genetic inactivation of VEGFA and VEGFC in the mouse results in embryonic death due to defects in the development of blood vessels and lymphatics vessels, respectively. Another important feature of VEGFA is that its expression is regulated by the oxygen tension in the tissue (Ferrara 2005).

In addition, several nonmammalian VEGF-related molecules, denoted VEGFE, VEGFF, and VEGFG, have been described (Shibuya 2011). Structurally, the VEGFs are homodimeric polypeptides arranged in an antiparallel fashion, presenting one receptor-binding domains at each "pole" of the dimer (Wiesmann et al. 1997).

The VEGFs bind to three different but structurally related receptor tyrosine kinases denoted VEGF receptors 1–3 (VEGFR1, VEGFR2, VEGFR3). Although their expression patterns are not exclusively restricted to the vasculature, VEGFR2 is preferentially expressed on blood vascular endothelial cells, while VEGFR3 is primarily expressed on lymphatic endothelial cells. However, VEGFR2 is also expressed on lymphatic endothelial cells, and VEGFR3 expression is induced in newly formed vessels during angiogenesis. VEGFR1 is more broadly expressed also on a range of non-endothelial cells and is essential in regulating the motility of leukocytes. However, much less is known about VEGFR1 than the other VEGF receptors, due to the poor kinase activation of VEGFR1 in response to VEGF and due to lack of good reagents such as highly specific antibodies. Thus, the role of VEGFR1 is more unclear although there are indications that it serves primarily as a negative regulator of VEGFR2. For a detailed review on VEGF receptors, see Koch et al. (2011).

VEGFA, VEGFB, and PlGF exist as alternative splice variants which regulate their interactions with heparan sulfate and other co-receptors such as the neuropilins (Vempati et al. 2014). Co-receptors are molecules that lack intrinsic

enzymatic activity, which bind VEGF family members and sometimes also the VEGF receptors, thereby stabilizing the ligand-receptor complex, prolonging its activity. Possibly co-receptors may also influence the folding of the tertiary structure of the ligands or receptors, thereby modulating the downstream signaling. Moreover, co-receptors may have other unique functions. Several splice variants of VEGFA, denoted VEGFA121, VEGFA165, and VEGFA189 (numbers indicating the number of amino acid residues in the splice variant), have been shown to differently interact with the VEGF co-receptors and, therefore, to induce different biological responses. Thus, expression of VEGFA120 (mouse numbering) alone results in delayed outgrowth and abnormal patterning of the retinal vascular plexus compared to the wild-type condition (Stalmans et al. 2002). VEGFC and VEGFD on the other hand undergo proteolytic processing, regulating interactions with the VEGF receptors (Vaahtomeri et al. 2017).

In conclusion, none of the VEGFRs bind all the VEGF family members. VEGFR1 binds VEGFA, VEGFB, and PlGF. VEGFR2 binds VEGFA and processed VEGFC and VEGFD. VEGFR3 binds both processed and mature VEGFC and VEGFD.

Binding of VEGF to its VEGF receptor leads to receptor dimerization, activation of the intracellular tyrosine kinase activity, and tyrosine phosphorylation of both the receptor itself and of intracellular substrates for the kinase, so-called signal transducers. Through transient molecular interactions between the phosphorylated receptor and the signal transducers, mediated through their relatively specific binding motifs such as Src homology 2 (SH2) domains that recognize phosphorylated tyrosine residues, signal transduction chains are created. Through such signal transduction chains, signals can be propagated from the activated receptor to different compartments in the cell, to eventually result in a cellular response. A number of phosphorylation sites in VEGFR2 have been identified (Matsumoto et al. 2005). Several of these phosphorylation sites have been studied in loss-of-function analyses by phenylalanine knock-in, in vitro using transfected cell lines or in vivo, using recombinant mice (Sakurai et al. 2005; Li et al. 2016). The Y949 site in the VEGFR2 kinase insert is critical in regulation of vascular leakage. It serves as a binding site for the SH2 domain of T cell-specific adaptor (TSAd), which uses its proline (P)-rich domain to bind to the cytoplasmic tyrosine kinase c-Src (Sun et al. 2012). c-Src is then translocated to endothelial cell junctions where it phosphorylates the adherens junction-specific protein vascular endothelial (VE)-cadherin (see Fig. 1). Other pathways involving p21-activated kinase (PAK) phosphorylation of VE-cadherin on serine residues have also been identified (Gavard and Gutkind 2006). Interestingly, at least in vitro, VEGFR2-dependent signal transduction is suppressed by intact adherens junctions and augmented when VE-cadherin's homophilic interactions are interrupted (Lampugnani et al. 2006).

VEGFR2's enzymatic activity can be induced by shear stress exerted by blood flow, in the apparent absence of ligand (Jin et al. 2003). Induction of VEGFR2 activity may be dependent on c-Src activity which also is induced by flow (Jalali et al. 1998). Whether the flow-activated VEGFR2 transduces a full downstream signaling effect as compared with the ligand-activated receptor is unclear. Together with platelet endothelial cell adhesion molecule-1 (PECAM-1) and c-Src, VEGFR2 forms a mechanosensing complex (Tzima et al. 2005). Engagement in such complexes may be a prerequisite for both VEGFR2 and c-Src to be activated by flow.

Replacement of tyrosine (Y) at position 949 for phenylalanine, thus preventing phosphorylation and downstream signal transduction, does not interfere with normal mouse development, but it renders endothelial junctions unresponsive to VEGFA (Li et al. 2016). In the wild-type, normal condition, exposure of endothelial cells to VEGFA results in increased leakage of solvent and molecules. In contrast, in a mouse expressing $Vegfr2^{Y949F/Y949F}$, the receptor is unable to couple to TSAd and relocate c-Src to endothelial cell junctions when exposed to VEGFA. The junctions remain closed, and there is no leakage of solvent or molecules (Li et al. 2016). *Tsad* gene inactivation, globally or specifically in endothelial cells, also makes endothelial junctions unresponsive to

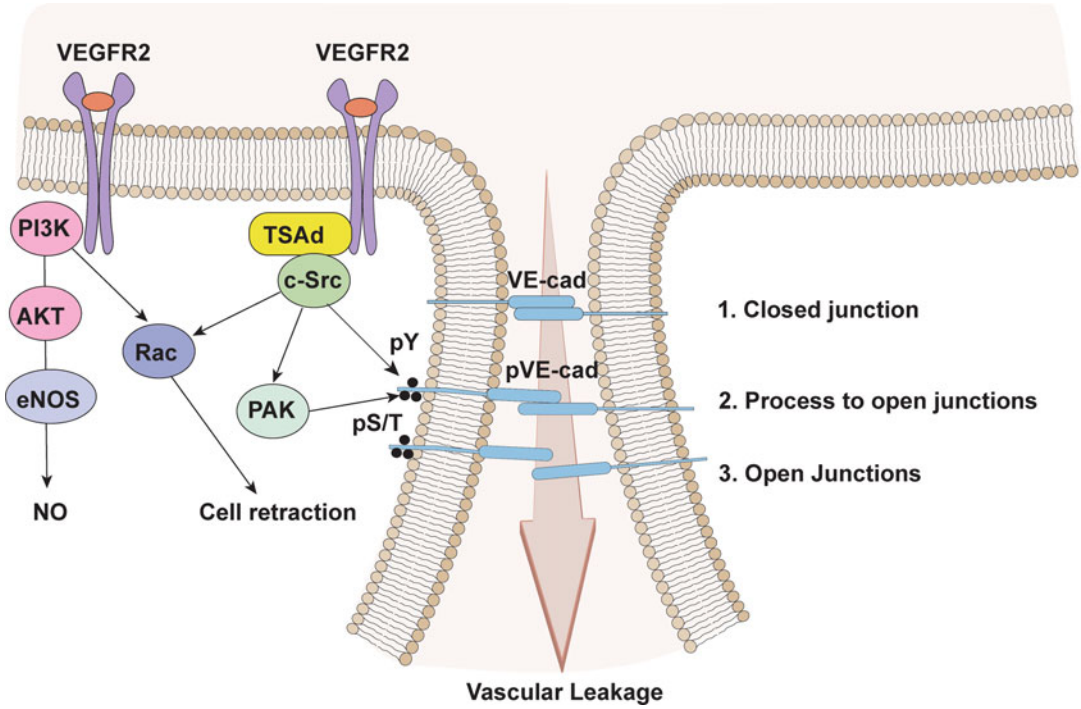

Fig. 1 Signal transduction regulating VEGFA-induced vascular leakage. VEGFR2 expressed on the surface of blood vascular endothelial cells becomes activated when binding VEGFA, resulting in induction of at least two main signal transduction chains, promoting opening of adherens junctions. One involves binding of TSAd/SRC, leading to increased tyrosine phosphorylation of VE-cadherin (VE-cad), interrupting its homophilic interactions. In another chain, PI3K promotes activation of AKT, leading to phosphorylation of eNOS and production of NO. PI3K also promotes activation of Rac which has multifaceted effects via the cell cytoskeleton leading to cell retraction. SRC can also regulate activation of PAK, leading to serine phosphorylation of VE-cadherin. For details, see the text. (*TSAd* T cell-specific adaptor, *PI3K* phosphoinositide 3'kinase, *eNOS* endothelial nitric oxide synthase, *PAK* p21-activated kinase)

VEGFA, resulting in loss of VEGF-induced vascular leakage (Sun et al. 2012).

Several studies from other laboratories implicate c-Src in phosphorylation of VE-cadherin (Weis et al. 2004; Eliceiri et al. 1999). According to the model, c-Src-induced phosphorylation of VE-cadherin disrupts VE-cadherin contacts between adjacent endothelial cells, followed by internalization and degradation or recycling of VE-cadherin (Fukuhra et al. 2006). c-Src may act to open adherens junctions not only by directly phosphorylating VE-cadherin. In a parallel signal transduction pathway, c-Src phosphorylates and activates focal adhesion kinase (FAK), which acts to anchor the actin cytoskeleton to focal adhesion sites. Focal adhesion sites are hotspots where integrin molecules in the plasma membrane bind specific extracellular matrix proteins, thereby anchoring the cell to the underlying substratum. The tension induced through the change in cell-matrix adhesion may pull on actin filaments, mediating retraction of the cell body, and pulling junctions apart.

The other VEGFR2 phosphorylation sites induce signaling pathways that may also contribute to vascular permeability regulation although this has not been directly addressed. These sites include Y1173 (Y1175 in the human VEGFR2) and Y1212 (Y1214 in the human VEGFR2). Phosphorylated Y1175 binds phospholipase C (Sakurai et al. 2005), as well as the SH2 domain molecule Shb (Funa et al. 2009). Potential binding partners for phosphorylated Y1212 have been less extensively studied. For details on their

downstream pathways, the reader is referred to Koch et al. (2011).

Whether other growth factors for which there are receptors on endothelial cells, such as PlGF (binding exclusively to VEGFR1), VEGFC/VEGFD (binding exclusively to VEGFR3), or fibroblast growth factors (FGFs, binding to FGFR1 and FGFR2), mediate acute or chronic vascular permeability has not yet been addressed in detail. A key question is whether effects are directly transduced by these factors or whether it is indirect and dependent on elevated production of VEGFA.

The angiopoietin receptor, Tie2, exerts negative regulation of VEGFA-induced vascular leakage in response to its ligand angiopoietin-1 (Ang1) (Brindle et al. 2006), through complex biology. One important effect of Ang1/Tie2 is to attract pericytes to increase the vascular support (Thurston et al. 1999). Ang1 may also stabilize junctions by promoting the recruitment of Tie2 to junctions (Saharinen et al. 2008).

The related Ang2 on the other hand may cause vessel disintegration resulting in loss of vascular integrity and massive vascular leakage, independent of VEGFA. Ang2 exerts antagonistic effect on vascular integrity in a manner dependent on Tie1. When Tie1 is lowly expressed or cleaved (Korhonen et al. 2016; Kim et al. 2016), Ang2 can act as a Tie2 agonist, rather than an antagonist. Other factors acting independently of VEGFA include inflammatory cytokines such as histamine and bradykinin, which are potent mediators of vascular leakage in inflammation. Histamine is produced by mast cells and binds to G-protein-coupled H1 and H2 histamine receptors (GPCRs) on endothelial cells (Marshall 1984). Bradykinin is cleaved from kininogen; it acts via GPCRs B1 and B2 (Sharma and Al-Dhalmawi 2003). Although other mechanisms have not been excluded, it is quite well established that exposure of vessels to either histamine or bradykinin results in activation of endothelial nitric oxide synthase (eNOS) and consequent production of NO which acts to relax vascular smooth muscle cells (Fig. 1). The relaxation results in a reduced vasotone, i.e., a widening of the vessel lumen.

The eNOS-NO pathway is strongly implicated also in VEGFA-regulated vascular leakage since ablation of eNOS expression attenuates responsiveness to VEGFA (Fukumura et al. 2001). NO may act directly on VE-cadherin to regulate its phosphorylation status, at least in vitro (Di Lorenzo et al. 2009). Another effect of NO that may affect vessel leakage is its S-nitrosylation of beta-catenin that will cause beta-catenin to dissociate from VE-cadherin, triggering the disassembly of adherens junctions (Thibeault et al. 2010).

Features Regulating Vessel Integrity

Basal Permeability

The CNS vascular barrier is guarded by specialized junctions that are impermeable in the healthy condition (see further below). In contrast, in non-CNS organs, there is a continuous basal vascular sieving (i.e., permeability) of solvent and small molecules from blood into tissues, which occurs in an "unstimulated" setting, i.e., in the absence of an elevated production of factors that promote leakage of blood constituents, such as VEGF (see below). Plasma proteins smaller than 40 kDa may extravasate spontaneously (Egawa et al. 2013), in a manner dictated by the glycocalyx (see below, "The Glycocalyx"), whereas leakage of larger molecules is restricted in a size-dependent manner. Passage of cells does not occur in the resting, unstimulated vasculature.

The mass of plasma solvent and solvents that crosses the vascular wall depends on at least three different factors as described previously (Dvorak 2010): (1) hemodynamic forces, i.e., blood pressure and blood flow, (2) concentration gradients of molecules and vascular area available for exchange, and (3) the intrinsic permeability of the vascular wall. Of note, solvent may also leak from tissues into the blood, dependent on these parameters.

In peripheral organs, blood vessels of all types may allow basal vascular permeability; however, it is probable that it is less prevalent in arteries as their intrinsic permeability is lower. The constant

sieving of solvent and small molecules is important in maintaining the interstitial pressure in the tissue. It also serves to maintain the immune surveillance function of the lymphatics as interstitial fluid collected by the lymphatics is carried via lymphatic capillaries to lymph nodes where foreign antigens will be exposed to the immune system (Cueni and Detmar 2008).

Whether lymphatic vessels also show a continuous basal sieving of solvent and small molecules has not been directly addressed, but it is likely to occur. In pathologies, lymphatic endothelial junctions become "leaky" in an Ang2-dependent manner, leading to changes in VE-cadherin phosphorylation (Zheng et al. 2014).

Endothelial Fenestrations

Endothelial cells in many vessels form an uninterrupted vasculature. In certain organs, however, the endothelial cells display specialized structures to facilitate rapid transport across the endothelium. See Tse and Stan (2010) for a detailed description. One example is the fenestrated endothelium that is present in vessels in endocrine glands, digestive tract mucosa, and kidney peritubular capillaries. Fenestrations are regions where the apical and basolateral endothelial membranes are fused to create circular pores that may be covered by a diaphragm. A key protein in the diaphragm is plasmalemmal vesicle protein-1 (PV1), organized in radial fibrils. Loss of PV1 does not prevent formation of fenestrae as such but results in loss of the diaphragm and severe leakage of plasma proteins (Stan et al. 2012).

There are naturally occurring fenestrae, or gaps, without diaphragm, i.e., in the kidney glomerulus (Tse and Stan 2010). The sinusoidal endothelium in the liver and the bone marrow also presents large gaps without a diaphragm. These gaps are heterogeneous but of larger diameter than the endocrine vessel fenestrae. Signaling through the actin cytoskeleton has been shown to regulate the diameter of these openings and thereby regulate vascular barrier function (Venkatraman and Tucker-Kellogg 2013; Braet et al. 1995).

The Glycocalyx

The glycocalyx is a carbohydrate-rich layer lining the vascular endothelium which long escaped detailed studies as it often was lost during fixation procedures in preparation for microscopy; moreover, endothelial cells in culture do not form a glycocalyx. While its exact composition has not been defined, the glycocalyx consists of a membrane-bound mesh of proteoglycans, glycoproteins, and glycosaminoglycans, which along with trapped plasma proteins and soluble glycosaminoglycans form an extensive three-dimensional structure extending into the vessel lumen. Rather than being static, the glycocalyx components are continuously turned over (Reitsma et al. 2007). The glycocalyx is vulnerable to insults such as inflammation, trauma, and hemorrhagic shock, which leads to exposure of the underlying endothelium to the insult. The glycocalyx influences mechanotransduction, hemostasis, and blood cell-vessel wall interactions. In particular, the glycocalyx is an important determinant in vascular permeability and selectivity properties of the vascular wall. Thus, the glycocalyx forms the principal molecular sieve at the endothelial wall, where the spacing between fibers in the glycocalyx allows penetration of molecules up to the size of albumin (Curry 2005).

The Blood-Brain Barrier (BBB)

The BBB is a unique barrier with the purpose of preventing the brain from exposure to the blood and the adverse consequence of edema, which may be detrimental for the tightly enclosed brain. The brain vasculature has, in addition to adherens junctions, also high resistance tight junctions and an abundant basement membrane. Perivascular components such as astrocytes, pericytes, and neurons participate functionally in creating the BBB (Paolinelli et al. 2011). A unique feature of the BBB is the transendothelial vesicular transport of a range of nutrients and metabolic waste products (Strazielle and Ghersi-Egea 2013). There is a keen interest from the pharmaceutical industry to find strategies to interrupt the BBB for

drug delivery. There is still limited information on to what extent the BBB can be transiently opened in response to growth factors and inflammatory cytokines (Hudson et al. 2014).

The Vesiculo-Vacuolar Organelle (VVO)

Based on the use of various tracers, for example, electron-dense ferritin, VVOs have been implicated as a possible pathway for macromolecular extravasation (Kohn et al. 1992). The VVO has been described and interpreted using transmission electron microscopy analyses, which have shown that VVOs are prominent structures in both tumor-supplying and normal vessel endothelial cells (Dvorak and Feng 2001).

There is general consensus on the notion that vesicular transport across the endothelium (transcytosis) is an important mechanism for delivery of macromolecules to tissues, in particular in the CNS. During transcytosis, caveolae, specialized regions in the plasma membrane (PM), "pinch off" from the PM to form discrete vesicular carriers that shuttle to the opposite side of the endothelium where vesicles fuse with the PM and discharge their cargo into the perivascular space. Endothelial transcytosis may occur in specialized vascular beds or under particular physiological conditions. Transcytosis has been described in the brain vasculature, and it is elevated under conditions when the BBB is disrupted due to pericyte deficiency (Armulik et al. 2010). VVOs may be one possible mechanism for transcytosis.

Vesicles and vacuoles that make up the VVO were originally thought to derive from caveolae. A main protein in caveolae is caveolin-1. While caveolin-1 knockout mice lacked caveolae and showed reduced permeability to macromolecules, the vasculature still contained VVOs (Chang et al. 2009). The exact composition of the VVO is therefore presently not known. A challenge in further analyses of VVOs is that they cannot be detected by conventional light microscopy. Moreover, there is at present no genetic loss-of-function model to study VVOs.

Endothelial Junctions in Lymphatic and Blood Vessels

Endothelial junctions play an important role in the regulation of passage of solvent, molecules, and cells across the vessel wall. In most organs, the endothelial cells form a dynamic barrier between the blood and the tissue. In resting conditions, the vasculature continuously leaks solvent and small molecules (basal sieving; see "Basal Permeability"), but restricts extravasation of larger molecules and cells. In many diseases, including cancer and chronic inflammatory conditions, the vascular barrier disintegrates, and leakage increases and may become chronic. The leakage of larger molecules and cells results in edema, inflammation, and, often, disease progression (Nagy et al. 2008).

In blood vessels, endothelial junctions consist of tight junctions and adherens junctions. Both types of junctions express proteins unique for blood endothelial cells as well as common junction proteins seen also in epithelial cell-cell junctions. Claudin-5 is preferentially although not uniquely expressed in endothelial cells. In the CNS, Claudin-5 has a critical function in maintaining the BBB (Argaw et al. 2009). In contrast, other tight junction proteins such as zona occludens1 (ZO1; also denoted tight junction protein-1) are more broadly expressed in endothelial and epithelial cells. There is still incomplete understanding of the composition of the endothelial tight junction, which may vary between different types of endothelial cells in arteries, capillaries, and veins and also between different vascular beds such as in the CNS and in peripheral organs. It is also unclear to which extent the tight junction barrier can be regulated by exogenous factors, i.e., made more or less stringent. There appears to be a molecular communication between adherens junctions and tight junctions, for example, via VE-cadherin and ZO1 in vitro (Tornavaca et al. 2015), but it remains to be shown that this communication occurs also in vivo.

The main component of the endothelial-specific adherens junction is VE-cadherin (Dejana et al. 1999). In contrast to tight junctions, adherens junctions can be induced to dissolve in

a specific and transient (in physiology) or chronic (in disease) manner, allowing leakage to occur (see below). The dissolution involves interruption of homophilic interactions between VE-cadherin molecules on opposing endothelial cells, followed by internalization of VE-cadherin. There are several VE-cadherin-associated molecules of critical importance for adherens junction maturation and stability: (1) p120-catenin which connects VE-cadherin to members of the Rho GTPase family (Kourtidis et al. 2013), (2) alpha-catenin which connects VE-cadherin with the actin cytoskeleton via binding to p120 and beta-catenin (Brieher and Yap 2013), (3) beta-catenin which when released from the VE-cadherin complex can act as a transcriptional regulator through the Wnt/Frizzled family of ligands and receptors (Valenta et al. 2012), and (4) plakoglobin (also denoted gamma-catenin). For details, see Dejana et al. (2008).

As mentioned above, the vasculature in the CNS is equipped with a particular strong barrier, the blood-brain barrier (BBB) (Paolinelli et al. 2011), to protect the brain parenchyma from detrimental edema. The detailed composition of the specialized tight junctions protecting the CNS vasculature is not yet known.

Junctions between lymphatic endothelial cells vary in morphology and function dependent on the vessel type (Baluk et al. 2007). Lymphatic capillary cell-cell contacts are denoted button junctions based on their discontinuous, oak-leaf morphology. They are considered leaky and can open up by mechanical pulling on lymphatic ligaments that extend radially from the lymphatic capillary (Leak and Burke 1966). Junctions in the collecting lymphatics are denoted zipper junctions, which are continuous junctions of higher integrity. There are also intermediary/transitory junctions in between the capillaries and collecting lymphatic vessels. While lymphatic endothelial cells also express VE-cadherin, as well as a range of tight junction molecules, it is unclear whether lymphatic junctions can be dissolved transiently in a similar manner to adherens junctions in blood vessels.

Adherens junctions dissolve in response to a number of stimuli; while the focus here is on VEGFA, inflammatory cytokines and other factors can also induce dissolution of endothelial adherens junctions. The causative factor may depend on the organ and particular biology, e.g., inflammation vs tissue growth. VEGFA causes a rift in VE-cadherin homophilic interactions, bridging between adjacent endothelial cells (Fig. 1). The rift is induced through a triggering signaling that involves hyperphosphorylation of VE-cadherin. However, in vivo, VE-cadherin is phosphorylated also in the basal, unstimulated state (Orsenigo et al. 2012; Li et al. 2016), possibly through flow-mediated activation of c-Src, which triggers VE-cadherin phosphorylation directly or indirectly (Fig. 1). The additional event induced by VEGFA causing VE-cadherin internalization remains to be identified, but may involve enhanced VE-cadherin internalization or particular intracellular trafficking. Mechanisms different from a direct phosphorylation of VE-cadherin by c-Src have been suggested (Gavard and Gutkind 2006).

VE-cadherin is phosphorylated on at least three tyrosine residues, Y658, Y685, and Y731, which are differently engaged in regulating junctional passage of molecules and cells. Phosphorylated Y685 is required for VEGFA-induced junctional leakage, whereas phosphorylated Y731 is required for passage of inflammatory cells, as deduced from studies of mice lacking individual phosphorylation sites (Wessel et al. 2014). The role of phosphorylated Y658 appears to be related to that of the pY685 site as they are regulated in a similar manner (Orsenigo et al. 2012).

When VEGFA is administered to the healthy tissue, the dissolution of adherens junctions is transient and the junctions will soon close again in part due to VE-cadherin recycling and reappearance on the cell surface (Fukuhra et al. 2006). Using transmission or scanning EM, junctions have been captured in their open state, revealing the kinetics of opening and closure in vivo (Baluk et al. 1997). In diseases characterized by excess vascular leakage, the regulation of junction dynamics is lost and the junctions remain open. This is denoted chronic permeability/leakage (Nagy et al. 2008); see further below.

Other mechanisms that may prevail in regulating junctional integrity in response to VEGFA include the rearrangement of the actin cytoskeleton in a manner that may involve c-Src-mediated activation of small GTPases such as Rac (Fig. 1). Phosphoinositide 3′ kinase (PI3K) activation in response to VEGFA may also orchestrate activation of small GTPases. Retraction of the endothelial cell body involving cytoskeletal rearrangements has been implicated in mediating increased vascular permeability (Majno et al. 1969). Thus, the action of intracellular motor proteins causes cells to contract in a manner that facilitates opening of paracellular junctions. However, the cell retraction hypothesis has been challenged, and the cell shape changes observed have been attributed to a natural recoil process occurring when cell-cell junctions are disassembled (Adamson et al. 2003; Waschke et al. 2004). The role of the actin cytoskeleton needs to be further studied.

Blood Flow Regulation: Implication for Basal Sieving

The basal rate of blood flow, most often estimated from the movement of erythrocytes, is an essential parameter dictating the rate of exchange across the vessel wall (Baskurt et al. 2004; Meininger and Davis 1992). Thus, with increased local blood flow, the basal sieving increases in the permissive vasculature. Blood flow is influenced by a number of parameters such as (1) the type of blood vessel including its mural support and its diameter, (2) the blood volume and its viscosity, and (3) the blood pressure, regulated by the renin-angiotensin II-aldosterone axis and influenced by the elasticity of the vessel wall and the tortuosity and branching of the vessel. A range of factors regulate the local blood flow by affecting the vessel diameter, hence its tone, through constriction or dilation of the arteriolar mural cell coat (Bergers and Song 2005). ATP, angiotensin II, endothelin, and adrenalin all induce vasoconstriction. Dependent on the context, adrenalin can also induce vasodilation. Other factors inducing vessel dilation include adenosine, prostaglandins, and NO. NO is regarded as an essential regulator of vascular permeability as well as vascular leakage, in response to inflammatory cytokines and VEGFA.

NO is produced from arginine by the enzyme eNOS (also denoted NOS3), which belongs to a family of three NOS members, eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). Both eNOS and iNOS are expressed in endothelial cells. While it is clear that eNOS exerts an important function in the vasculature, it has not been shown whether iNOS has a similar role. VEGFA and inflammatory cytokines activate eNOS through phosphorylation by the serine/threonine kinase AKT (Phung et al. 2006; Fulton et al. 1999; Fukumura et al. 2001; Dimmeler et al. 1999) (see Fig. 1). AKT is not the only serine/threonine kinase that can phosphorylate and activate eNOS, but it is the best studied pathway. NO is a potent regulator of the vascular tone; it mediates vasodilation by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells, which causes their relaxation (Forstermann and Sessa 2012).

Local regulation of blood flow is moreover thought to be controlled through precapillary sphincters. In the mesentery, precapillary smooth muscle sphincters have been described, consisting of folds of smooth muscle cells, arranged concentrically and distinct from the perineural coat, located at the point where a capillary branch leaves an arteriola. Based on electron microscopy analyses and a thorough investigation of the literature, Sakai and Hosoyamada concluded that precapillary sphincters are missing from a wide range of other capillary beds (Sakai and Hosoyamada 2013). Thus, how arteriolar resistance is exerted is still a matter of debate.

VEGF-Induced Transient Vessel Leakage

The vasculature is protected from uncontrolled leakage in different manners dependent on the vessel type and anatomical location. Thus, large molecules and cells require an active disruption of the vascular barrier in order to extravasate to the surrounding tissue. Such induced leakage takes

place in peripheral (non-CNS) organs preferentially in postcapillary venules (Majno et al. 1969; Kohn et al. 1992), but capillaries and larger venules may also leak (Roberts and Palade 1995). In contrast, arteries and arterioles do not leak. Overall, studies on the regulation of vascular leakage often suffer from the lack of physiological readouts, that is, instead of following leakage of endogenous substances, various tracers are followed that may or may not be representative of physiological leakage. It is clear, however, that leakage of molecules and cells to some extent is differently regulated.

Leakage of Molecules

Plasma contains three main molecular constituents: albumin, globulins, and fibrinogen (Adkins et al. 2002). Extravasation of macromolecules serves diverse purposes, for example, to maintain the balanced blood and interstitial pressures and to carry other molecules, such as hormones and lipids, across the vessel wall. Extravasated fibrinogen, processed to fibrin, may form a provisional matrix on which new blood vessels extend (Dvorak et al. 1987). Extravasated plasma molecules in peripheral tissues are believed to preferentially pass through opened endothelial junctions.

Leakage of Cells

Junctional gaps appear to be required also for extravasation of inflammatory cells; however, the preferred route of exit for leukocytes and immune cells has been difficult to unequivocally sort out (Vestweber et al. 2014). Inflammatory cells adhere to the endothelium through binding to specific adhesion molecules on the endothelial surface. The cells can then transmigrate directly through the thin endothelial wall or through endothelial junctions (Vestweber 2012; Phillipson and Kubes 2011; Nourshargh et al. 2010). The route of choice might depend on the stimulus, type of leukocyte, and vascular bed. Interestingly, expression of a fusion protein between VE-cadherin and α-catenin in mice resulted in a complete sealing of junctions to macromolecular extravasation (Schulte et al. 2011). Inflammatory cell extravasation was however not completely restricted. Indeed, the extent of immune cell extravasation appeared not to be affected (Schulte et al. 2011). It is possible that different inflammatory cells extravasate through different mechanisms or that the cells are sufficiently plastic to adopt to the possibilities offered in the particular situation. Finally, exit of inflammatory cells may be differently regulated in acute and chronic inflammation.

Vascular Leakage in Disease

Vascular Leakage and Cancer

The tumor vasculature displays a spectrum of morphological and functional abnormalities including loss of vessel hierarchy, increased tortuosity, poor perfusion, instability, and increased vascular leakage (McDonald and Baluk 2005). To a considerable extent, the tumor vessel phenotype is a consequence of hypoxia-driven persistent VEGFA production (Liao and Johnson 2007). Anti-angiogenic treatment, e.g., using VEGFA-blocking antibodies or VEGFR kinase inhibitors, therefore induces a more normal tumor vessel morphology and attenuates the exaggerated permeability (Jain 2005). The therapeutic benefit of anti-angiogenic treatment in prolonging progression-free and overall survival depends on the cancer diagnosis. The reader is referred to in-depth recent reviews on this important matter; see, e.g., Singh and Ferrara (2012). To what extent the potential benefit of anti-angiogenic therapy on growth of the primary tumor and suppression of metastatic spread primarily depends on suppression of vascular leakiness or whether other effects of the treatment, e.g., on neoangiogenesis in the tumor, are more important is very challenging to distinguish.

The excess vascular leakage in cancer has a range of deteriorating effects on the microenvironment of the tumor including increased interstitial pressure leading to impaired therapeutic delivery (Azzi et al. 2013). Moreover, the leaky vasculature may facilitate both leukocyte infiltration into the tumor and escape of tumor cells into the blood to establish distant metastases.

Vascular Leakage and Myocardial Pathology

Tissue damage in myocardial infarction (MI) is triggered by tissue ischemia as a consequence of vessel occlusion and poor blood flow. This in turn leads to induction of VEGFA production and an acute increase in vascular leakage and a consequent tissue edema, impairing the ability of the heart to pump efficiently. Moreover, the increased vessel leakage is manifested as increased infiltration of inflammatory cells in the acute phase after vessel occlusion (Nagy et al. 2008; Weis 2008). One of the first cell types to enter the infarcted myocardium is the neutrophil (Carbone et al. 2013). Neutrophils contribute to tissue damage, e.g., by producing several enzymes that produce reactive oxygen species (ROS) and other tissue-damaging metabolites such as nitrosylated products. Such enzymes include nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and myeloperoxidase (MPO) (Carbone et al. 2013). Elevated MPO levels predict the risk of heart disease in subgroups otherwise associated with low risk (Meuwese et al. 2007; Karakas et al. 2012). Elevated MPO levels also independently predict the early risk of future cardiovascular events in patients with acute coronary syndromes (Baldus et al. 2003; Cavusoglu et al. 2007).

Vascular Leakage in Ocular Diseases

The vasculature in the eye is protected by the blood-retinal barrier (BRB), which is maintained by tight junctions between retinal capillary endothelial (RCE) cells and retinal pigment epithelial (RPE) cells, which form the inner and outer BRB, respectively (Barar et al. 2009). RCE cells possess intercellular tight junctions, which are formed by RCE and glial cells (Gardner et al. 1999). Loss of normal BRB function is a common feature to many retinal degenerative disorders including age-related macular degeneration, diabetic retinopathy, and retinal vein occlusions (Stewart 2012). Age-related macular degeneration patients present focal ischemia in the outer retina with associated inflammation, which induces VEGF production and angiogenesis resulting in vessel leakage. Prolonged elevation of blood sugar concentrations in diabetic patients causes endothelial apoptosis, basement membrane thickening, and pericyte loss, accompanied by increased VEGF production and transvessel passage. Retinal vein occlusions can be attributed to hemodynamic disturbance (increased coagulation, impaired flow properties) resulting in ischemia and increased VEGF synthesis (see Stewart 2012 for details). The common aspects of many eye diseases are therefore ischemia, increased VEGF production, and vascular leakage (Miller et al. 2013). The excess leakage has been attributed both to the overstimulated, abnormal vasculature and to changes in the phosphorylation of tight junction proteins such as occludin and ZO1 (Antonetti et al. 1999).

Lymphatic Neoangiogenesis and Cancer

Lymphatic drainage in the healthy tissue is regulated by the interstitial pressure, opening up the lymphatic capillaries, and possibly by signaling in the blood vasculature resulting in release of cytokines regulating signaling in the lymphatic endothelium (Fig. 2). In cancer, drainage is inadequate in spite of the elevated interstitial pressure and elevated signaling in the blood endothelium, possibly due to the persistent and dysregulated nature of the signaling. Moreover, cancer lymphatic vessels are often collapsed due to the excessive interstitial pressure further exacerbating the edema (Stacker et al. 2014). Several cell types in the cancer produce lymphatic growth factors, including VEGFC that binds and activates VEGFR3 (Adams and Alitalo 2007). Similar to the overstimulated and dysfunctional blood vasculature, the lymphatics may undergo neoangiogenesis in cancer, which would facilitate draining of the tumor edema on the one hand but also provide a route for spread of the cancer via the lymphatics. However, the relationship

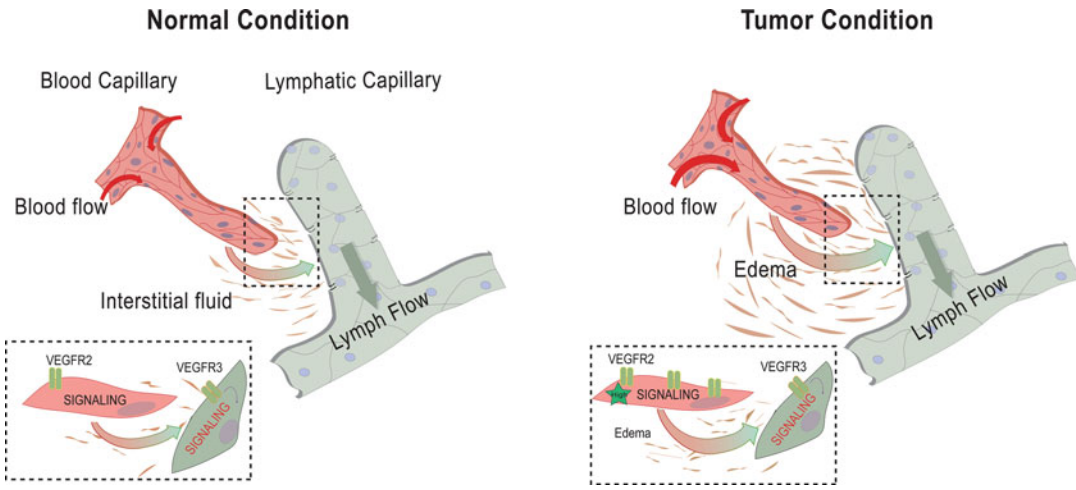

Fig. 2 Communication between the blood and lymphatic vasculature in normal and tumor conditions. Interstitial fluid accumulates as a consequence of basal sieving from the blood vasculature and is drained by the lymphatics (left part of the panel). Endothelium in blood and lymphatic vessels may also communicate by VEGFA/VEGFR2 signaling resulting in production of factors regulating lymphatic endothelial signaling (boxed to the left). In cancer (right part of the panel), excessive VEGFA/VEGFR2 signaling leads to elevated and chronic vascular leakage and increased interstitial accumulation of fluid. Due to poor drainage by the lymphatics, edema builds up. The lymphatic flow is impaired due to collapse of the lymphatic vessel and possibly through exaggerated and dysregulated signaling (indicated by star) from the blood endothelium communicating with the lymphatic endothelium (boxed to the lower right)

between formation of new lymphatic vessels and metastatic spread in cancer is as yet incompletely understood.

Imaging Vascular Flow and Integrity

Recent advances in microscopy techniques combined with computational analysis have created a paradigm shift in studying vascular flow and permeability. Powerful imaging systems have been developed to monitor microvasculature dynamics in vivo, including various tomography techniques such as Doppler ultrasound, dynamic contrast-enhanced magnetic resonance imaging, and optical imaging methods (Jennings et al. 2008). Optical imaging techniques are most commonly used for non-clinical and in-depth study of vascular flow and leakage.

Fluorescent tracer dyes as well as variable-sized fluorescent probes combined with intravital microscopy provide a more detailed understanding of vascular flow and permeability under normal and diseased conditions (Fukumura et al. 2010). Upright imaging using normal epifluorescence (Pink et al. 2012) and multiphoton imaging (Egawa et al. 2013, Brown et al. 2001) are the two most common techniques of optical imaging of blood vessels. The former, i.e., wide-field microscopy, is often limited by the depth of penetration and resolution, while these limitations can be overcome using a multiphoton microscope. Near infrared imaging (NIR) using fluorescent indocyanine green and molecular probes also provides an in-depth understanding of vascular as well as lymphatic permeability under normal and tumor conditions. Conducted at near infrared wavelengths (650–900 nm), NIR has advantages of enhanced tissue penetration, decreased tissue absorption, and decreased autofluorescence (Proulx et al. 2013).

Vascular flow and permeability data obtained from the microscopy techniques combined with biophysical modeling can provide insights and predictions to flow. Such models provide useful insights to the understanding of blood flow in tumors (Soltani and Chen 2013), interstitial

pressures and metastasis (Jain et al. 2007), and transport of nanoparticle therapy (Stapleton et al. 2013). A combined approach of imaging and modeling would therefore provide an increased understanding of changes in blood flow during tumor development and could also help predict the efficacy of drug transport.

Perspectives

Excess vascular permeability resulting in edema and swelling of the tissue (in latin; *tumor*) was noted already in the encyclopedia *De Medicina* by Aulus Cornelius Celsus (25 BC–50 AD) as one of the four cardinal signs of inflammation (*tumor, rubor, calor, dolor*). A focus of interest today is whether specifically suppressing excess vascular permeability is therapeutically beneficial in a range of diseases. Thereby, tissues engaged in the disease would be less edematous, and the interstitial pressure would be lower, allowing more efficient delivery of conventional therapeutics, such as chemotherapy to treat cancer. A more efficient delivery of chemotherapeutics, perhaps at a lower, less toxic dose, is obviously of considerable interest clinically. It would be expected that the barrier presented by non-leaky vessels would provide better perfusion and thereby facilitate tissue homeostasis and promote healing.

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Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Imaging Tumor Angiogenesis
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Benefits and Pitfalls of Tumor Vessel Normalization

Jin-Sung Park, Intae Park, and Gou Young Koh

Contents

Introduction 52
Lessons and Questions from Anti-VEGF-A/VEGFR2 Therapy 52
Hallmarks of Tumor Vessel Normalization 53
Inducing Tumor Vessel Normalization 54
Benefits of Tumor Vessel Normalization 59
Pitfalls of Tumor Vessel Normalization 60
Translational Implications 62
Challenges for Clinical Application 64
Summary 65
Cross-References 66
References 66

Abstract

Tumor vasculature has been intensively studied not only to understand its role in tumor progression and metastasis but also to discover regulatory pro- and anti-angiogenic molecules and cells. Until now, numerous anti-angiogenic agents have been developed, with more than ten agents currently being administered or tested to treat patients with various types of cancers. Despite high hopes for success, recent clinical trials have shown that these anti-angiogenic agents are not as effective as other drugs with different targets in terms of increasing patient survival when used as a single agent. These unsuccessful trials have led researchers to reevaluate the nature of tumor vasculature and the dynamic consequences that arise from anti-angiogenic treatments. Subsequently, a new hypothesis was introduced, where tumor vessels were sought to be tamed and harnessed to our advantage rather than

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simply attempting to eliminate them, which, by itself, has shown only marginal survival benefit. Thus, a new avenue of research was revealed, and the concept "tumor vessel normalization" has gained considerable attention ever since. However, our knowledge in this field is still rather rudimentary, and much still needs to be accomplished in order to overcome the pitfalls and relish the benefits of normalizing tumor vessels for anticancer therapy.

Keywords
Tumor vessel normalization · Tumor vasculature · Tumor microenvironment · Enhanced drug delivery · Enhanced perfusion · Reduced hypoxia · Anti-angiogenesis · Tie2 activators

Introduction

Angiogenesis is a critical process that is driven with the purpose of providing every living cell with adequate passages for nutrient and oxygen supplementation and waste removal. In cancer, angiogenesis is constantly activated to meet the unending demand of new blood vessels to match the unregulated growth of tumor mass, marking angiogenesis as one of the most evident phenotypic hallmarks of cancer (Hanahan and Weinberg 2011). Tumor growth and metastasis are largely dependent on the accompanied growth of tumor vasculature, so called tumor angiogenesis. Indeed, tumor angiogenesis has been an appealing target for antitumor therapy, which has been proposed more than four decades ago (Carmeliet 2005; Fenton et al. 2004; Folkman 1971; Jain 2005; Kerbel and Folkman 2002). Ever since, numerous strategies have been devised to block tumor angiogenesis or destroy pre-existing tumor vessels. Anti-angiogenic therapies basically reduce or "prune" growing tumor vessels. However, this strategy turned out to be less effective than anticipated, mostly because the underlying cause of the pro-angiogenic drive in cancer, severe hypoxia, is actually worsened by anti-angiogenic strategies. Removing tumor vessels exacerbates tumor hypoxia, which ultimately generates a rebound increase in pro-angiogenic force, ending in treatment resistance and failure. To circumvent this situation, focus has shifted to alleviating hypoxia, rather than destroying tumor vasculature altogether (Jain 2014). Tumor vessel normalization is a concept that has emerged to overcome the shortcoming of current anti-angiogenic strategy (Carmeliet and Jain 2011b). Here, we discuss the current knowledge and understanding of numerous ways and mechanisms to normalize tumor vasculature, why tumor vessel normalization is advantageous over tumor vessel destruction, and its potential benefits and pitfalls in real-world applications.

Lessons and Questions from Anti-VEGF-A/VEGFR2 Therapy

The most widely studied strategy to block tumor angiogenesis is by inhibiting the interaction between vascular endothelial growth factor (VEGF) and its receptor VEGF receptor 2 (VEGFR2), which stimulates endothelial proliferation, migration, permeability, and survival and together forms the strongest agonistic axis for new vessel formation (Chung et al. 2010; Nagy et al. 2007). More than ten drugs that target VEGF-VEGFR2 axis have been developed and approved for treating patients with various types of cancers. However, despite the dramatic responses shown in multiple preclinical animal studies, recent clinical trials using VEGF-VEGFR2-blocking agents yielded rather disappointing results; randomized phase III clinical trials showed only a minimal survival benefit in patients with a monotherapy of anti-angiogenic agents (anti-VEGF drugs) (Giantonio et al. 2007; Jain et al. 2006; Gligorov et al. 2014; Gilbert et al. 2014). These trials clearly demonstrated the limitation of anti-angiogenic strategy when it is implemented as a single treatment modality. However, the addition of cytotoxic chemotherapeutic drugs to the VEGF-A-blocking antibody, bevacizumab, led to improved patient outcomes in those with colorectal, breast, and lung cancer (Hurwitz et al. 2004; Sandler et al. 2006). These puzzling results raised important questions about the use of anti-angiogenic agents for treating

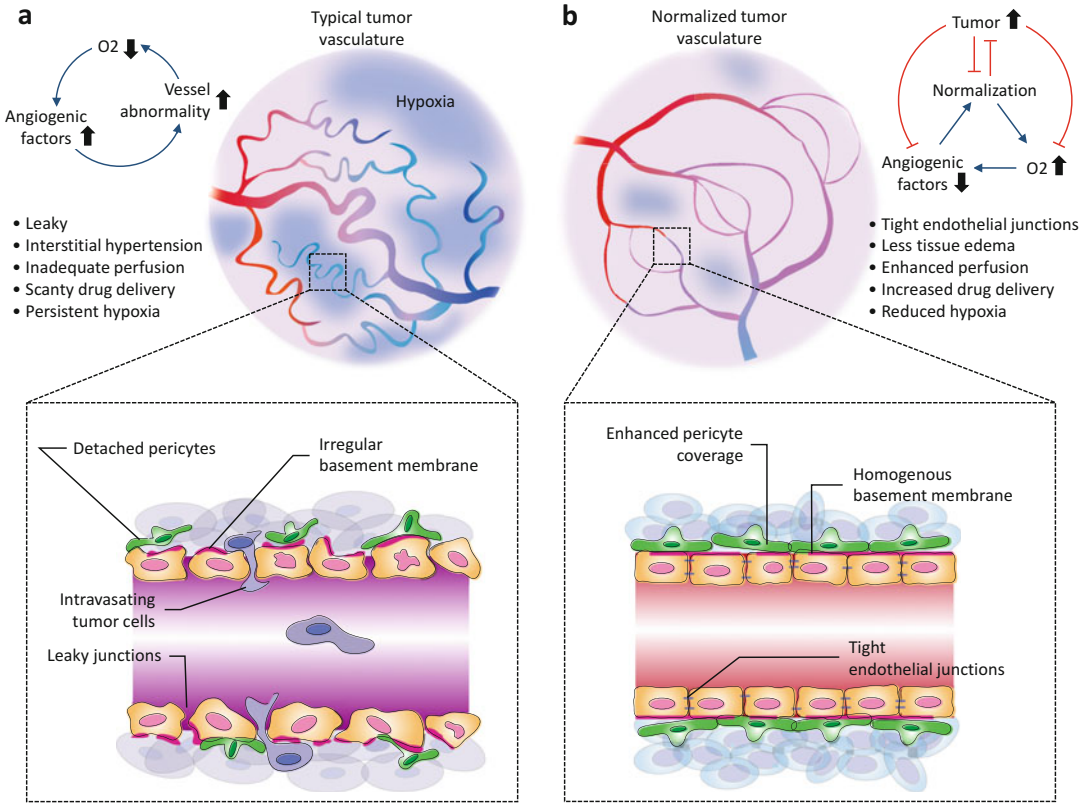

Fig. 1 Comparisons of typical and normalized tumor vasculature. (**a**) In established tumors, vessels are tortuous, dilated, and haphazardly interconnected (upper left panel). Endothelial cell junctions are loose and leaky, pericytes are detached, basement membrane is irregular, and tumor cells can easily intravasate into vessel lumen (lower left panel). These structural abnormalities lead to functional impairment that gives rise to severe hypoxia (blue shaded area in upper panel) within tumors. Hypoxia induces overexpression of pro-angiogenic factors, which aggravate vessel abnormalities to further exacerbate hypoxia, forming a vicious cycle. (**b**) Normalization partly restores structural and functional integrities of tumor vasculature, exemplified by smooth, regularly patterned vessels (upper right panel) with enhanced pericyte coverage, homogenous basement membrane, and tight endothelial junctions (lower right panel). Normalization enhances perfusion and reduces hypoxia, which lowers the expressions of angiogenic factors. Decrease in angiogenic factors can induce normalization, and normalization itself can slow down tumor growth. However, continued tumor growth tips the balance back to pro-angiogenic force, thus making normalization transient

cancer. Why was anti-VEGF effective in combination with cytotoxic drugs, while it was unable to produce survival benefits as a monotherapy in randomized trials? Shouldn't anti-VEGF agents destroy tumor vessels and hinder the delivery of chemotherapeutic drugs? These seemingly paradoxical results led researchers to investigate the molecular mechanism of anti-angiogenic therapy and its true effect on tumor vasculature, eventually giving rise to a novel working model of tumor vasculature's response to anti-angiogenic force, namely, "tumor vessel normalization."

Hallmarks of Tumor Vessel Normalization

Tumor vasculature is impaired in both structure and function compared with normal blood vessels, featured by leaky, hyper-permeable, and tortuous vessels that have random interconnections without proper hierarchy (Fig. 1). The junctions between endothelial cells (ECs) are disconnected, pericytes that cover endothelial lumen are loosely attached or absent, and the basement membrane is

discontinuous, reduced, or absent. These structural abnormalities hinder adequate blood flow and create spatiotemporal heterogeneity within tumor microenvironment. Moreover, leakiness in vessels leads to increased interstitial fluid, which acts in concert with proliferating cancer cells to increase physical pressure and compress blood and lymphatic vessels. These abnormal features of tumor vasculature contribute heavily to the formation of a characteristic tumor microenvironment that is featured by interstitial hypertension, hypoxia, and acidosis. Interstitial hypertension acts as a barrier that hinders the delivery of therapeutics to the central region of tumor mass. Hypoxia makes tumor cells resistant to radiation therapy and also induces numerous genes that make tumor cells resilient to cytotoxic drugs. It also causes genetic instability within tumor cells and triggers genetic mutations that make the tumor cells more malignant and prone to metastasis. Acidosis, combined with hypoxia, weakens the cytotoxic functions of infiltrated immune cells. Essentially, structural and functional abnormalities of the tumor vasculature and the resulting harsh tumor microenvironment work in tandem to hinder the effectiveness of cancer therapy. This implies that tumor vasculature plays important roles in generating a hostile tumor microenvironment and also suggests the possibility of improving such hostility in order to maximize cancer therapy by managing and adjusting the structure and function of tumor vasculature.

Hallmarks of tumor vessel normalization are reduced mean vascular density, increased pericyte coverage, and less tortuous structure and morphology, which subsequently lead to functionally improved blood flow within tumor mass and reduced interstitial pressure and edema (Goel et al. 2012; Park et al. 2016; Viallard and Larrivee 2017; Carmeliet and Jain 2011b). Because of these structural and functional changes of tumor vasculature, tumor vessel normalization has been gathering attention as a viable alternative to conventional anti-angiogenic therapy (Cully 2017; Rivera and Bergers 2015). Instead of abolishing the blood vessels and thus the nutrients and oxygen supply to tumors, which aggravates tumor characters and antagonizes other treatments, it was sought to reinforce them to promote synergism with other treatment modalities. In other words, normalizing tumor vessels will pave the path for better delivery of drugs and oxygen, leading to therapeutic success. Tumor vessel normalization not only enhances the delivery of drugs and oxygen but also facilitates a more uniform and concentrated distribution of these therapeutics in tumor mass to ensure that a larger fraction of tumor cells is in contact with them. Furthermore, it can alleviate the inhospitable tumor microenvironment to generate a friendlier setting in which anticancer immune cells can function better. In sum, normalization alters the tumor microenvironment and creates a battleground more amenable for treatment.

Inducing Tumor Vessel Normalization

In normal angiogenesis, stimulators of angiogenesis temporarily outweigh inhibitors to tip the balance between pro-angiogenic and anti-angiogenic stimuli to prompt new vessel growth. Once vessel growth is completed and tissue is sufficiently vascularized, the level of angiogenic inhibitors becomes more dominant and vessels become quiescent and mature (Carmeliet and Jain 2011a; Potente et al. 2011). In tumors, rapid growth of tumor cells generates a chronically hypoxic microenvironment that acts as the major driving force for the production of pro-angiogenic activators, and thus the balance is skewed in favor of new vessel formation. More importantly, unlike physiologic angiogenesis, this imbalance between pro-angiogenic and anti-angiogenic stimuli persists, because tumor angiogenesis generates abnormal vessels that cannot completely resolve the underlying tissue hypoxia. This persistent hypoxia in turn generates more pro-angiogenic stimulators, and tumor vessels become increasingly abnormal, thereby creating a vicious cycle (Ziyad and Iruela-Arispe 2011; Jain 2014). Thus, it is reasonable to assume that, by blocking pro-angiogenic stimulators or modulating different components that affect the structure and function of tumor vessel walls, one can restore the balance of pro-angiogenic and

anti-angiogenic stimuli and promote normalization of tumor vessels.

Mechanisms affecting tumor endothelium: Many preclinical studies demonstrated that high levels of VEGF within tumor can induce tumor vessel abnormalities (Jain 2005, 2008). Therefore, it is reasonable to speculate that targeting VEGF signaling by direct or indirect modulators will decrease structural and functional abnormalities of tumor vessels. As expected, treatment with a VEGF-blocking antibody induced transient tumor vessel normalization, which was demonstrated by enhanced pericyte coverage, reduced vessel size and tortuosity, and normalized basement membrane (Yuan et al. 1996; Tong et al. 2004; Winkler et al. 2004; Baffert et al. 2006; Kamoun et al. 2009). Mechanistically, VEGF blockade induces upregulation of angiopoietin 1 (Angpt1), which acts to promote tightening of EC junctions and stabilize ECs (Winkler et al. 2004). However, the source and underlying mechanism for the upregulation of Angpt1 are still unclear. VEGF also affects platelet-derived growth factor receptor β (PDGFRβ) signaling in smooth muscle cells, which are known to be critical for pericyte recruitment and coverage (Greenberg et al. 2008). In various preclinical studies, transient vessel normalization induced by VEGF blockade resulted in reduced interstitial fluid pressure and tissue edema (Tong et al. 2004; Kamoun et al. 2009; Dickson et al. 2007; Tailor et al. 2010), increased perfusion along tumor vessels (Dickson et al. 2007; Myers et al. 2010), and enhanced oxygen and drug delivery to the tumor core (Tong et al. 2004; Winkler et al. 2004; Dickson et al. 2007; Myers et al. 2010). During this transient normalization window, VEGF blockade synergistically inhibits tumor growth with chemotherapeutic drugs and radiation therapy, since the delivery of drugs and oxygen to the tumor core is enhanced. However, excessive vascular regression by prolonged or overdosing of VEGF blockade might compromise the synergistic effect and antagonize the response to radiation and chemotherapy (Fenton et al. 2004; Ma et al. 2001; Murata et al. 1997). Considering the lengthy chemo- or radiotherapy regimens in the clinic, this dose-dependent effect and narrow normalization window by VEGF blockade is an active area of research.

Another example of a molecule that regulates vessel disorganization is the oxygen sensor molecule prolyl hydroxylase domain-containing protein 2 (PHD2) (Aragones et al. 2009; Majmundar et al. 2010). Due to the absolute requirement of oxygen supply for cell sustenance, it is not surprising that a sophisticated mechanism to sense oxygen levels was devised by ECs. PHD2 is an oxygen-sensing enzyme which hydoxylates the transcription factors, hypoxia-inducible factors (HIFs), for proteasomal degradation when oxygen concentration is sufficient (Aragones et al. 2009; De Bock et al. 2009). Under hypoxic conditions, PHD2 is inactivated and transcription factors HIFs are activated to induce gene expressions to increase oxygen supply, partly via angiogenesis since VEGF-A is a well-known target gene of HIF1α (Rey and Semenza 2010). In the aspect of HIF1α, PHD2 downregulation should upregulate VEGF-A and subsequently induce vessel abnormalization. Perplexingly, however, endothelial PHD2 haplodeficiency by genetic modification in mice induced tumor vessel normalization without significantly affecting vascular density or size, while physiologic angiogenesis was largely unaffected (Mazzone et al. 2009; De Bock et al. 2013). In normalized vessels, vascular leakage and remodeling were reduced, whereas endothelial junction tightening and vessel maturation were increased, leading to increased tumor perfusion and reduced hypoxia. In addition, endothelial junctions formed a tight barrier against tumor cell intravasation and reduced metastasis (Mazzone et al. 2009). These vascular changes did not affect primary tumor growth, but reduced distant metastasis and also improved response to chemotherapy. Mechanistically, the molecular changes by PHD2 haplodeficiency induced an upregulation of vascular endothelial cadherin, a critical component of endothelial junctions, and also induced an upregulation of soluble FLT1, which acts as a decoy receptor that traps soluble VEGF-A (Mazzone et al. 2009; De Bock et al. 2013).

Even methods that may not seem to target tumor vasculature can also induce normalization. Metronomic chemotherapy, a method of drug

delivery which administers suboptimal dose of chemotherapeutic drugs at frequent intervals, has also been shown to induce tumor vessel normalization. The mechanism seems to involve enhanced expression of thrombospondin 1, which is a well-known inhibitor of angiogenesis (Kerbel and Kamen 2004). Aerobic exercise has also been shown to induce normalization in preclinical model by activating calcineurin-NFAT-TSP1 signaling pathway (Schadler et al. 2016). Very interestingly, a study revealed that the deletion of RhoJ, a Rho GTPase enriched in tumor ECs, not only inhibited tumor angiogenesis but also induced vascular disruption (opposite to tumor vessel normalization) in established tumor vessels using various murine tumor models (Kim et al. 2014), leading to significantly increased hypoxia and necrosis that, as a result, delayed tumor growth. Thus, RhoJ in tumor ECs plays an important role in maintaining tumor vessel integrity, and enhancing RhoJ activity could induce tumor vessel normalization.

Mechanisms which affect pericyte coverage and tumor vessel maturation: Typical structural characteristics of normalized tumor vessels include enhanced pericyte coverage, and thus molecules that stimulate or promote mural cell coverage of endothelial lumen can serve as important regulators of tumor vessel normalization (Carmeliet 2003; Jain 2003). The most widely studied signaling pathway on pericyte coverage is PDGFRβ signaling axis. Its ligand PDGF-B is released from ECs and recruits perivascular mural cells expressing PDGFRβ. Another well-known pericyte marker is neuron-glial antigen 2 (NG2), also known as chondroitin sulfate proteoglycan 4 (CSPG4), which is a membrane proteoglycan found on plasma membrane of diverse cell types. Genetic depletion of NG2 generated abnormal tumor vessels with reduced pericyte and basement membrane coverage, which resulted in reduced perfusion and increased tumor hypoxia (Huang et al. 2010). Moreover, many preclinical studies showed that depletion of pericytes from tumor vasculature promotes metastasis, as vessel walls without pericytes form loose barrier that cannot block dissemination of tumor cells (Gerhardt and Semb 2008). Lack of pericyte coverage also correlates with metastasis in clinical settings (Yonenaga et al. 2005), and a clinical trial using PDGFRβ blockade showed excessive fluid leakage (Jayson et al. 2005). Interestingly, while overexpression of PDGF-D facilitates tumor growth and lymph node metastasis, it normalized tumor vasculature and enhanced drug delivery (Liu et al. 2011). Clearly, further studies are required to clarify the benefits and pitfalls of PDGF blockade in cancer treatment and vessel normalization.

Another important molecular pathway involved in vessel maturation and tumor vessel normalization is the Angpt-Tie receptor axis, which plays crucial roles in the formation of stable vasculature (Winkler et al. 2004; Augustin et al. 2009; Saharinen et al. 2017). As briefly mentioned, binding of Angpt1 – which is known to be released by pericytes – to its receptor Tie2 tightens endothelial junctions and promotes EC survival, whereas Angpt2 acts as a context-dependent antagonist of Tie2 that destabilizes EC and disrupts endothelial junctions (Augustin et al. 2009; Saharinen et al. 2017). Blockade of Angpt2 induces junctional tightening of endothelial barrier and enhances pericyte coverage, while reducing tumor growth and metastasis (Falcon et al. 2009; Nasarre et al. 2009). Simultaneous inhibition of Angpt2 and VEGF-A by a bispecific trap, double anti-angiogenic protein (also known as "DAAP"), also substantially induces vessel normalization and markedly reduces vessel leakage in the ovarian cancer ascites model (Koh et al. 2010). On the other hand, several studies sought to activate Tie2 directly rather than inhibiting its antagonist, Angpt2, to normalize tumor vessels. However, activating Tie2 has been much more challenging compared with blocking Angpt2, since native Angpt1 is prone to aggregation and is largely insoluble (Cho et al. 2004; Koh 2013); it was only recently that a potent activator of Tie2 that has a long systemic half-life and minimal toxicity was developed (Park et al. 2016; Han et al. 2016). Activation of Tie2 in tumor ECs by an Angpt1 analog, VE-PTP inhibitor, or an activating antibody induces tight endothelial junctions and enhances pericyte coverage, which alleviates hypoxia and enhances the effects of cytotoxic drugs (Hwang et al. 2009; Goel et al.

2013; Park et al. 2016). Tie2 activation also reduces tumor growth and metastasis, which is thought to be the result of reduced hypoxia within the tumor core and enhanced pericyte coverage that provides a stable barrier against tumor cell extravasation (Park et al. 2016). Another advantage of Tie2 activation stems from its unique relationship with Angpt2. Destabilized ECs like tumor ECs overexpress angiogenic genes like Angpt2, ESM 1, and VEGFR2 by transcriptional activation of FOXO1, and Tie2 activation inhibits the expression of these angiogenic genes (Daly et al. 2004, 2013; Park et al. 2017). While treatment of Angpt2-blocking antibody leads to a rebound increase in Angpt2 expression (Mazzieri et al. 2011), Tie2 activation stably downregulates the angiogenic genes expressed by destabilized ECs by harnessing the inhibitory pathway built within ECs. The recently developed Tie2 activating antibody, angiopoietin-2 binding and Tie2 activating antibody (ABTAA), takes advantage of the paradoxical relationship between Angpt2 and Tie2 to induce relatively profound vessel normalization (Park et al. 2016). The Tie2-activating ability of ABTAA is dependent on Angpt2, whereby it binds and clusters Angpt2 to switch it from a Tie2 antagonist to an agonist. Subsequently, Tie2 is activated by the ABTAA-Angpt2 complex, which downregulates the expression of Angpt2 and breaks the pro-angiogenic cycle. Because of this unique mode of action, adequate Tie2 activation is guaranteed even without tight regulation of antibody concentration. Therefore, continuous hyper-activation of Tie2, which has been shown to promote tumor metastasis (Holopainen et al. 2009), is inherently impossible because any further expression of Angpt2 is inhibited by ABTAA-induced Tie2 activation; thus any excess ABTAA left in the system will be inactive. For these reasons, Tie2 activators are gathering attention as a safer alternative to anti-angiogenic agents and for tumor vessel normalization (Fig. 2).

Regulator of G-protein signaling 5 (RGS5) also affects vessel maturation, and inhibition of RGS5 can induce vessel normalization. RGS5 is produced by ECs in hypoxic condition or by activated pericytes (Hamzah et al. 2008). RGS molecules block G protein-coupled receptor (GPCR) signaling, and loss of RGS5 in pancreatic islet cancer model normalizes tumor vessels with reduced leakage (Hamzah et al. 2008). RGS5-deficient tumors show uniformly distributed vessels, and pericytes have more mature phenotype. Influx and penetration of immune cells are also increased, and adoptive transfer of immune effector cells prolongs the survival of tumor-bearing mice (Hamzah et al. 2008; Nisancioglu et al. 2008). Finally, a recent study highlights the importance of metabolic profile of tumor ECs and its role in tumor vessel normalization. Tumor cells acquire a unique metabolic profile known as "Warburg effect," which is one of the characteristic hallmarks of cancer (Hanahan and Weinberg 2011). Just like tumor cells, tumor ECs also exhibit a unique metabolic profile that is significantly different from normal ECs (Cantelmo et al. 2016). Tumor ECs show hyperglycolytic metabolism, and one of the enzymes involved in this process, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), is closely linked to tumor vessel normalization. Genetic deletion or pharmacologic inhibition of PFKFB3 from tumor ECs led to improved tumor vessel maturation and perfusion (Cantelmo et al. 2016). PFKFB3 inhibition reduced the endocytosis of a junctional molecule, VE-cadherin, hence generating a tight endothelial barrier that consequently reduced tumor metastasis. PFKFB3 inhibition also enhanced pericyte coverage and reduced the expression of adhesion molecules, which is critical in regulating tumor cell intravasation/extravasation (Cantelmo et al. 2016).

Mechanisms involving various types of immune cells: Immune cells comprise a major component of tumor microenvironment and have recently been highlighted for their roles in promoting or suppressing tumor growth (De Palma et al. 2017). Trafficking and infiltration of immune cells is largely dependent on ECs, and immune cells also interact closely with ECs to maintain its proper function. The most widely studied immune cell type is tumor-associated macrophages (TAMs), which affect tumor angiogenesis, growth, metastasis, and also clinical outcome

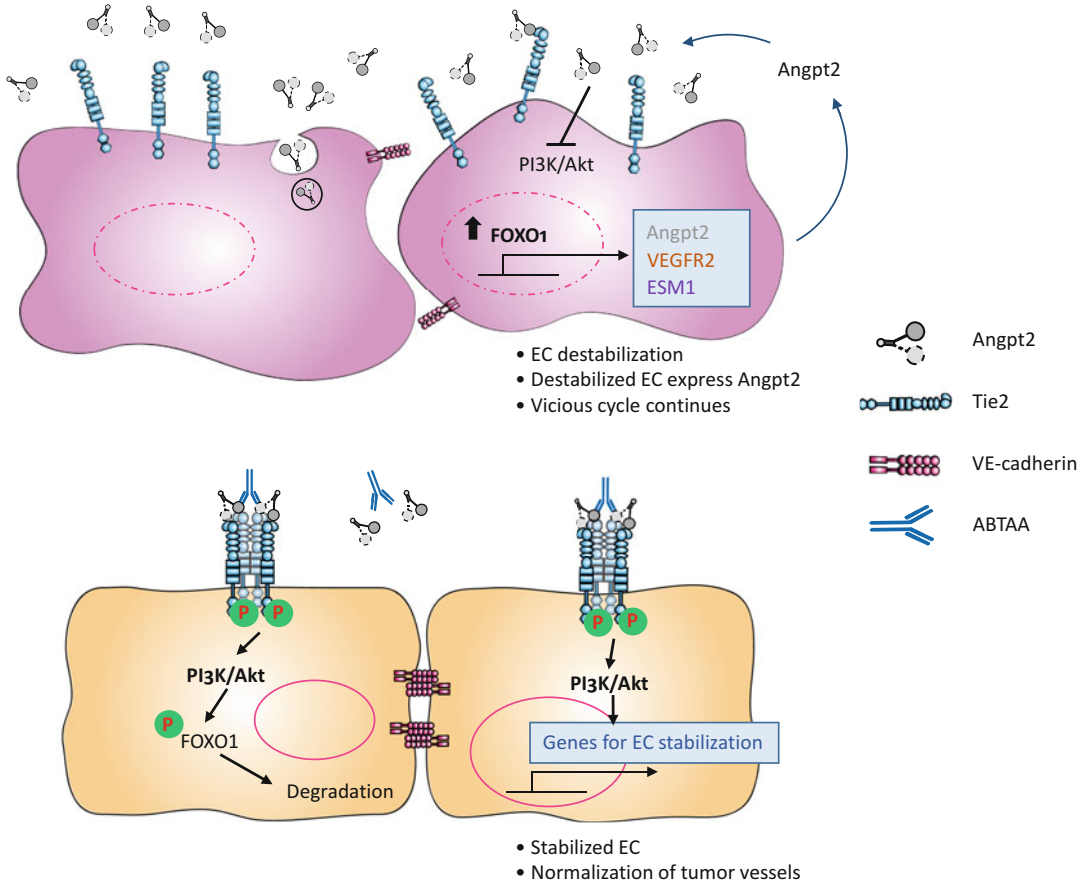

Fig. 2 Tie2 activator takes center stage of tumor vessel normalization. Schematic diagram depicting how Tie2 activation leads to EC stabilization and tumor vessel normalization. Tumor ECs have insufficient pericyte coverage and lack Tie2 activation, leading to attenuated PI3 kinase-Akt signaling and enhanced FOXO1-induced upregulation of pro-angiogenic genes. Of the upregulated genes, the overexpressed Angpt2 acts as an endothelial destabilizing factor, and destabilized ECs further stimulate FOXO1 and its downstream pathway to produce Angpt2, creating a vicious pro-angiogenic cycle. These consequently lead to impaired vessel integrity and increased permeability. The newly developed Tie2 activating antibody, ABTAA (Angpt2 binding and Tie2 activating antibody), breaks this vicious pro-angiogenic cycle by binding to Angpt2 and activating Tie2. Tie2 activation by phosphorylation (P) triggers the PI3 kinase-Akt signaling, which phosphorylates FOXO1 for degradation. Overall, the blockade of endothelial destabilizing factor, Angpt2, and activation of Tie2 for suppression of FOXO1 improves endothelial stability with increased tight junction formation by VE-cadherin

(De Palma et al. 2017). TAMs can change their character according to external stimuli, and their polarization states (either M1 or M2) largely determine their role in tumor angiogenesis. TAMs that are polarized to M2-like phenotype are known to promote tumor angiogenesis as well as increase its malignancy. They can be stimulated to secrete various types of pro-angiogenic cytokines, and some subset of TAMs, especially TIE2-expressing monocytes, remain closely attached to tumor ECs, where they modulate tumor angiogenesis and largely affect the integrity of tumor vasculature (Mazzieri et al. 2011).

Deletion of VEGF from myeloid lineage cells has been shown to induce tumor vessel normalization, enhance tumor oxygenation, and enhance the response to chemotherapy (Carretero et al. 2015; Stockmann et al. 2008). Another important angiogenic factor of the VEGF family, placental growth factor (PlGF), is also known to affect

tumor vasculature, at least in part by modulating TAMs. Genetic deletion of PlGF increases pericyte coverage and perfusion of tumor vessels while reducing tumor vessel leakage and vessel remodeling. PlGF downregulation by histidine-rich glycoprotein (HRG) inhibits tumor growth and metastasis and improves the efficacy of chemotherapy. By substantially downregulating PlGF, polarization of TAM can be skewed from M2-like phenotype to the antitumor M1-like phenotype without affecting the number of TAMs (Rolny et al. 2011).

Eosinophil is another type of immune cell that is closely linked to cancer. Tumor-associated eosinophilia is frequently observed in patients with cancer (Ishibashi et al. 2006; Nielsen et al. 1999), but their role is still rather ambiguous. A recent study revealed that intravenous transfer of eosinophils can induce tumor vessel normalization and polarize TAMs to M1-like phenotype (Carretero et al. 2015). Eosinophils also secrete various chemoattractants to enhance the migration of cytotoxic T cells into tumor and play an important role in cancer rejection (Carretero et al. 2015). Neuropilin-1-expressing monocyte (NEM) is another subset of monocytes with a role in tumor vessel normalization (Carrer et al. 2012). NEMs secrete various cytokines to promote pericyte recruitment and vascular smooth muscle cell proliferation, which leads to enhanced pericyte coverage of tumor vessels. Tumors injected with NEMs show reduced tumor growth and hypoxia and enhanced perfusion and pericyte coverage. These data clearly demonstrate that NEMs represent a novel subpopulation among monocytes that can induce tumor vessel normalization and inhibit tumor growth (Carrer et al. 2012). T lymphocytes (helper T cells, cytotoxic T cells, regulatory T cells, etc.) are also gaining considerable attention as an integral part of cancer therapy, especially after recent successes with immune checkpoint inhibitors in cancer treatment (Huang et al. 2017; Reck et al. 2016). Tumor vasculature interacts closely with T lymphocytes for their recruitment and infiltration, and it has recently been shown that normalized tumor vessels actually enhance the delivery of cytotoxic T cells into tumor mass (Zhao et al. 2017). Another recent study also showed that T lymphocytes actively participate in the process of tumor vessel normalization (Tian et al. 2017). The mutual regulatory loop formed by helper T cells and tumor endothelium highlights the importance of immune cells and their role in normalization of tumor vasculature.

Benefits of Tumor Vessel Normalization

Tumor vessel normalization is anti-angiogenic in nature: The process of tumor vessel normalization, whether by anti-VEGF agents or other numerous methods described earlier, is thought to be anti-angiogenic in nature (Winkler et al. 2004; Maes et al. 2014; Carmeliet and Jain 2011b). The pro-angiogenic drive that accelerates tumor angiogenesis is mainly due to hypoxia within tumor mass, and severe hypoxia leads to enhanced expression and secretion of various pro-angiogenic, destabilizing growth factors, including VEGF-A and Angpt2, which promote new vessel formation. By normalizing tumor vessels, blood flow is increased; hence the delivery of oxygen to the tumor core is also increased. Therefore, by significantly reducing tumor hypoxia, secretion of pro-angiogenic growth factor will be reduced, ultimately generating a net anti-angiogenic effect. There are concerns, however, that enhanced perfusion to tumor core might actually aid the progression and proliferation of tumor mass, because more oxygen and nutrients will be supplied by the normalized tumor vessels. On the contrary to this seemingly plausible idea, numerous preclinical studies to date concur on the fact that tumor growth tends to slow down or halt progression instead (Carmeliet and Jain 2011b). These results can be explained by the reduction in mean vascular density and the net anti-angiogenic effect of tumor vessel normalization, despite the increase in perfusion through normalized vessels. Another important factor to be considered is tumor cells' response to hypoxia. Normal cells cannot tolerate persistent hypoxia, so it is natural to assume that alleviating hypoxia by tumor vessel normalization should aid tumor growth. However, the hallmarks of tumor cells enable them to develop

resistance against hypoxic damage, and a large number of studies indicate that hypoxia can actually promote cancer growth (Eales et al. 2016; Semenza 2012). Finally, hypoxia triggers numerous growth factors including FGFs and IGFs along with VEGFs, which are all major drivers of tumor and EC growth. Accordingly, alleviating hypoxia can eventually reduce the level of growth factors that tumors feed upon and ultimately lead to reduction of tumor growth. Conclusively, tumor vessel normalization generates an environment that opposes tumor growth as well as provides a net anti-angiogenic effect.

Tumor vessel normalization synergizes with conventional chemotherapeutic drugs and radiation therapy: Another obvious and primary benefit of tumor vessel normalization strategy is that it can significantly increase the delivery of oxygen and conventional chemotherapeutic drugs, thus creating synergistic effects in terms of tumor reduction and hopefully patient survival (McGee et al. 2010; Batchelor et al. 2013). High interstitial pressure created by leaky tumor vessel is a major physical barrier that obstructs the delivery of chemotherapeutic drugs through disorganized tumor vessels. Non-efficient perfusion and heterogeneity of tumor vessels also hinder homogeneous and effective delivery of drugs throughout tumor mass. The same applies for oxygen supply, with most tumor regions becoming hypoxic. Since the efficiency of radiation therapy depends heavily on generating reactive oxygen species and fixation of DNA damage by oxygen molecules, both of which need ample amount of oxygen atoms inside tumor mass, hypoxia is directly responsible for resistance against radiotherapy (Barker et al. 2015). For these reasons, tumor vessel normalization can help overcome these obstacles by reducing vessel leakage through tightening of endothelial junctions and enhancing pericyte coverage, thus enabling even perfusion and distribution of drugs and oxygen throughout the tumor mass.

Tumor vessel normalization reduces metastasis: Vessel normalization typically involves tightening of endothelial junctions and enhanced coverage of pericytes, thereby strengthening vessel walls. While metastasis of tumor cells is a complex process involving numerous steps, the critical initial step is tumor cell intravasation, which is defined as penetration of tumor cells into the bloodstream by passing through endothelial barriers (Reymond et al. 2013; Valastyan and Weinberg 2011). Since tight endothelial junctions and pericyte coverage make endothelial barriers much more solid, normalized tumor vessels are less susceptive to tumor cell intravasation compared with the initial unstable tumor vessel. Furthermore, tumor vessel normalization also contributes to reducing distant metastasis of tumor cells by generating a milder, less hostile tumor microenvironment with less hypoxia and lactic acidosis. Hypoxic tumor condition triggers genetic mutations in tumor cells that turn them to be more committed to distant metastasis (Bristow and Hill 2008; Nguyen and Massague 2007). Thus, tumor vessel normalization lessens genetic instability of tumor cells by alleviating hypoxia, ultimately decreasing the likelihood of the relatively milder tumor cells metastasizing to other organs.

Tumor vessel normalization assists the functions of antitumor immune cells: The actions of tumor vessel normalization do not end in simply changing the structure and function of tumor vasculature, but the improved perfusion and oxygenation statuses contribute to significantly alter the whole tumor microenvironment, including immune cells (Park et al. 2016). Hypoxia within tumor mass generates an immune-suppressive microenvironment, which inhibits the effects of cytotoxic $CD8^+$ T cells and polarizes TAMs into the pro-angiogenic M2-like phenotype. Also, the migration and extravasation of immune cells within tumor mass depend heavily on intact ECs (Zhao et al. 2017). Indeed, tumor vessel normalization strategy has been demonstrated to synergize with immune checkpoint inhibitor therapy (Schmittnaegel et al. 2017), increase extravasation of adoptively transferred T cells into tumor (Shrimali et al. 2010), and sway the TAM polarization to the antitumor M1-like phenotype (Park et al. 2016).

Pitfalls of Tumor Vessel Normalization

Tumor vessel normalization is transient: One of the major drawbacks of tumor vessel normalization strategy is that the effect is transient in nature.

Tumor vessels are embedded within tumor microenvironment, which is vastly heterogeneous and undergoes dynamic changes; pro- and anti-angiogenic signaling similarly undergoes such radical changes, and thus it is hard to keep up with the changes and adequately modulate the angiogenic signaling to maintain a stable and constant state of tumor vessel within tumor mass (Fig. 3). Regardless, the transient normalization effect creates a narrow window of opportunity for effective combination treatment. However, considering that a general therapeutic regimen lasts at least a month in the clinic, the few days of normalization window proven in preclinical studies is short-handed for clinical application. The biggest challenges to overcome for this strategy to have translational value are easy monitoring and identification of these normalization windows in clinical settings.

Noninvasive methods for easy monitoring of the normalization status are still lacking: Related to the aforementioned pitfall, a solid evidence of tumor vessel normalization effect in patients is still missing. Some noninvasive imaging techniques like magnetic resonance imaging (MRI) or positron emission tomography (PET) imaging with 18-fluoromisonidazole provide some hints of vessel function in patients (Emblem et al. 2014; Hormigo et al. 2007; Hernandez-Agudo et al. 2016), but they only provide a single parameter of many that indicate successful tumor vessel normalization. In addition, the inconvenience, expense, and lengthy duration of these imaging techniques leave them impractical for repeated

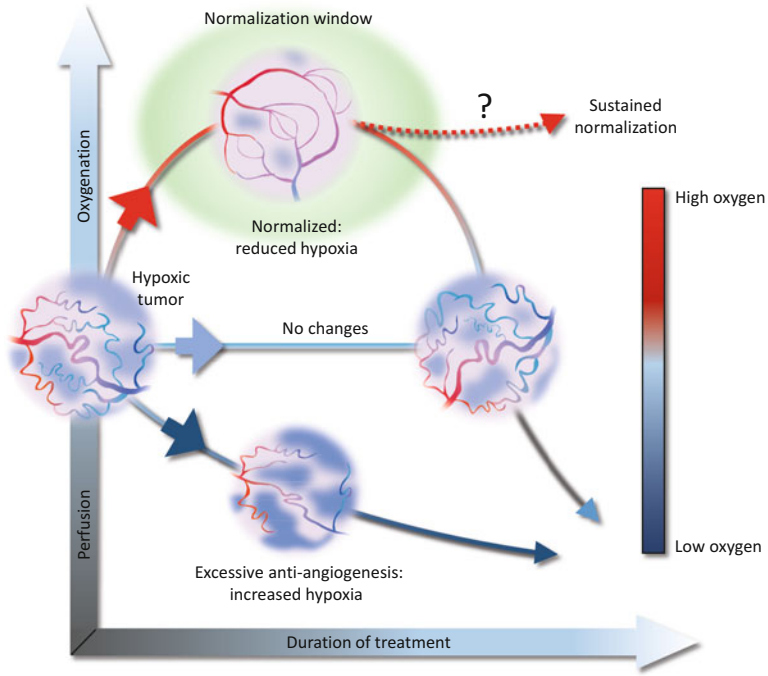

Fig. 3 Effects of tumor vessel normalization on perfusion and oxygenation of tumor. Tumor vasculature is abnormal both in structure and function because pro-angiogenic signals outweigh anti-angiogenic signals. This vascular abnormality initiates a vicious cycle as shown in Fig. 1, which ultimately generates a hypoxic tumor microenvironment. Enhancing anti-angiogenic signaling or inhibiting pro-angiogenic signaling can transiently restore the balance between pro- and anti-angiogenic stimuli within tumor, resulting in normalization of tumor vessels. Depending on the extent of anti-angiogenesis versus normalization, perfusion and oxygenation through tumor vessels may increase, decrease, or remain largely unchanged. The transient period when tumor perfusion and oxygenation increase by the normalized vessels is known as "normalization window." Whether tumor vessel normalizing agents can generate sustained normalization and maintain high perfusion status for an extended period of time remains to be seen

use. Live imaging of tumor vessels *in vivo* is another potential tool for visualization and monitoring of normalization status, but it is only applicable in very specific situations and cannot easily be translated into human practice.

Dose-dependent effect of anti-VEGF therapy: VEGF-blocking agents show different outcomes in terms of vessel normalization depending on the administered dose (Jain 2013; Sorensen et al. 2012). Several preclinical trials using other normalization agents had a similar problem (Maes et al. 2014; Zhang et al. 2015). This dose-dependent effect of vessel normalization poses another challenge in applying these strategies into diverse tumor models or patients. Each tumor has a unique microenvironment with greatly varying percentages of tumor vasculature, dependency on VEGF signaling, and level of angiogenic molecules among not only tumor models but also individual patients. There are some tumors that are more responsive to anti-angiogenic therapy and some that are more resistant. Moreover, the same tumor can have different responses to anti-angiogenic drugs according to their current status (Bagri et al. 2010). Therefore, identifying the optimal amount of treatment is critical to maximize the normalization effect, which requires tremendous effort and makes it difficult to generalize the strategy unlike other treatment options. In addition, VEGF blockade also affects normal vasculature, and suboptimal dose or administration schedule of VEGF-blocking agents can cause adverse effects on cardiovascular, endocrine, and nervous systems and can also increase the risk of arterial thromboembolism (Jain et al. 2006).

No reliable serum marker: One of the reasons that anti-VEGF therapy showed limited benefit in clinical trials is that there is no reliable serum marker to monitor the effect of anti-angiogenic therapy during treatment. Likewise, it is impossible to preselect patients who would benefit more from anti-angiogenic therapy while excluding those who would be resistant. Same is true for tumor vessel normalization strategy. Due to the absence of any method to monitor or predict tumor vessel normalization status in the clinic, it is impossible to accurately time any other therapeutic modalities as a combination so that it is given exactly within the normalization window. Novel biomarkers that are sensitive and specific to changes in structural and functional aspects of tumor vessel would be ideal. Although numerous preclinical studies and candidate molecules have been suggested, a successful target is yet to be identified in the clinic.

Optimal time to induce vessel normalization is still unclear: Although most preclinical studies concur on the fact that tumor vessel normalization generally does not enhance tumor growth, this effect can differ depending on tumor size and progression stages (Goel et al. 2013). Some preclinical studies indicate that, while normalization strategy is effective in early stages of tumor development, no apparent differences in tumor growth are observed when it is applied in later stages where tumor mass is considerably large; some normalization strategies even promote tumor growth in later stages of tumor development (Hamzah et al. 2008). This dependency on tumor stages poses a difficult question in translating normalization into clinical settings. Again, there are no absolute selection criteria to be certain that patients receiving normalization agents will actually benefit from the treatment. Another aspect to consider is that the benefits of normalization, namely, enhanced delivery of chemotherapeutic drugs and oxygen and reduced metastasis, may not coincide. Many preclinical studies demonstrate several of many features of tumor vessel normalization, and not necessarily all parameters are examined in a single study. In this regard, there is a possibility that different features of normalization are more evident in different stages; normalization in early stages may have the highest drug delivery enhancement effect, whereas normalization in later stages may be most effective at reducing distant metastasis. A more thorough investigation with preclinical models is required to describe the definite selection criteria for application of normalization strategy.

Translational Implications

It is interesting to note that the concept of tumor vessel normalization had been introduced at around 2001 (Jain 2001), but its importance was

never really addressed until very recent years. In comparison, anti-angiogenic therapy was proposed more than four decades ago, and more than ten drugs have been approved for clinical use. This vast time gap may explain why most evidence of tumor vessel normalization is still rooted in preclinical studies and why most clinical studies of vessel normalization have applied anti-angiogenic drugs. This suggests that the translational potential of tumor vessel normalization is still in its infancy and is not yet fully explored.

Another important point to consider for the lack of translational progress of tumor vessel normalization strategy is related to the fact that it is hard to obtain tumor samples from patients during cancer treatment, which is essential to monitor maturation status of tumor vessels. It is also crucial to consider the therapeutic goals that we hope to achieve with the tumor vessel normalization concept. For example, should normalization be used as a stand-alone, first-line therapy for cancer, or, more likely, should it be used exclusively as a combination treatment modality with current cytotoxic therapy? Should normalization be applied to all stages of cancer progression, or should it be limited to certain stages of cancer development, for instance, at the early stages? Accumulating evidence provide hints and directions, but we still do not have a clear, direct answer to these intriguing questions. Most importantly, is there any evidence of tumor vessel normalization in patients with cancer? Biopsy samples from human tumors show vascular abnormalities similar to those seen in preclinical models using mice, and it is a well-known fact that human tumors are embedded in hypoxic and acidic conditions with increased interstitial pressure (Willett et al. 2004, 2009 Bullitt et al. 2004; Wagemakers et al. 2010). However, in a limited set of clinical trials, anti-VEGF therapy has been shown to induce certain features that suggest tumor vessel normalization, including reduced number of vessels, increased pericyte coverage, and reduced edema; this was observed after treatment with bevacizumab in advanced rectal cancer patients (Willett et al. 2004, 2005, 2009). Although these parameters are similar to those observed in animal models following anti-VEGF treatment, these data must be interpreted with caution and not be generalized, as the number of patients was quite limited. Also, even if these results suggest the existence of normalization phenotype in bevacizumab-treated human cancer, it is unclear whether it is the normalization or anti-angiogenic effect that is responsible for tumor growth inhibition.

Studies using MRI also provide some evidence of tumor vessel normalization in patients treated with anti-VEGF agents. For instance, administration of cediranib (receptor tyrosine kinase inhibitor of VEGF receptors) to patients with recurrent glioblastoma showed reduced brain edema as measured by MRI (Batchelor et al. 2007, 2010). In this clinical trial, tumor vessel normalization was demonstrated by measuring vessel diameter and permeability. In case of brain tumors, increased permeability and breakdown of blood-brain barrier by the growing tumor can be life-threatening, as it results in increased intracranial pressure. Vessel normalization can restore the blood-brain barrier and reduce edema, which can reduce the risks of serious complications. MRI studies further demonstrated that patients with recurrent glioblastoma treated with cediranib showed correlation between patient survival and vascular normalization index (changes in vascular permeability, blood flow, microvascular volume, and circulating collagen IV, which indicates remodeling of basement membrane) (Sorensen et al. 2009). Nevertheless, the effect of anti-VEGF agents on tumor shrinkage and its contribution to overall survival remain to be determined. This is a particularly difficult question to answer in the clinic because current imaging techniques are unable to discriminate between decrease in contrast (which is correlated with permeability of vessels) and decrease in tumor size (Sorensen et al. 2008). However, there are preclinical studies indicating that anti-VEGF therapy prolongs overall survival of tumor-bearing mice by reducing intracranial pressure, even though tumor continues to grow (Kamoun et al. 2009; Claes et al. 2008). Other important questions include whether the reduced permeability by anti-VEGF therapy can enhance the efficacy of radiation therapy for patients with glioblastoma

and whether tightening of BBB reduces or enhances delivery of chemotherapeutic drugs to tumor mass. Thus, the clinical benefit of tumor vessel normalization in patients with glioblastoma requires further studies.

Similarly, in patients with hepatocellular carcinoma, inhibition of VEGFR resulted in decreased blood flow and tumor progression and thus raised the question of whether normalization index can predict the efficacy of therapy (Zhu et al. 2009). In addition to imaging, blood tests revealed that the level of soluble Flt1 in plasma correlates with degree of tumor regression in patients with rectal cancer who received anti-VEGF therapy and neoadjuvant chemoradiation therapy (Duda et al. 2010). Another situation where tumor vessel normalization by anti-VEGF therapy was beneficial was when VEGF blockade was combined with conventional chemotherapeutic drugs. Other than glioblastoma, the clinical use of bevacizumab in patients with solid cancer is only approved as a combination treatment with cytotoxic drugs. The superior benefit conferred by combination therapy was attributed to numerous mechanisms, one of which is the sensitization of tumor ECs to cytotoxic damage (Carmeliet 2005; Jain 2008). Enhanced delivery resulting from vessel normalization was also proposed to explain the overall benefit of combination therapy. This explanation is supported by preclinical studies showing that VEGF blockade induces deeper penetration of molecules by reducing hydrostatic pressure barrier across the vessel wall and also by enabling more even distribution of blood flow along the tumor vessels (Jain 2005; Tong et al. 2004; Dickson et al. 2007;Wildiers et al. 2003).

These evidence strongly imply that tumor vessel normalization is a viable option as a combination that can be applied to clinical practice. Yet its potentials that were demonstrated in preclinical studies have not been explored in humans. For example, in addition to cytotoxic drugs, anti-VEGF therapy improves immunotherapy in preclinical models by enhancing the accessibility of immune cells into the tumor (Shrimali et al. 2010; Schmittnaegel et al. 2017). As well as affecting the drug delivery and immune cell infiltration, tumor vessel normalization can also make tumor cells more sensitive to chemotherapy by, for example, reducing hypoxia and making the tumor cells that have been exposed to drugs more proliferative and thus more susceptible to drug-induced damage (Jain 2005; Willett et al. 2004, 2009). In addition, normalization can improve tumor oxygenation and thus enhance the effect of radiation therapy, for which oxygen is crucial for the production of reactive oxygen species and DNA damage fixation. However, caution is needed when translating preclinical data into patient care since the effects seen in animal models may not occur in human cancer. For example, enhanced drug delivery by VEGF blockade is not a universal phenomenon observed in all preclinical models (Tailor et al. 2010). Furthermore, it is still not clear whether partial oxygen pressure (pO2) actually changes in human cancers before or after VEGF blockade, since measurement methods and data are lacking. It is also unclear how long the normalization effect sustains in patients with different types of cancer and whether the effect is the same in different stages of cancer. The precise mechanisms and benefits of normalization by bevacizumab treatment thus need to be investigated further.

Taken together, these clinical trials and preclinical studies provide some indirect but optimistic evidence indicating the possible therapeutic potential of tumor vessel normalization in patients with cancer. Clearly, additional randomized trials involving patients with numerous types of cancers and larger patient populations need to be performed to confirm these preliminary findings. In addition, whether tumor vessel normalization can actually increase oxygenation and drug delivery in patients needs to be validated thoroughly. The future challenge would be to explore whether the aforementioned novel methods to normalize tumor vessels (other than anti-VEGF therapy) can promote normalization in patients and whether its effects are superior and more persistent compared with anti-VEGF therapy.

Challenges for Clinical Application

The approval of the VEGF blockade for clinical use has taught us several valuable lessons, the most important of them being the fact that VEGF-blocking agents are mostly effective only

when combined with cytotoxic drugs in terms of having significant effects on patient survival (Jain et al. 2006; Gligorov et al. 2014; Taal et al. 2014). Increasing the dose of anti-angiogenic agents may exhibit toxicity in normal tissues, increase tumor hypoxia, and impair drug delivery by removing too much blood vessels from tumor mass. However, optimal administration dose and scheduling can induce tumor vessel normalization without noticeable damage to normal tissues. There are few major challenges that must be resolved before tumor vessel normalization strategy can be successfully translated into the clinic.

The first challenge is to validate which among the numerous normalization agents tested in preclinical trials is actually effective in patients. As numerous preclinical trials showed, any therapy that can restore the balance between pro-angiogenic and anti-angiogenic force can normalize tumor vessels – in theory. Whether these strategies could also induce normalization in human cancer remains to be shown. Ongoing translational research should help us reduce the gaps in this aspect of our current understanding of tumor and its vasculature. Important point to consider is that most clinical trials are designed to measure gross changes like tumor size or overall survival and do not pay serious attention to vascular changes after treatment. More specific and clever designs of clinical trials are needed to shed light upon the vascular biology of tumor.

The second challenge is to find a way to easily monitor and measure normalization status in patients using surrogate serum markers or more advanced imaging technologies. Identifying normalization window and vascular response to normalizing agents are paramount in planning combination treatments and reducing potential side effects. Measuring blood vessel density with biopsy samples is too invasive and may not provide functional information of tumor vessels. Current imaging techniques are expensive and have a hard time tracking subtle changes, but they can measure vascular permeability, blood perfusion, and uptake of certain drugs to provide some insight on normalization status in a noninvasive manner. PET scan with 18-fluoromisonidazole and MRI images can provide some indication of tumor oxygenation status, which might be useful in tracking normalization window. Some studies indicate that the number of circulating ECs and their progenitors decrease after VEGF blockade (Duda et al. 2006; Willett et al. 2004, 2005), but it is not clear whether this decline coincides with vessel normalization. Serial blood sampling and measurement of molecules known to be involved in vessel maturation during the course of normalization therapy can potentially identify surrogate markers of normalization. However, the lack of accurate biomarkers and the impracticality of these methods make clinical translation difficult.

The third challenge is to gain more comprehensive knowledge on molecular and cellular mechanisms involved in tumor vessel normalization. It is still unclear whether the effects of normalizing agents depend on tumor size and developmental stages, whether different tumor stages require different mechanisms for normalization, and whether different tumor stages are differently affected by tumor vessel normalization. Although there are concerns and few evidence of accelerated tumor growth by enhanced vessel function, the ultimate benefit that can be achieved by combining tumor vessel normalization with cytotoxic drugs as compared with cytotoxic drug alone must be considered. A growing number of preclinical evidence are piling up, and we need more innovative ideas on how to translate the knowledge gained from preclinical studies into actual patient care.

Summary

Tumor needs to generate new blood vessels to support its growth and to invade or metastasize into other organs. This dependency on neovascularization led to the development of numerous therapeutic agents targeting tumor vasculature. The initial approach was to destroy or inhibit tumor angiogenesis, thereby starving tumor of oxygen and nutrients. However, after numerous failures in clinical trials to provide significant survival benefits, focus has been shifted to an alternative approach. Here, we discussed the advent and recent progress of the collective treatment tenet known as “tumor vessel

normalization." Much like the shift in the paradigm of oncoimmunology whereby research now focuses on removing the breaks rather than pushing the accelerators, the concept of tumor vessel normalization is a clever detour to the original anti-angiogenic concept, whereby the tumor vasculature is harnessed and utilized to our advantage, rather than destroying it. Numerous drugs and pathways have been revealed to induce tumor vessel normalization, and by normalizing the tumor vasculature, numerous side effects of conventional anti-angiogenic therapy can be circumvented. However, tons of work still remain in order to translate precious insights from preclinical studies into actual patient care. Whether these numerous regimens and drugs are equally effective against different types of cancers and on patients with different stages of cancer progression needs to be determined. Also, whether there is an optimal agent to induce normalization in different types of cancers altogether or if normalization strategies must be tailored according to individual patient is another important hurdle that needs to be conquered. For immediate clinical application, it would be ideal to develop a strategy that is persistent and not dose-dependent, given that the current technology cannot accurately observe the characteristics of tumor vessels and there are no biomarkers to help us calculate the optimal dosage for each patient. A better insight into the molecular mechanisms and development of optimal methods to induce and observe vessel normalization will result in more potent and compelling therapies for numerous types of cancer.

Cross-References

- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Biomarkers for Anti-angiogenic Therapy
- Combination of Anti-angiogenics and Other Targeted Therapies
- Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis
- Endothelial Cell-Cell Junctions in Tumor Angiogenesis
- Imaging Tumor Angiogenesis
- Mechanisms of Anti-angiogenic Therapy
- Mechanisms of Tumor Angiogenesis
- Pathology of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- The Role of VEGF in Controlling Vascular Permeability

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The Impact of Endothelial Transcription Factors in Sprouting Angiogenesis

Melanie Hofmann and Joerg Heineke

Contents

Introduction 74

Pro-angiogenic Transcription Factors 75
The ETS Family of Transcription Factors: Essential for Endothelial Cell Development 75
The cAMP Response Element-Binding Transcription Factors 75
The GATA Family: Highly Expressed in Endothelial Cells 76
Hypoxia-Inducible Factors (HIFs) 78
The Transcriptional Regulation by YAP/TAZ 78
MYC 79
The Nuclear Factor (NF) κB 79

Anti-angiogenic or Endothelial Quiescence-Promoting Transcription Factors 80
The Forkhead Transcription Factors 80
The Kruppel-Like Factors 2 and 4 82
The Nuclear Factor-Erythroid 2 p45-Related Factor (NRF2) 82
The p53 Transcription Factor 82
The Recombining Binding Protein Suppressor of Hairless (RBPJ) 84

Conclusions and Future Directions 84

References 84

Abstract

Sprouting angiogenesis by endothelial cells is the main mechanism of new vessel formation in fetal development and in postnatal disease, where either exaggerated (e.g., in cancer, inflammation, and eye diseases) or inadequate vessel growth (e.g., in ischemic diseases like stroke, myocardial infarction, or neurodegeneration) drives the progression of pathology. Endothelial cells receive signals (such as hypoxia, growth factors, or mechanical cues) from the tissue environment and respond by adjusting sprouting angiogenesis and related

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processes like the adhesion of inflammatory cells, the permeability of intercellular junction, or cellular differentiation in order to maintain tissue homeostasis. Endothelial transcription factors are located at a strategically important nexus to control the expression of specific gene groups and thereby coordinate the endothelial responses to external stimuli. We review here mainly evidence from studies in model organisms (especially mice and zebrafish) regarding the function of pro- and anti-angiogenic transcription factors, their important target genes, and how they are regulated by upstream cytosolic signaling pathways.

Keywords

Sprouting angiogenesis · Transcription factors · ETS · ERG · CREB · GATA · HIF · NFκB · MYC · FOXO · p53 · KLF · RBPJ · YAP/TAZ

Introduction

The vascular system builds early during embryonic development, before blood circulation starts. This process is called vasculogenesis and describes the de novo formation of blood vessels from angioblasts that give rise to endothelial cells. Angioblasts develop from hemangioblasts, which differentiate from mesodermal stem cells (Risau 1995, 1997; Schmidt et al. 2007). The term vasculogenesis specifically describes the formation of the first vessels in the embryo, whereas angiogenesis comprises blood vessel growth during development and disease and describes the formation of new blood vessels from pre-existing ones. It is accomplished either by endothelial cell sprouting or by intussusceptive angiogenesis (splitting of one blood vessel in two) (Ribatti 2006).

In 1977 sprouting angiogenesis was described for the first time by Ausprunk and Folkman (Ausprunk and Folkman 1977), as the growth from the pre-existing vascular network toward an angiogenic stimulus. Sprouting angiogenesis is usually initiated in poorly perfused tissues, where oxygen-sensing mechanisms detect a hypoxic environment that demands the formation of a new vasculature to maintain tissue homeostasis. In the case of low oxygen levels, parenchymal cells secrete pro-angiogenic stimuli to guide the proliferation and migration of endothelial cells, which form new capillaries. The hypoxia inducible factor HIF1α induces the transcription of more than 60 genes, including vascular endothelial growth factor (VEGF) (perhaps the most famous angiogenic growth factor), and therefore plays a crucial role for the response of different tissues to hypoxia (Gerhardt 2008).

The distribution of VEGFA stimulates the formation of specialized endothelial cells, called tip cells, and directs them through the capillary basement membrane by the formation of filopodia that secrete large amounts of proteolytic enzymes (Horowitz and Simons 2008). Besides tip cells, endothelial stalk cells, which follow behind a tip cell, are also important for blood vessel development. Stalk cells are very proliferative (while tip cells are not) and lead to capillary sprout elongation and tube and branch formation (van Hinsbergh and Koolwijk 2008). When two or more tip cells of different capillary sprouts reach the source of the VEGFA stimulus, they fuse together and create a vascular lumen to enable blood flow and therefore oxygen supply. When blood flow is ensured, shear stress and other mechanical stimuli emerge that lead to network maturation and stabilization by the recruitment of pericytes (Chien 2006).

Besides VEGF, Delta-Notch signaling is a key regulatory component of sprout formation between cell-cell contacts. Notch receptors and most of its ligands are transmembrane proteins. VEGFA signaling drives the expression of the Notch ligand delta-like 4 (DLL4) in tip cells leading to an activation of Notch receptors at the membrane of adjacent stalk cells. Notch receptor activation in stalk cells suppresses their VEGF receptor (VEGFR2) expression leading to a lower VEGF responsiveness and lower sprouting capacity compared to tip cells (Suchting et al. 2007).

This chapter focuses on how endothelial cell transcription factors influence endothelial cell sprouting mechanisms in conjunction with important signaling pathways.

Pro-angiogenic Transcription Factors

The ETS Family of Transcription Factors: Essential for Endothelial Cell Development

ETS transcription factors are essential for many biological processes such as hematopoiesis, angiogenesis, wound healing, cancer, and inflammation (Sharrocks et al. 1997). Based on their structural domains, the family of ETS (E-26 transformation specific) transcription factors can be divided into 11 subfamilies. Of the 28 described mammalian ETS family members, at least 19 are expressed in the human endothelium (Randi et al. 2009) and 13 during embryonic hematopoietic or vascular development. All share an 85-amino acid conserved DNA binding domain (ETS domain), which is a winged helix-turn-helix motif, often located in the C-terminal half of the protein. The ETS domain binds to a DNA core consensus motif 5′GGA(A/T)3′ associated with one or more unrelated transcription factors such as AP1, MafB, and CBP (Verger et al. 2001). Although ETS binding sites occur in 5–15% of all gene promoters (Hollenhorst et al. 2007), including many of housekeeping genes, nearly all endothelial enhancers and promoters contain ETS DNA-binding motifs (Fig. 1). Consequently, ETS transcriptian factors are essentially involved in the transcriptional programs that control enothelial cell development (De Val et al. 2008).

Compared to other ETS transcription factors, Etv2 has the strongest impact in vascular development, because Etv2-null mutant or morpholino knockdown zebrafish embryos show an almost complete loss of vasculogenesis (Sumanas and Lin 2006; Pham et al. 2007). Furthermore, Etv2 interacts with FoxC2 to regulate expression of multiple vascular endothelial genes such as VegfR2 and Pecam1 (De Val et al. 2008) as well as the endothelin-converting enzyme-1 (Ece1), which is essential for the regulation of the vascular tone during embryonic development (Robinson et al. 2014). These strong ETS factor-dependent defects in early vasculogenesis mostly recover at later stages of development, mainly due to a redundancy between Etv2 and Fli1b, whereby Craig et al. (2015) revealed that only double Etv2 and fli1b loss-of-function zebrafish embryos develop profound defects in angiogenesis.

Another important ETS transcription factor for endothelial cell development is ERG, mainly implicated in the regulation of hematopoiesis (Loughran et al. 2008; Kruse et al. 2009) and associated with acute myeloid leukemia and Ewing's sarcoma (Giovannini et al. 1994; Martens 2011; Tomlins et al. 2013). ERG mediates expression of VE-cadherin and controls junctional integrity (Asano et al. 2010). In 2015, Birdsey et al. (2015) demonstrated that constitutive deletion of ERG in endothelial cells causes embryonic lethality with vascular defects in retinal angiogenesis of mice. Furthermore, they found that loss of ERG appears in tumors with decreased vascular stability resulting from a disruption of Wnt/β-catenin signaling. ERG directs the Wnt/β-catenin pathway through transcriptional control of the Wnt-receptor frizzled4 (Fzd4) and VE-cadherin, which increases β-catenin levels leading to enhanced vessel stability, EC survival, and proliferation (Fig. 1).

The ETS transcription factors Ets1 and Ets2 are strongly redundant to each other. In this regard, only double-knockout mouse mutants of these two factors show defects in angiogenesis and enhanced cellular apoptosis, while individual mutants do not show vascular phenotypes (Hollenhorst et al. 2004). The Elf1 and Elf2 ETS transcription factors are controlling the expression of the endothelial markers Tie1 and Tie2 in cultured endothelial cells (ECs), which both play a crucial role in angiogenesis (Dube et al. 2001; Gaspar et al. 2002).

The cAMP Response Element-Binding Transcription Factors

The cAMP response element-binding (CREB) proteins are key transcriptional mediators of stimulus-induced nuclear responses that underlie the development and function of diverse cell types. They belong to the family of basic leucine zipper proteins and bind to a specific cAMP response element (CRE) in the promoter region

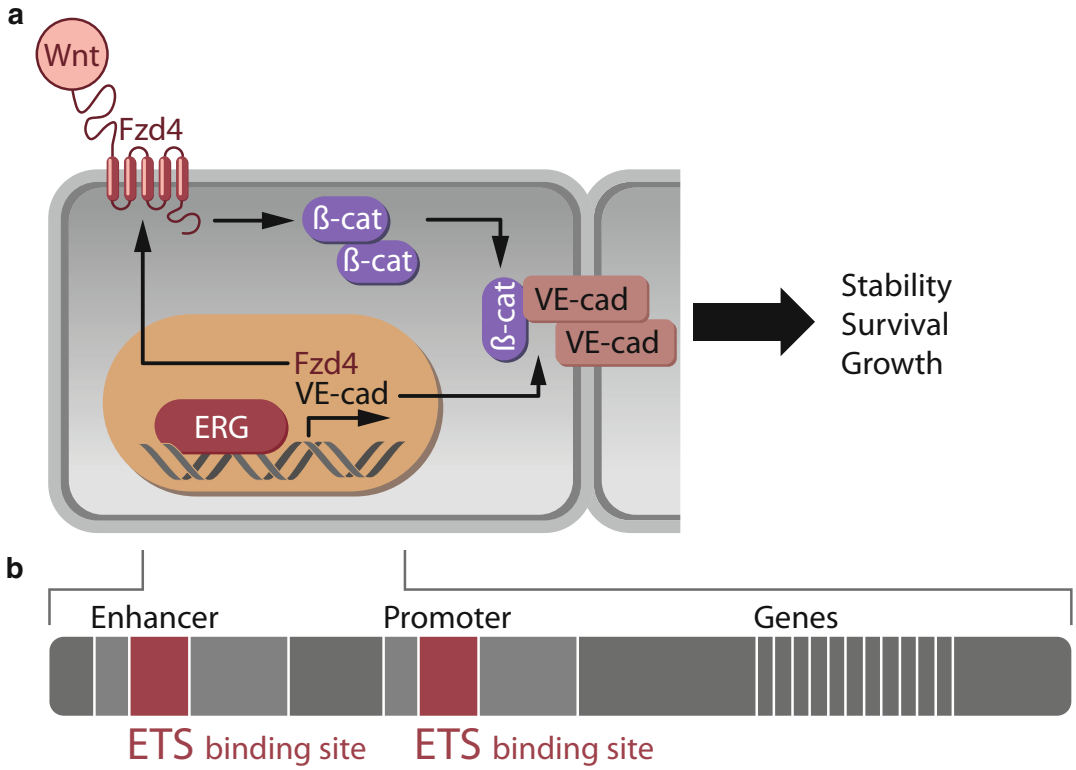

Fig. 1 Promotion of blood vessel growth and stability by the endothelial transcription factor ERG through Wnt/β-catenin signaling. (**a**) ERG drives the expression of Wnt-receptor Fzd4, which stabilizes β-catenin complexes in the cytoplasm. Together with the expression of VE-cadherin that is also promoted by ERG. VE-cadherin and β-catenin together are responsible for vessel stability. Activated Wnt signaling enhances angiogenesis. (**b**) ERG is activating gene expression by binding to the ETS binding motif, which can be found in almost all endothelial enhancer and promoter regions of endothelial genes

of target genes, and they enhance expression of target genes (Shaywitz and Greenberg 1999). In response to hypoxia, CREB is activated by phosphorylation of serine 133 by protein kinase C (PKC), protein kinase A (PKA), and calmodulin kinase (Wen et al. 2010). In endothelial cells, CREB regulates a number of genes including VEGFA, FGF2, and HGF (Morishita et al. 1995; Hoot et al. 2010; Kottakis et al. 2011) and thereby promotes cell survival, angiogenesis, and endothelial barrier function (Suehiro et al. 2010; Chava et al. 2012).

Furthermore, Singh and colleagues (Singh et al. 2015) reported that VEGFC promotes the proliferation and migration of hypoxic retinal endothelial cells upstream of CREB: VEGFC triggers the phosphorylation of p38-MAPK, thereby activating CREB, which upregulates DLL4 and Notch1 and induces tip cell formation and angiogenesis (Singh et al. 2015).

The GATA Family: Highly Expressed in Endothelial Cells

Based on sequence homology and expression pattern, the zinc-finger GATA family of transcription factors can be divided in 2 subfamilies. The hematopoietic group, which includes GATA1, GATA2, and GATA3 (Orkin 1992) is prominently expressed in hematopoietic stem cells. The "cardiac group," including GATA4, GATA5, and GATA6, is known to be expressed within various mesoderm- and endoderm-derived tissues but mainly in the heart and gut (Laverriere et al. 1994; Shivdasani and Orkin 1996). Several

studies have revealed that all GATA factors (with the exception of GATA5) are generally required for prenatal development (Tsai et al. 1994; Pandolfi et al. 1995; Soudais et al. 1995; Fujiwara et al. 1996; Tsai and Orkin 1997; Koutsourakis et al. 1999). The most highly expressed GATA factors in endothelial cells are GATA2, GATA3, and GATA6 (Umetani et al. 2001). GATA transcription factors bind the DNA sequence (A/T) GATA(A/G).

Among all GATA proteins that are expressed in endothelial cells, most is currently known about GATA2, which is the most abundantly expressed GATA factor in endothelial cells (Lee et al. 1991). The expression of GATA2 is regulated by activation of the Notch Receptor 1 in the dorsal aorta of mice (Robert-Moreno et al. 2005), and it is in turn required for the expression of Runx1, which is essential for the transdifferentiation of the aortic endothelium into hematopoietic stem cells (Gao et al. 2013). The role of GATA2 in hematopoietic development is well established as homozygous GATA2-deficient mouse embryos die by E10.5 and exhibit strong anemia (Tsai et al. 1994). Nevertheless, a normal embryonic vasculature development was found at that point. Conditional EC deletion of GATA2 in mice suggests that GATA2 mainly promotes lymphatic development and vascular integrity during embryonic development (Lim et al. 2012; Johnson et al. 2012). Numerous endothelial enhancers contain GATA binding sites and are directly bound by GATA2 (Linnemann et al. 2011). Lugus et al. reported in 2007 (Lugus et al. 2007) that the overexpression of GATA2 in embryonic stem cells enhanced the generation of Flk1+/Tal1+ hemangioblast-like cells and the induction of endothelial-specific genes. Another report did show that GATA2 knockdown in HMVECs (human dermal microvascular endothelial cells) results in significant decrease of endomucin and KDR/VEGFR2 (all pro-angiogenic genes) while enhancing mesenchymal genes like SM-actin and epithelial SNAIL (Kanki et al. 2011), indicating that endothelial GATA2 is important for the maintenance of endothelial identity.

GATA3 is highly expressed in endothelial cells of large vessels (Song et al. 2009). Functional analyses revealed that GATA3 deletion in HUVECs (human umbilical vein endothelial cells) inhibits angiopoietin 1 (Ang1)-mediated AKT signaling, cell migration, survival, and tube formation by binding to the Tie2 promoter (Song et al. 2009).

GATA4 is mainly described to play a role in cardiac development (Singh et al. 2010), but some publications indicate a role for GATA4 in vascular development. For instance, GATA4 is described to control murine liver sinusoidal endothelial specification and function (Géraud et al. 2017). Liver sinusoids contain highly specialized, discontinuous endothelial cells, which lack a basement membrane, show fenestrations, and contain specialized junctional complexes with high permeability for solutes. Geraud et al. demonstrated that liver endothelial GATA4 is crucial for sinusoidal cell (LSEC) differentiation since ablation of GATA4 in these cells switched the discontinuous LSECs to normal continuous capillary ECs. This capillarization of LSECs caused liver hypoplasia, fibrosis, and impaired colonization by hematopoietic stem cells and embryonic mortality.

The GATA family member GATA5 is well characterized in the regulation of cardiovascular cell expansion and differentiation (Pikkarainen et al. 2004). It has been shown that GATA5 is necessary for differentiation of cardiogenic precursors into endothelial or endocardial cells (Nemer and Nemer 2002). Interestingly endothelial-specific GATA5-null mice exhibit increased blood pressure, endothelial dysfunction, and age-dependent end-organ damage, which are all features of human hypertension (Messaoudi et al. 2015).

GATA6, which also belongs to the cardiac subgroup of GATA transcription factors, is expressed in a wide range of tissues (heart, lung, liver, kidney, pancreas, spleen, ovary, and small intestine) in humans (Suzuki et al. 1996). GATA6 is also described to be highly expressed in quiescent vascular smooth muscle cells, where it contributes to the maintenance of the contractile phenotype of these cells (Wada et al. 2000; Nishida et al. 2002). In a pathological model of pulmonary arterial hypertension, Ghatnekar et al.

(2013) revealed endothelial GATA6 as an important player in the development of this disease through regulating the expression of EDNRA and CX3CL1 (fractalkine) leading to the recruitment of inflammatory cells. Furthermore, GATA6 was also shown to play a crucial role in angiogenesis and endothelial cell survival in primary human endothelial cells in vitro (Froese et al. 2011).

In conclusion, endothelial GATA transcription factors function to maintain endothelial differentiation and function, although their contribution to pathology and disease currently remains poorly defined.

Hypoxia-Inducible Factors (HIFs)

Hypoxia sensing is a fundamental biological process, which is implicated in the pathogenesis of cancer, ischemic heart disease, stroke, chronic lung disease, and many other disorders. At the center of the cellular responses to hypoxia are HIF proteins, which belong to a family of oxygen-sensitive basic helix-loop-helix transcription factors. Mammals express three different isoforms: HIF1α is expressed ubiquitously in all cells, while HIF2α and HIF3α are expressed more selectively, for example, in pneumocytes, renal interstitial cells, liver parenchymal cells, and vascular endothelial cells (Kaelin and Ratcliffe 2008; Bertout et al. 2008). They all consist of a heterodimerized protein structure with an oxygen-sensing α-subunit and a stable β-subunit, which enables the recognition and binding to the hypoxia response element (HRE) with the consensus sequence G/ACGTG in the genome (Mole et al. 2009). In normoxia, prolyl hydroxylase domain proteins (PHD1–3) use oxygen to hydroxylate the HIFs and target them for proteasomal degradation, while in hypoxia HIF transcription factors become more abundant and thus function as master regulators of the transcriptional response to hypoxia, which is especially important for the navigation of sprouting vessels (Coulon et al. 2010).

It is well established that HIF1α and HIF2α transcription factors promote angiogenesis as a response to hypoxia by the regulation of key angiogenic genes such as VEGFA (Hu et al. 2003). Mice with endothelial cell-specific deletion of HIF1α exerted defective blood vessel growth with skin wounds and xenograft tumors under hypoxic conditions (Tang et al. 2004). Interestingly, isolated mutant endothelial cells of these mice showed disrupted endothelial proliferation and migration with defective VEGFR2 activation. HIF2α is also highly expressed in endothelial cells during embryological development (Ema et al. 1997). Endothelial cell-specific HIF2α knockout mice showed impaired and abnormal tumor angiogenesis and reduced tumor growth in comparison to control mice (Skuli et al. 2009). Importantly, whereas HIF1α promotes angiogenesis via VEGFA, VEGFA expression is not regulated by HIF2α in endothelial cells (Tang et al. 2004; Skuli et al. 2009).

Migration and proliferation of endothelial cells require a fast supply of nutrients and energy, which is provided by metabolic activity. Under anaerobic (hypoxic) conditions, HIF1α is promoting the cellular glucose uptake and glycolysis through upregulation of VEGFA, glucose transporter type 1 (GLUT1), and glycolytic enzymes, such as lactate dehydrogenase A (LDHA) and PFKFB3 (Ebert et al. 1996; Fukasawa et al. 2004; Obach et al. 2004). PFKFB3 is a glycolytic enzyme that provides energy for cytoskeletal remodeling during filopodia and lamellipodia formation. Its promoter is directly activated by HIF1α binding. Additionally, some publications (Kim et al. 2007; Chen et al. 2013; Fu et al. 2016) revealed synergies between HIF transcription factors, especially HIF1α, and the dysregulated MYC oncogene in cancer, which accelerates glycolytic metabolism and angiogenesis through the induction of key proteins, HK2, PDK1, and VEGFA (Kim et al. 2007).

The Transcriptional Regulation by YAP/TAZ

The mammalian transcriptional coactivator YAPX (yes-associated protein) and its vertebrate paralog TAZ (transcriptional coactivator with

PDZ-binding motif), which do not directly bind DNA by themselves, promote gene regulation by interacting with TEAD/TEF family of transcription factors. YAP and TAZ are negatively regulated by Hippo signaling. The Hippo pathway consists of upstream kinases (such as MST and LATS) that inhibit YAP/TAZ activity through phosphorylation-dependent cytoplasmic retention and destabilization and thereby play crucial roles in regulating proliferation, differentiation, and migration of cells, tissue growth, and organ morphogenesis (Piccolo et al. 2014; Yu et al. 2015). Interestingly, liver-specific YAP transgenic overexpression in mice entails remarkable phenotypes of liver overgrowth, enhanced stem cell content, and reduced cellular differentiation (Dong et al. 2007). Moreover, several studies revealed Hippo pathway components to be involved in vasculogenesis and angiogenesis during embryonic development (Morin-Kensicki et al. 2006; Lee et al. 2008; Oh et al. 2009; Dai et al. 2013).

In this context, J. Park et al. published in 2017 that YAP/TAZ is critical for sprouting angiogenesis and vascular barrier maturation. They show that endothelial-specific deletion of YAP/TAZ in mice produced blunted, aneurysm-like tip endothelial cells with less and dysmorphic filopodia at the vascular front, a hyper-trimmed vascular network, reduced and disarranged distributions of tight and adherent junction proteins, disrupted barrier integrity, subsequent hemorrhage in growing retina and brain vessels, and reduced pathological choroidal neovascularization. Mechanistically, YAP/TAZ promotes Cdc42 and MLC2 activation and thereby actomyosin contractility, which is essential for filopodia formation and cell migration in tip ECs. Furthermore, YAP/TAZ upregulates expression and activity of MYC transcription factors and thereby promotes cell proliferation in stalk ECs and maturation in barrier endothelial cells forming the blood-brain barrier (Park et al. 2017) (Fig. 2).

MYC

The transcription factor MYC is a proto-oncogene, which is well known as driver of cell growth, proliferation, anabolic metabolism, and cancer development. It is a helix-leucine-zipper transcription factor that heterodimerizes with MYC-associated protein X (MAX), which is itself under tight regulation by a network of protein-protein interactions with MAX dimerization protein (MAD, also known as MXD1) and MAX interactor 1 (MXI1, also known as MAD2) (Grandori et al. 2000; Baudino and Cleveland 2001). Mechanistically, MYC functions downstream of growth factor signaling cascades such as extracellular-regulated kinase ERK that phosphorylates and increases its abundance by inhibiting proteasomal degradation (Dang et al. 2012).

The importance of MYC for endothelial cell function was found by Wilhelm et al. in 2016, who identified the MYC protein as key target of endothelial FOXO1 function. Myc is highly expressed in sprouting vessels where its depletion reduces glycolysis, mitochondrial activity, and proliferation of endothelial cells. Constitutively active FOXO1 inhibits MYC at several levels by suppressing MYC expression directly and by increased expression of negative regulators of MYC activity, such as MXI1 and FBXW7. MYC overexpression restores metabolism and proliferation in endothelial cells and repairs vascular defects with constitutively active FOXO (Wilhelm et al. 2016).

The Nuclear Factor (NF) κB

The pro-inflammatory transcription factor NFκB belongs to a family of five proteins (NFκB1, (NFκB2, RelA, RelB, and cRel) which all share a structural homology with the retroviral oncoprotein v-Rel (Nabel and Verma 1993; Ghosh and Hayden 2008). NFκB promotes the inflammatory response and the endothelial adhesion of leukocytes by triggering the expression of pro-inflammatory cytokines such as TNFα, IL1β, or ROS, bacterial endotoxins (Collins et al. 1995), and other pro-inflammatory genes like E-selectin, VCAM1, ICAM1, and IL6 (Bach et al. 1997). The NFκB pathway activation depends on the IKKα/β-NEMO complex leading to IκBα

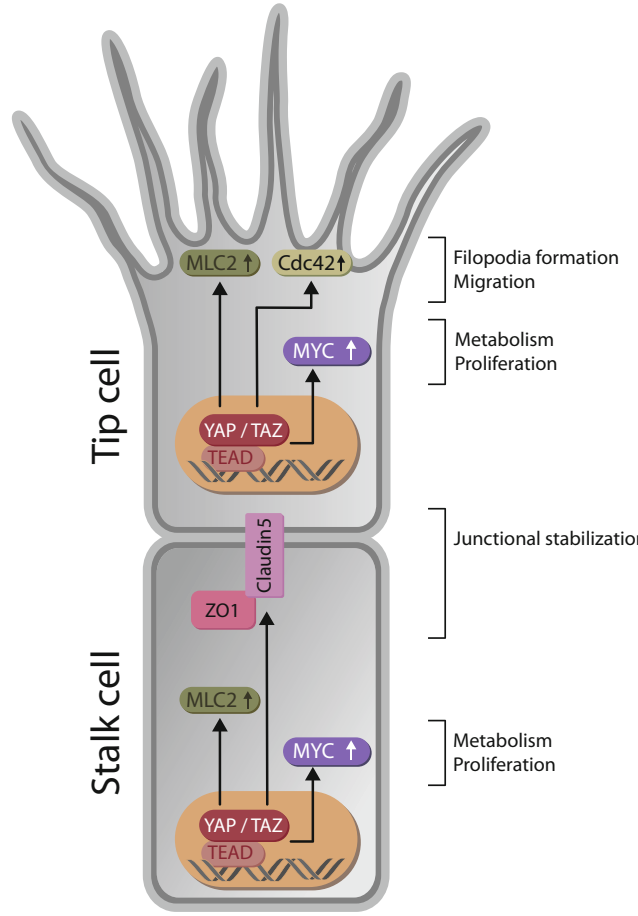

Fig. 2 Transcriptional regulation by Yap/TAZ in sprouting angiogenesis. YAP/TAZ binds to TEAD transcription factors and thereby promotes expression of MLC2 and Cdc42, which are essential for filopodia formation and endothelial cell migration in tip cells. Furthermore, YAP/TAZ regulates endothelial metabolism and growth by positively regulating the expression and activity of MYC signaling, which is important in tip and stalk cells. In quiescent stalk cells, YAP/TAZ is responsible for junctional stabilization by upregulation of junctional protein expression

phosphorylation and its subsequent degradation that releases NFκB from the inhibition by IκBα and induces its nuclear translocation (Perkins 2007).

Besides the pro-inflammatory response, NFκB is also activated by PKCε to protect human vascular endothelial cells. By activating ERK1/2, PKCε redirects the NFκB pathway away from inducing pro-inflammatory gene expression toward promoting protective cell survival and proliferative genes including anti-apoptotic Bcl2, cyclin D, and A20 (Dumont et al. 2012).

Interestingly, a recent in vitro proteomic analysis of Anderson et al. (2016) identified a key role of NFκB signaling to mediate mesenchymal stem cell (MSC) exosome-induced angiogenesis. As MSC are known to facilitate healing of ischemic tissue-related diseases, exosomal NFκB might be a potential treatment target in this disease.

Anti-angiogenic or Endothelial Quiescence-Promoting Transcription Factors

The Forkhead Transcription Factors

The forkhead transcription factors belong to a family of five different subfamilies, FOXO, FOXC, FOXF, and FOXH, which are known to be expressed in endothelial cells (De Val and Black 2009). They all share a conserved DNA binding domain and recognize the consensus sequence 5'-TTGTTTAC-3′ (Park et al. 2013).

The forkhead box O (FOXO) family contains four members, FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX), and FOXO6, of which FOXO1, FOXO3, and FOXO4 are highly homologous to each other (Carter and Brunet

2007). In the absence of growth factors, cellular FOXO transcription factors are localized in the nucleus where they regulate gene transcription. In response to growth factors (such as VEGFA) FOXO proteins get phosphorylated by AKT or serum/glucocorticoid-regulated kinase (SGK) leading to nuclear exclusion, shuttling to the cytoplasm, and subsequent FOXO degradation via the ubiquitin-proteasome pathway (Greer and Brunet 2005) (Fig. 3). FOXO transcription factors play important roles in the control of cell proliferation and survival, cell cycle progression, DNA repair, oxidative stress resistance, energy metabolism, and cell differentiation (Accili and Arden 2004; Arden 2008).

Interestingly, FOXO3- and FOXO4-deficient mice are viable, whereas FOXO1-null embryos die at embryonic day (E)11 due to defective vascular development (Castrillon et al. 2003; Hosaka et al. 2004; Furuyama et al. 2004). In 2016, Wilhelm et al. (2016) identified FOXO1 as essential regulator of endothelial proliferation and metabolic control. Endothelial FOXO1 overexpression in mice restricts vascular expansion, leads to vessel thinning, and reduced branching. FOXO1 also acts as a gatekeeper of endothelial quiescence by decelerating metabolic activity and reducing glycolysis and mitochondrial respiration through suppression of MYC signaling.

The members of the C subgroup of forkhead transcription factor family, FOXC1 and FOXC2, are also expressed in the developing vasculature, and FOXC1 as well as FOXC2 knockout mice die prenatally (Winnier et al. 1999; Petrova et al. 2004). FOXC1-deficient mouse embryos show coarctation of the aortic arch (Winnier et al. 1999), while FOXC2-deficient mice exert disturbed lymphangiogenesis with a lack of valves and increased smooth muscle cell layer (Petrova et al. 2004). Compound FoxC1/FoxC2 deletion in mice produced embryos with arteriovenous malformations due to reduced Dll4 and Notch expression (Kume et al. 2001; Seo et al. 2006). Mechanistically, FOXC1 and FOXC2 induce Dll4 through direct binding and activation of the promoter (Seo et al. 2006; Park et al. 2013).

The remaining subclasses of forkhead transcription factors FOXF and FOXH also have a

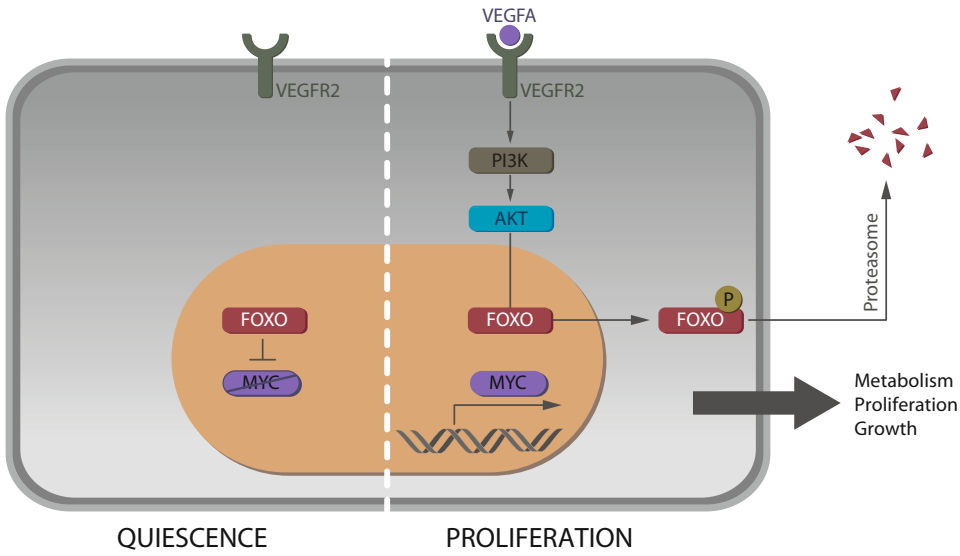

Fig. 3 Transcriptional regulation of endothelial metabolism by forkhead transcription factor FOXO and MYC. Without growth factor signaling, the transcription factor is located in the nucleus of (endothelial) cells where it regulates gene expression such as the downregulation of MYC. In the presence of growth factors (such as VEGFA), FOXO becomes phosphorylated by the protein kinase B (AKT) leading to nucleus exclusion and proteasomal degradation. Therefore, FOXO acts as a gatekeeper of endothelial metabolism between quiescence and proliferation by the regulation of MYC signaling

relevance in endothelial gene regulation. FOXH1 is described as a negative regulator of vascular development: Its overexpression in zebrafish interferes with vessel formation in part due to negative regulation of flk1 expression (Choi et al. 2007).

The Kruppel-Like Factors 2 and 4

Fluid shear stress in the endothelium is critical to maintain vascular homeostasis and can be categorized in laminar shear stress, which promotes endothelial cell quiescence, while oscillatory shear stress, mostly found at branch points of blood vessels, promotes endothelial dysfunction such as atherosclerosis (Chiu and Chien 2011).

The Kruppel-like factors 2 and 4 (KLF2 and KLF4) belong to a family of 17 zinc-finger transcription factors (Dekker et al. 2002) that are described in the literature as atheroprotective factors (Doddaballapur et al. 2015). Together with NFE2-related factor-2 (NRF2), KLF2 and KLF4 are central for the maintenance of endothelial homeostasis in response to shear stress (Mason 2016).

In a study where cultured endothelial cells have been exposed to unidirectional shear stress, both KLF2 and KLF4 were highly induced (Dekker et al. 2002; Hergenreider et al. 2012). KLF2 and KLF4 critically promote the expression of cytoprotective genes including eNOS, thrombomodulin, and HO1 (Dekker et al. 2002; Parmar 2005; Hamik et al. 2007; Ali et al. 2007; Villarreal et al. 2010). Mechanistically, the KLF2 promoter is activated by myocyte enhancer factor-2 (MEF2) in response to laminar shear stress downstream of MEK5/ERK5 (Parmar 2005). Activated KLF2 suppresses downstream transcription of glycolytic genes, including HK1, PFKFB3, and PFK1 (Wellen and Thompson 2012) and therefore promotes the quiescent state in endothelial cells (Boon et al. 2011). Notably, a similar metabolic state of quiescence is caused by FOXO1 as described above. In addition, KLF2-null mice die by embryonic day (E)14.5 due to hemorrhage caused by defective vessel stabilization and perturbed tunica media formation (Kuo et al. 1997; Lee et al. 2006). In addition, hemizygous deficiency of KLF2 resulted in enhanced atherogenesis in a murine model (Atkins et al. 2008).

The Nuclear Factor-Erythroid 2 p45-Related Factor (NRF2)

The transcription factor nuclear factor-erythroid 2 p45-related factor (NRF2) is a basic region leucine zipper and known as a master regulator of the antioxidant response. Nrf2 exists in complex with Kelch-like ECH-associated protein 1 (Keap1) in the cytosol until oxidative stress, toxins, growth factors, or laminar shear stress leads to the dissociation of the Nrf2-Keap1 complex. As consequence, Nrf2 translocates to the nucleus where it binds to the antioxidant response element (ARE) mediating the expression of a group of genes encoding phase II detoxification enzymes and antioxidant proteins, such as glutathione reductase (GR), heme oxygenase-1 (HO1), peroxiredoxin 1 (Prx1), nicotinamide adenine dinucleotide phosphate (NADPH), and quinone oxidoreductase-1 (NQO1) (Ishii et al. 2000; Kobayashi and Yamamoto 2005; Niture et al. 2014).

It has been reported that in endothelial cells exposed to oscillatory disturbed shear, but not to unidirectional laminar shear flow patterns, Nrf2/ARE redox signaling is inhibited, leading to the activation of the proinflammatory transcription factors nuclear factor-κB (NFκB) and activator protein-1 (AP1) ultimately resulting in activation of inflammatory genes to enhance expression of adhesion molecules and chemokines promoting the adhesion of monocytes (Hosoya et al. 2005; Warabi et al. 2007; Dai et al. 2007; Zakkar et al. 2009; Takabe et al. 2011).

The p53 Transcription Factor

The p53 protein, also known as "the guardian of the genome," is the most famous key transcription factor involved in cellular stress responses and the protection against cancer (Carson and Lois 1995). Since its discovery in 1979, researchers revealed p53 as the most frequently altered gene in human

cancers (Hollstein et al. 1991). Evidence on p53 as a tumor suppressor mainly emerged from the findings that p53-deficient mice are susceptible to spontaneous tumorigenesis (Donehower et al. 1992). In normal cells, p53 is continuously degraded by the E3 ubiquitin ligase MDM2 to maintain a low level of the transcription factor. Cellular stress, such as nucleotide deprivation, DNA damage, hypoxia, or oncogene signaling, leads to posttranslational modification of p53, resulting in inhibition of p53 degradation and consequent stabilization of p53 levels (Ho and Benchimol 2003) (Fig. 4).

Most of the characterized target genes of p53 are on one hand preventing cell cycle progression, such as p21, and on the other hand inducing apoptosis, such as BAX, PUMA, or NOXA (Vousden and Lu 2002). Several studies on clinical tumor sample gave evidence that there are also p53 target genes that regulate metastasis or angiogenesis. For instance, two independent studies of human prostate cancer have shown that tumors harboring p53-mutated genes caused significant higher blood vessel density than tumors expressing wild-type p53 (Yu et al. 1997; Takahashi et al. 1998). The same influence on tumor vascularization could have been shown in studies of colon cancer (Faviana et al. 2002) and breast cancer (Gasparini et al. 1994).

One of the major pathways how p53 limits tumor angiogenesis is the regulation of HIF in response to hypoxia. HIF is a dimeric transcription protein (more details in section "Hypoxia-Inducible Factors (HIFs)") comprised of HIF1α and HIF1β. Under normal physiological conditions, HIF1α levels are kept low by proteasomal degradation. Under hypoxic conditions, HIF1α is stabilized and dimerizes with HIF1β to activate pro-angiogenic target gene expression (Ivan et al. 2001; Jaakkola et al. 2001). In an oncogenic situation or during cellular stress, p53 is stabilized and inhibits pro-angiogenic HIF1α activity by directly binding HIF1α and targeting it for degradation (Ravi et al. 2000).

Besides the HIF pathway, p53 is found to have a central role by inhibiting several other important pro-angiogenic proteins, including VEGF (Pal et al. 2001), bFGF (Ueba et al. 1994), and COX2 (Subbaramaiah et al. 1999). Furthermore, it is published that p53 inhibits the PI3K/AKT/mTOR pathway in osteosarcoma and thereby exerts an anti-angiogenic effect (Song et al. 2015).

In addition to the inhibition of several pro-angiogenic proteins, p53 has been shown to reduce vascular proliferation by inducing the expression of anti-angiogenic genes such as thrombospondin-1 (TSP1) (Dameron et al. 1994), brain-specific angiogenesis inhibitor 1 (BAI1) (Nishimori et al. 1997), ephrin receptor A2 (EPHA2) (Dohn et al. 2001; Brantley et al. 2002), and certain collagens that have a negative angiogenic effect (Teodoro et al. 2006). The activation of these target genes is dependent on the recognition of a specific DNA-binding motif,

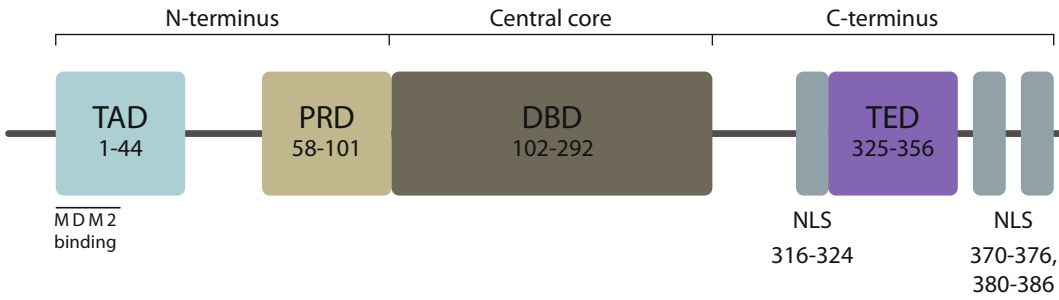

Fig. 4 Scheme of the protein domain structure of p53 transcription factor. The protein is composed of four functional domains: an N-terminal transactivation domain (TAD), where MDM2 is able to bind and inhibit its transactivation function, a proline rich domain (PRD), a central DNA binding core domain (DBD), and a C-terminal tetramerization domain (TED), through which p53 is able to form highly symmetrical tetramers. In addition, the C-terminal region contains three nuclear localization signals (NLS)

called the "p53 response element," by p53 in their promoter region.

The Recombining Binding Protein Suppressor of Hairless (RBPJ)

The recombining binding protein suppressor of hairless (RBPJ), also known as CSL, is the most important transcription factor in the regulation of genes downstream of the DLL-Notch signaling pathway. The gene is located on chromosome 5 and is expressed ubiquitously in mammalian tissues (Hamaguchi et al. 1992). Endothelial cells use the Notch pathway for their communication over cell-cell contacts to coordinate blood vessel sprouting. By the activation of Notch receptors at the surface of stalk cells by Dll4, which is expressed by tip cells with high sprouting activity, the VEGF receptor expression in stalk cells is downregulated resulting in a low sprouting capacity in these cells (Suchting et al. 2007).

The transcription factor RBPJ can act either as a transcriptional activator or repressor. In the absence of activated Notch, the RBPJ transcription factor is located in the nucleus and functions as a transcriptional repressor through complexing with the SMRT/SKIP corepressor proteins (Zhou and Hayward 2001). Activation of Notch by one of its ligands (Dll1, Dll3, Dll4, Jagged1, or Jagged2) leads to proteolytic cleavage of the receptor, which releases the intracellular domain of Notch (NICD) that is now able to enter the cell nucleus. Inside the nucleus, NICD binds RBPJ leading to a switch from the RBPJ repressor to an activator function by the recruitment of the coactivator mastermind (Kovall and Blacklow 2010). Besides a broad range of genes, the Notch/RBPJ complex induces the expression of the hairy/enhancer of split (HES) and HES-related genes (HEY, CHF, HRT, HESR), which serve as important Notch signaling effectors in the developing vasculature (Nakagawa et al. 2000). A study with loss of RBPJ in mice results in embryonic lethality prior to E10.5 through defects in sprouting angiogenesis, arterial/venous specification, and vascular smooth muscle cell organization (Krebs et al. 2004).

Conclusions and Future Directions

Endothelial transcription factors are powerful regulators of sprouting angiogenesis and of endothelial cell differentiation. By fulfilling these functions, they not only regulate the state of the vasculature but also have a strong impact on organ development, homeostasis, and function. This is best illustrated by the recent finding on the liver sinusoidal endothelial-specific knockout of GATA4, which changes the differentiation of these highly specialized, fenestrated, endothelial cells into normal continuous capillary endothelial cells leading to embryonic death due to liver hypoplasia, fibrosis, and impaired colonization by hematopoietic endothelial cells. In the light of these important new findings, it seems likely that more organ-specific endothelial cell-specific programs and their upstream transcriptional regulators will have to be identified to understand the contributions of endothelial cells to the function of various organs. In addition, a potential link to diseases needs to be established: Does reduced GATA4 in liver sinusoid contribute to fibrotic liver disease? Similarly, are there endothelial-specific gene programs that contribute to heart, kidney, or neurological disease? Are there endothelial-specific transcription factors that are dysregulated in these diseases? The advent of powerful methods to selectively isolate cell populations from different organs, to analyze the whole transcriptome by (single-cell) RNA sequencing, and to conduct advanced epigenetic and proteomic OMICS analyses in combination with time- and cell-specific genetic manipulation in model organisms will provide answers to these questions. Finally, when disease-specific gene programs and their regulators are identified, tools are needed that will allow therapeutic targeting of these entities to improve the outcome of human disease.

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Endothelial Cell-Cell Junctions in Tumor Angiogenesis

Quentin Roux and Julie Gavard

Contents

Introduction 92

Molecular Basis of Tumor Angiogenesis 92
Formation of the Vascular Network During Development 92
Overview on Tumor-Induced Angiogenesis 95
Vascular Leakage in Tumor Angiogenesis 97
Endothelial Cell-Cell Junctions 99
The Endothelial Tight Junctions 100
The Endothelial Adherens Junctions 101
VE-Cadherin in the Endothelial Barrier 102

Endothelial Junctions in Tumor-Induced Angiogenesis 107
Dynamics of Endothelial Junctions in Migration and Sprouting 107
Endothelial Cell-Cell Junctions in Polarity 108
Remodeling of Endothelial Junctions in Tumor Vascular Permeability 109
Involvement of Endothelial Junctions in Tumor Vascular Aberrations 111

Future Directions 113

Cross-References 113

References 113

Abstract

Angiogenesis is a complex and tightly regulated multistep process whose deregulations induce an aberrant growth of blood vessels, strongly associated with cardiovascular pathologies and also with tumor progression in most of the solid cancers. Tumor vessels are essentially smaller, disorganized, and leaky. In this scenario, the endothelial cells that mat the inner side of the vascular wall are excessively activated and exhibit higher proliferation rate and enhanced migratory phenotype. The loss of endothelial barrier integrity is one of the most striking phenotype of the tumor vasculature and contributes to exacerbate angiogenesis, tissular damage, stromal abnormalities,

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perivascular inflammation, and poor drug delivery.

Physiologically, the endothelial barrier controls the bidirectional passage and the flux of fluids, molecules, and cells from the blood stream to the irrigated tissues. In the tumor microenvironment, this barrier is strongly permeable, allowing thereby unrestricted, anarchic movements across the endothelium. Molecularly, the dismantlement of the endothelial cell-cell junctions, notably those formed by the cell-cell adhesion molecule VE-cadherin, supports vascular leakage in the tumor microenvironment.

There is now growing evidence that restoring the function of endothelial cell-cell junctions could help normalizing the tumor vasculature and further support the use of anti-permeability agents as potent means to interfere with tumor-driven angiogenesis.

Keywords

Adherens junctions · Endothelial barrier · Tight junctions · Tumor angiogenesis · Vascular leakage · Vascular permeability · VE-cadherin

Introduction

During embryonic development, the vasculature is established through de novo formation of blood vessels (termed as vasculogenesis) followed by the stereotyped organization of the vascular network from pre-existing vessels (termed as angiogenesis). High concentration of angiogenic factors, such as vascular endothelial growth factor (VEGF), drives neo-angiogenesis in embryos and adults. This morphogenetic program is also aberrantly reactivated in the tumor microenvironment to supply tumor cells with nutrients, oxygen, and growth factors. However, the tumor vasculature is comparatively disharmonized and rather inefficient.

Endothelial cells that line the luminal face of blood vessels sustain both vascular homeostasis and bidirectional exchanges with irrigated organs. The main functions of the endothelium are not only to act as transport tubes that fuel organs but also to form a physical yet flexible barrier. In functional vessels, adherens and tight junctions seal the endothelial cells together and orchestrate the endothelial barrier. Both adherens and tight junctions take the lead role in controlling the exchanges between blood and tissues. Notably, VE-cadherin, a cell-to-cell adhesion protein exclusively expressed in the endothelium, sets at the cornerstone in the assembly and disassembly of endothelial junctions, in health and diseases.

One striking feature of tumor blood vessels is the loss of barrier integrity and the abnormal elevation of vascular permeability. This is associated with enhanced vascular sprouting and many other abnormalities concerning origin, organization, and fate of tumor endothelial cells. In this chapter, we will summarize current knowledge in (1) tumor-induced angiogenesis mechanisms, (2) the organization of endothelial cell-cell junctions, and finally (3) how endothelial cell-cell junctions contribute to the different processes of tumor-induced angiogenesis, with an emphasis on the mechanisms involved in the disruption of the endothelial barrier and the increase of vascular permeability.

Molecular Basis of Tumor Angiogenesis

Formation of the Vascular Network During Development

The vascular system is established during embryogenesis and gives rise to a dense, structured blood vessel network of arteries, arterioles, veins, venules, and capillaries that ultimately perfuses all tissues throughout the body and fuels cells with oxygen and nutrients. The vascular tree originates from the primary vascular plexus in which mesoderm-derived progenitor cells differentiated into angioblasts (Carmeliet 2005). This embryonic vascular structure is formed upon vasculogenesis, which corresponds to de novo formation of a primitive vascular network. It is progressively remodeled by angiogenesis, allowing the formation and maturation of new

blood vessels from the pre-existing network (Carmeliet 2005). Although the vascular system is considered as mostly quiescent after birth, developmental angiogenesis program can be reactivated to form new blood vessels and adapt the network to cells' needs, such as postnatal retinal vascularization, pregnancy, body growth, etc. (Carmeliet 2005).

Blood vessels consist of an endothelial cell monolayer covered by a basement membrane that anchored perivascular muscle cells and smooth muscle cells for arteries and veins, and pericytes for capillaries, which together maintain the integrity of the vascular wall and allow contraction. Endothelial cells orchestrate the vascular barrier and form a stable, dynamic monolayer acting as a selective filter between the blood compartment and the irrigated tissues (Dejana 2004; Gavard 2013). Barrier properties are largely modulated by both adherens junctions, enriched in the VE-cadherin cell-cell adhesion molecule, and tight junctions with claudins, occludin, and junction adhesion molecules (JAMs), that bridge neighboring endothelial cells together and maintain the cohesiveness of this tissue. The adhesive properties of these molecules and their intracellular signaling capabilities are essential for endothelium homeostasis, with key roles in adhesion, migration, proliferation, division orientation, and adapted, coordinated responses to external cues (please see section "Endothelial Cell-Cell Junctions").

Angiogenesis mostly refers to sprouting angiogenesis during which pre-existing capillaries bud and form a neo-vessel that migrate and invade the avascular space, and further maturate and integrate within the vascular network (Fig. 1) (Potente et al. 2011). In hypoxic conditions, cells secrete growth factors to stimulate surrounding vessel sprouting, among which is the vascular endothelial growth factor family (VEGF), and more specifically VEGF-A_{165} (Olsson et al. 2006). Briefly, VEGF signaling pathway is implicated in several developmental processes, in the vascular system but also in the nervous and lymphatic systems (Olsson et al. 2006; Ferrara et al. 2003). Secreted by tissues in hypoxic conditions, VEGF-A can notably interact with its cognate receptors VEGF-R1 and VEGF-R2 expressed at the plasma membrane of endothelial cell. VEGF-A can also be presented to VEGF-R via the transmembrane binding receptor neuropilin-1 (NRP1) (Koch et al. 2014). VEGF-A binding to VEGF-R2 induces the dimerization and autophosphorylation of the receptor, allowing recruitment of SH2 (Src Homology 2) domain kinases that further activate intracellular signaling pathways implicated in cytoskeleton rearrangement, migration, and cell survival (Olsson et al. 2006). Through these signaling cascades, VEGF regulated the different steps of neo-vessel formation including endothelial differentiation, proliferation, and migration and controls as well vessel permeability by modulating the composition and localization of endothelial cell-cell junctions. Contrarily to VEGF-R2, VEGF-R1 bears a weak tyrosine kinase activity and instead may operate as a competitive inhibitor for VEGF-R2, limiting thereby endothelial cell responses to the growth factor. From the study of knockout mice, VEGF-A and VEGF-R appear essential for vascular development, VEGF knockout leading to the in utero death of embryos between days 8.5 and 10.5 because of severe defects in the establishment of the vascular network (Carmeliet et al. 1996; Dumont et al. 1994; Ferrara and Kerbel 2005).

In response to VEGF, pericytes located on the external face of capillaries detached and the basement membrane are degraded (Armulik et al. 2005; Betsholtz et al. 2005). Subsequently, VEGF signaling induces endothelial activation and differentiation in two separate phenotypes with nonredundant cellular functions and distinct genetic expression profiles: tip and stalk cells (Blanco and Gerhardt 2013). Tip cells notably develop numerous cytoplasmic protrusions termed as filopodia and lead and guide the vascular sprouts. Thus, tip cells allow endothelial cells to migrate, sense, and explore the environment and determine the orientation of the sprout. Guided by the tip cell, stalk cells proliferate at the rear and subsequently form the lumen of neo-vessels (Blanco and Gerhardt 2013).

From a molecular standpoint, two major pathways are implicated in this differentiation step: (i) the VEGF pathway that induces filopodia

formation and promotes the tip cell phenotype and (ii) the Notch/Dll4 pathway operating as a controller pathway that orientates endothelial cell toward the stalk phenotype (Fig. 1). The Notch signaling pathway is highly conserved through evolution and is implicated in numerous developmental processes and cell fate determination. Modulators of this pathway are Notch receptors (Notch 1 to Notch 4 in Vertebrates) expressed at cell membrane and their transmembrane ligands, namely, the Delta like ligands (DLL1, DLL3, DLL4) and Jagged ligands (Jag1 and Jag2) (Blanco and Gerhardt 2013). The membrane localization of ligands and receptors defines the Notch pathway as an intercellular contact-dependent pathway. Briefly, Notch activation in angiogenesis is initiated with the ligand DLL4 harbored at the membrane of neighboring endothelial cells. This DLL4/Notch interaction results in the proteolytic cleavage of the Notch intracellular domain (NICD) that translocates into the nucleus and activates Notch target genes including genes encoding for VEGF receptors (Blanco and Gerhardt 2013; Guruharsha et al. 2012). Consequently, Notch balances VEGF signaling in endothelial cells. Of note, mice knockout for Notch1, Notch4, or the ligand DLL4 die in utero because of vascular anomalies and an inability to remodel the primary vascular plexus into a hierarchized network (Xue et al. 1999; Krebs et al. 2000).

To ensure the formation of a stereotyped vessel network, endothelial cell phenotype is thus strictly controlled, and number of tip cells and sprouts remains limited during developmental and postnatal physiological angiogenesis.

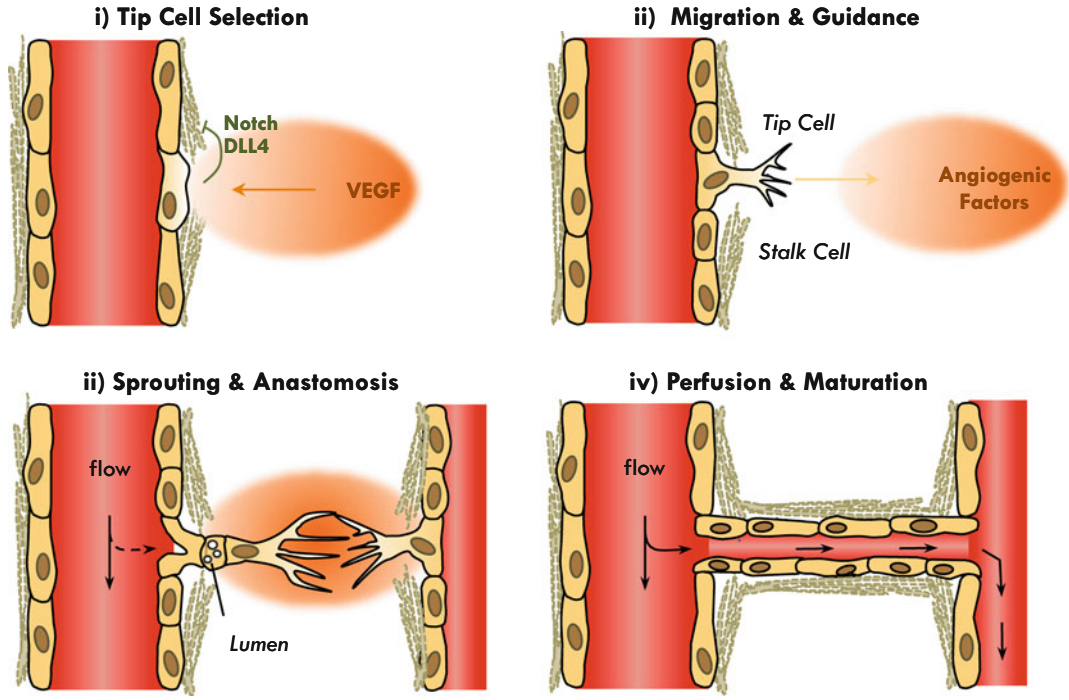

Fig. 1 Multistep processes of sprouting angiogenesis. (i) VEGF (vascular endothelial growth factor) gradient concentration allows DLL4 (delta-like ligand 4) expression in the receiving endothelial cell. Neighbor endothelial stalk cells express DLL4 receptor Notch. Basal matrix is locally degraded. (ii) Tip cell is selected to migrate and lead the sprout, as the endothelial cell with high VEGF-R2 activation, low VEGF-R1, and low Notch activation, opposed to proliferating stalk cells at the rear. (iii) Stalk cells multiply and allow nascent vessel expansion. At the front, tip cells between two adjacent vascular buds join. Vacuolization is initiated to drive lumen formation. (iv) Upon lumen formation and perfusion, a neo-vessel is functionally formed. Maturation includes strengthening of endothelial junctions, matrix deposit, and pericyte recruitment

Endothelial cell specification is initiated by the gradient of VEGF-A and VEGF/VEGF-R2 signaling that induces the formation of filopodia, tip cell differentiation, and an increased expression of DLL4 at the endothelial tip cell membrane (Hellstrom et al. 2007; Jakobsson et al. 2009, 2010; Bentley et al. 2009). Tip cells are migratory cells, characterized by high levels of DLL4 and VEGF-R2, while stalk cells have a proliferative phenotype and express less DLL4 and VEGF-R2. Instead, stalk cells express high levels of Notch Jagged ligand (Jag1) and VEGF-R1 (Hellstrom et al. 2007; Phng et al. 2013). Concurrently, VEGF induces the transcriptional activation of the DLL4 promoter in tip cells. The interaction between DLL4 (expressed by tip cell**s**) and Jag1 (expressed by stalk cells) leads to Notch activation in the neighboring stalk cells. Consequently, Notch signaling is activated through DLL4 in the endothelial cells surrounding the tip cell. Notch activated-endothelial cells adopt a stalk cell phenotype and proliferate to elongate the sprout. Downstream, Notch activation in stalk cells increases the expression of the low kinase activity VEGF receptor VEGF-R1, while decreasing VEGF-R2 levels, ultimately desensitizing stalk cells from VEGF signaling and therefore modulating proliferation versus migration phenotype (Hellstrom et al. 2007; Phng et al. 2013). Thus, the activity of the Notch pathway is inversely regulated in tip and stalk cells. Experimental loss of DLL4 expression results in an excessive sprouting and branching phenotype due to excessive number of formed tip cells and endothelial proliferation (Hellstrom et al. 2007). Notch-depleted endothelial cells indeed adopt features of tip cells and present migratory phenotype by sprouting and branching, whereas Notch activation in stalk cells promotes proliferation (Blanco and Gerhardt 2013). Recently, Notch activity, but not DLL4, was reported to contribute to specify arterial endothelial cell phenotype (Pitulescu et al. 2017). Notch signaling is thus essential for the establishment of a hierarchical and functional vascular network, by restricting endothelial cell tip specification but also by coupling angiogenesis to arteriogenesis during blood vessel formation.

Sprout progression is guided by the tip cell in response to attractive and repulsive guidance molecules allowing the formation of a vessel adapted to the need and the topology of the perfused tissue (Michaelis 2014). Attracted toward the same gradient of pro-angiogenic factors, migrating sprouts fuse via anastomosis and connect their respective lumen. In turn, blood perfusion reduces VEGF tissue expression and orientates endothelial cell toward a quiescent state notably through activation of the shear stress responsive transcription factor KLF2 (Kruppel-Like Factor 2), further promoting endothelial cell survival and strengthening of cell-cell junctions (Dekker et al. 2006; Wu et al. 2008). A fully mature branch is established after deposition of the basement membrane, recruitment of mural cells through PDGF-B (platelet-derived growth factor B), and stabilization of endothelial cell-cell junctions that maintain endothelial cell quiescence, polarity, and survival (von Tell et al. 2006; Armulik et al. 2005). In case of abnormal or not suitable vessel formation, angiogenesis can be reversed and vessel pruned (Ferrara and Kerbel 2005) while ensuring the integrity and the functionality of the vascular network.

Overview on Tumor-Induced Angiogenesis

Following Judah Folkman's postulate in 1971, the development of a dense and anarchic vascular network within tumors was shown to be essential for exponential tumor growth and metastasis. This pioneer work demonstrated the need for solid tumors to develop a specific vasculature and be perfused to grow over a limited size of 2 mm^3. This paves the way for the anti-angiogenic concept in clinics, i.e., anticancer strategy by which therapeutic drugs are designed to block neo-vessel formation and inhibit tumor progression (Folkman 2006). For instance, the crucial role for VEGF signaling in tumor growth justifies the development of anti-angiogenic therapies based on blocking this mechanism (Folkman 2006; Ferrara and Kerbel 2005). Notably, bevacizumab, a humanized mouse anti-VEGF antibody, is the first clinically approved

anti-angiogenic drug and has proven efficiency in combination with standard chemotherapies in non-small cell lung cancers and advanced colorectal cancers (Folkman 2006; Ferrara and Kerbel 2005). However, blocking angiogenesis does not appear adequately effective to starve tumor cells and may even be deleterious for patients, suggesting that instead of destructing the vasculature, normalizing its function might prove better clinical benefits (Jain 2005). Indeed, tumor vasculature consists in a dense, badly structured and poorly functional network of leaky capillaries that fail to maturate. The formation of this network relies on the unreasonable reactivation of developmental processes, sprouting angiogenesis being the more studied and most likely the more prevalent. Alternate strategies of neo-vessel formation rely on reciprocal interaction between cancer cells and endothelial cells, in processes defined as co-option, mimicry, and transdifferentiation (Fig. 2).

Growing tumors rapidly develop a hypoxic core where oxygen concentration is not sufficient to sustain inordinate cell expansion. Similarly to normal conditions, hypoxia-regulated pathways induce pro-angiogenic factor secretion to stimulate surrounding vessel growth. Tumors release considerable amounts of VEGF that aberrantly activate neighboring endothelial cells, multiplying tip cell number, filopodia development, and network ramifications. Interestingly, tumor-emanating VEGF is disseminated and made available to the microenvironment, under multiple forms including within extracellular vesicles (Skog et al. 2008; Andre-Gregoire and Gavard 2017; Feng et al. 2017). VEGF signaling in endothelial cells is tightly regulated during physiological angiogenesis. VEGF controls endothelial differentiation and guides sprout migration in the activated endothelium, while in quiescent endothelial cells, VEGF autocrine and paracrine signaling are required to maintain vascular homeostasis, endothelial cell survival, and junctional stability (Lee et al. 2007). In the tumor microenvironment, high concentration of VEGF results in the growth of numerous unstable tumor capillaries, where endothelial cells fail to establish stable cell-cell junctions, recruit perivascular cells, and form permeable blood vessels sustaining limited tumor oxygen and nutrient needs (Carmeliet and Jain 2011). According to its pivotal function in tumor-induced angiogenesis, VEGF is the primary target for anti-angiogenic therapy (Folkman 2006; Ferrara and Kerbel 2005; Carmeliet and Jain 2011), as exemplified by the anti-VEGF monoclonal blocking antibody bevacizumab, whose efficacy has been proven as adjuvant therapy in certain cancer types, although individual responses vary between patients and are opposed to resistance.

In addition to sprouting angiogenesis, other mechanisms such as the recruitment of endothelial progenitors from the bone marrow can be initiated by cancer cells to develop their vascular network, mimicking thus vasculogenesis developmental program (Folkins et al. 2009). New branches can also be formed by induction of intussusception to split existing blood vessels (Fig. 2).

Moreover, the plasticity of cancer cells allows them to become integral components of the tumor vasculature. Although marginal, cancer cell-based vascular-like structures actively participate in tumor blood supply, dissemination, and resistance to anti-angiogenic therapies. In a process defined as vascular or vasculogenic mimicry, cancer cells acquire the ability to form de novo a tubular network perfused with plasma and red blood cells within the tumors (Kirschmann et al. 2012). This phenomenon is observed in a large variety of high-grade cancers, including melanoma, sarcomas, carcinomas, and glioma. Tumor cells adopt an endothelial phenotype and display modified gene expression of the vascular (VE-cadherin, EphrinA2, VEGF-R1) and embryonic/stem cell (Nodal, Notch4) repertoires (Folberg et al. 2000). The activation of endothelial-specific pathways, including the ectopic expression of VE-cadherin normally restricted to the endothelial lineage, induces the establishment of cell-cell junctions between cancer cells and endothelial cells, resulting in endothelial-like cancer cells integration within the endothelium (Fig. 2). In response notably to the activation of tumor-derived VEGF/VEGF-R1 pathway, endothelial-like cancer cells form

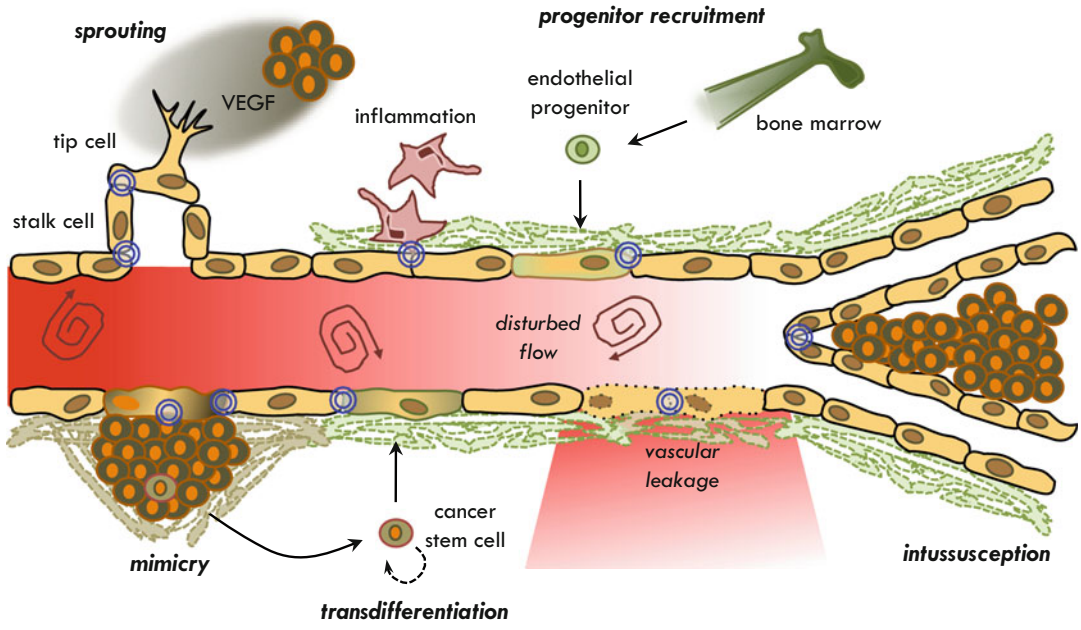

Fig. 2 Cell-cell interactions in tumor-induced angiogenesis. Neovascularization of tumor mass can occur via multiple mechanisms: (i) reactivation of developmental sprouting angiogenesis, (ii) homing of circulating endothelial progenitor cells in a process reminiscent of vasculogenesis, (iii) vascular mimicry where tumor cells adopt a endothelial-like phenotype and associate with tumor vessel, (iv) transdifferentiation is the ability of cancer stem-like cells to recapitulate endothelial differentiation program and integrate tumor vessels, and (v) intussusception and co-option correspond to the ability of tumor cells to employ the existing vessel network to their own benefit by splitting and sequestrating it, respectively. Tumor vasculature features abnormal number of tip cells and sprouts, perivascular inflammation, vascular leakage, disturbed blood flow

pseudo-vascular structures (Seftor et al. 2012; Kirschmann et al. 2012).

Cancer stem-like cells are also a rare, self-sustained population of cancer cells with multipotency properties. They reside in tumor vascular niche and are in tight interaction with endothelial cells (Lathia et al. 2015). For instance, experimental animal models for brain tumors suggest that human cancer cells bearing stem properties can differentiate and integrate the host vasculature (Ricci-Vitiani et al. 2010; Wang et al. 2010; Cheng et al. 2013). Such cells could be tracked and identified as pseudo-endothelial and pericyte-like cells. How much they contribute to tumor vascularization and promote vascular functionality is not completely clear.

Finally, cancer cells can also be fueled through the pre-existing vasculature notably in highly perfused organs (lungs, brain, and liver) where tumors can be initiated and grow without inducing new vessel formation in a process termed as vessel co-option (Leenders et al. 2002). For cancer cells, co-option consists in maintaining tissue vessels quiescent and fully functional, forming thus a stable and efficient blood supply beneficial for tumor growth.

All these different tactics elaborated by cancer cells to enhance vascularization have to be considered when designing anti-angiogenic therapies, as they may impact directly on response to treatment and development of resistance.

Vascular Leakage in Tumor Angiogenesis

The vascular endothelium forms a semipermeable barrier separating blood stream from surrounding tissues while permitting constant, directional passages between the two compartments. The

vascular system represents indeed a 5000 m^2 exchange surface between the blood and the irrigated organs. This barrier controls the passage of plasma molecules and solutes, as well as circulating cells into the adjacent tissue. Reciprocal movements across the endothelium can occur via two different routes, namely, a transcellular and paracellular transport, i.e., through and between endothelial cells, respectively (Fig. 3).

The transcellular transport takes place through endothelial cells and operates as follows via (i) passive diffusion of ions or lipophilic molecules; (ii) gradient diffusion from high to low concentrations; (iii) active diffusion which requires energy to transport large molecules, such as fatty acids or vitamins; and (iv) transcytosis where macromolecules are transported within a membrane-bound carrier from one side of the cell to the other (Vestweber 2012; Azzi et al. 2013). This latter mode occurs either through a system of clustered vesicles, called vesicular vacuolar organelles (VVOs), which direct link vascular lumen and albumen, or through more typical intracellular vesicles (caveolae) (Kiss 2012).

The paracellular transport corresponds to the molecular and cellular fluxes between adjacent endothelial cells. This requires the weakening of endothelial cell-cell junctions that are normally sealed by cell-to-cell adhesion molecules. For instance, low vascular permeability blocks delivery and diffusion of xenobiotics and drugs, as observed at the blood-brain barrier (Zlokovic 2008). Conversely, high endothelial permeability occurs in demand to specialized functions, such as blood filtration in the kidney where glomerular endothelial cells are doted of fenestrations, i.e., transcytoplasmic holes that allow crossing the glomerular capillary wall (Satchell and Braet 2009). The selectivity of the endothelial barrier is thus adapted to the tissue needs. The expression profile of adhesion molecule and their organization can be modified, as observed in the context of the highly selective, protective blood-brain barrier and conversely to facilitate exchanges in lung capillaries. The integrity of the endothelial junctions has to be tightly regulated, since barrier dysfunctions can directly alter the homeostasis of perfused tissues and blood flow. Physiologically, endothelial cell-cell junctions mechanically mediate adhesion between neighboring endothelial cells. This sealed contact is not permeable to albumin (69 kDa) and other large molecules and presents as well selectivity toward the passage of much smaller molecules (<1 kDa) (Vestweber 2012; Azzi et al. 2013). Additionally to endothelial cell-cell adhesive contacts, blood-tissue permeability is controlled by endothelial cell/extracellular matrix and endothelial cell/pericyte interactions that constitute an additional filter (Vestweber 2012; Betsholtz et al. 2005).

Permeability elevation is a hallmark of neo-angiogenesis. While transient and regulated in normal angiogenesis, excessive angiogenesis frequently correlated with uncontrolled vascular

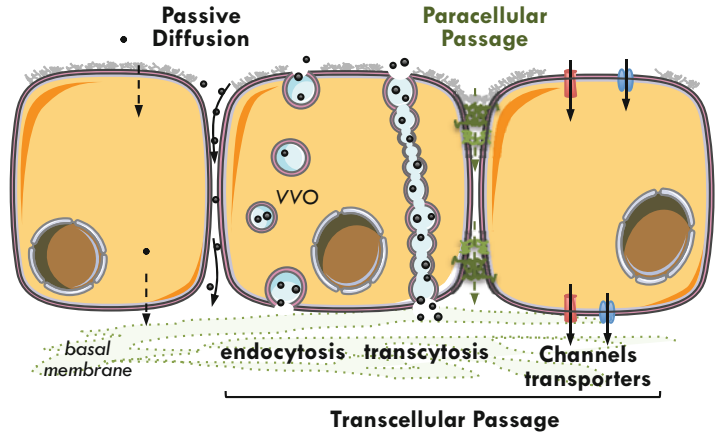

Fig. 3 Routes through the endothelial barrier. There are two major ways to cross the endothelial barrier: (i) the paracellular passage in between the endothelial cells and (ii) the transcellular pathway via passive and active diffusion, via transcytosis, or via a specific network of intracellular organelles, named as vesiculo-vacuolar organelles (VVO)

leakage (Weis and Cheresh 2005). In this context, the constantly elevated VEGF concentration found in the tumor microenvironment is the principal cause of formation, instability, and high permeability of capillaries. VEGF affects pericytes and smooth muscle cells in which VEGF signaling induces detachment from the basement membrane and gives rise to barely covered tumor capillaries (von Tell et al. 2006). Pericytes are indeed loosely attached to tumor vessels, and this therefore contributes to disproportionate permeability increase (Goel et al. 2011). Loss of pericytes induces rapid degradation of the basement membrane further destabilizing endothelial cells and impairs mural-endothelial Ang1/Tie2 signaling that usually maintain endothelial quiescence and junction stability in covered vessels (Saharinen et al. 2008; Gavard et al. 2008). In tumor capillaries, endothelial junctions are also directly impaired by elevated activation of VEGF/VEGF-R2 signaling that induces VE-cadherin destabilization and internalization (Gavard and Gutkind 2006; Gavard et al. 2008).

The resulting increased tumor vessel permeability contributes to deviant neo-angiogenesis directly profitable to tumor cells that receive an unrestrained, yet not optimal access to nutrients, oxygen, and growth factors (Fig. 4) (Le Guelte et al. 2011). Elevated permeability manifests early in the angiogenic process and serves for endothelial cell sprouting out of the vascular bud. Administration of monoclonal antibodies engineered against immature vessels with high permeability and relaxed junctions (Corada et al. 2002; May et al. 2005) decreases tumor vascularization and decelerates tumor growth in animal models (Corada et al. 2002). Moreover, this loss of tumor vessel integrity hijacks regulated leukocyte transmigration pathways (diapedesis), increasing immune cell recruitment within the tumor and exaggerating the inflammatory responses, a hallmark of tumor microenvironment that again benefits to tumor growth, progression, and metastasis (Grivennikov et al. 2010; Vestweber 2012). Blood flow is also particularly altered in the tumor vasculature, vessel permeability being associated with an increased interstitial pressure leading to interstitial fluid accumulation in the tumor microenvironment (Azzi et al. 2013). This vascular leakage is thus a limiting factor for chemotherapeutic and other blood-delivered treatments (Jain 2005).

Endothelial Cell-Cell Junctions

The endothelial cell-cell junctions rely on transmembrane cell-cell adhesion molecules that

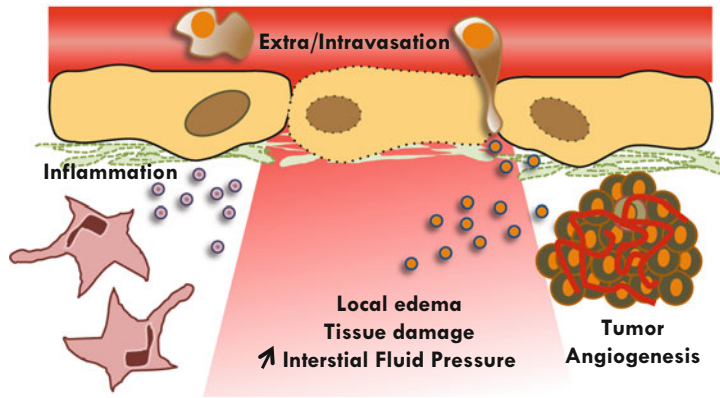

Fig. 4 Impact of vascular leakage on the tumor microenvironment. Enhanced, uncontrolled vascular permeability is a hallmark of tumor vessels. The loss of barrier integrity can affect tumor growth by encouraging: (i) metastasis and dissemination of cancer cells in and out of the blood stream (intra- and extravasation); (ii) inflammation process where macrophages and neutrophils are recruited into the perivascular bed; (iii) extravasation of plasma solutes and fluids that contribute to edema, tissue damage, and increased interstitial fluid pressure; and (iv) tumor angiogenesis

orchestrate the vascular barrier in a selective and dynamic manner. Two interrelated structures are found at the endothelial-endothelial junctions: the tight junctions and the adherens junctions (Fig. 5). Tight junctions are highly impermeable cell-cell contact structures that play a role in barriers and cell polarity. They are organized around a heterogeneous family of cell-cell adhesion molecules: occludin, claudins, and junctional adhesion molecules (JAM). Adherens junctions are believed to form more dynamic contacts than tight junctions and rely on four main cell-cell adhesion molecules, namely, VE-cadherin, PECAM, Nectin, and JAMs.

The Endothelial Tight Junctions

Occludin is a four membrane-spanning domain protein with two extracellular loops and intracellular N- and C-terminal parts (Furuse et al. 1993). The C-terminal tail connects plasma membrane proteins to the actin cytoskeleton through intracellular adaptors, such as the zona occludens family proteins (ZO). The C-terminal tail harbors multiple tyrosine, serine, and threonine phosphorylation motifs that modulated the interaction between occludin and ZO. For instance, Src-mediated tyrosine phosphorylation of occludin unleashes ZO-1 from the tight junction protein and therefore destabilizes cell-cell contact (Elias et al. 2009). Conversely, chronic shear stress promotes the recruitment of tight junction proteins to the plasma membrane and the increase of their transcription (occludin and claudin-5) (Walsh et al. 2011). The stabilization of cell-cell contacts via chronic shear stress involves the inhibition of occludin tyrosine phosphorylation, favoring in turn the interaction between occludin and ZO. This ultimately connects tight

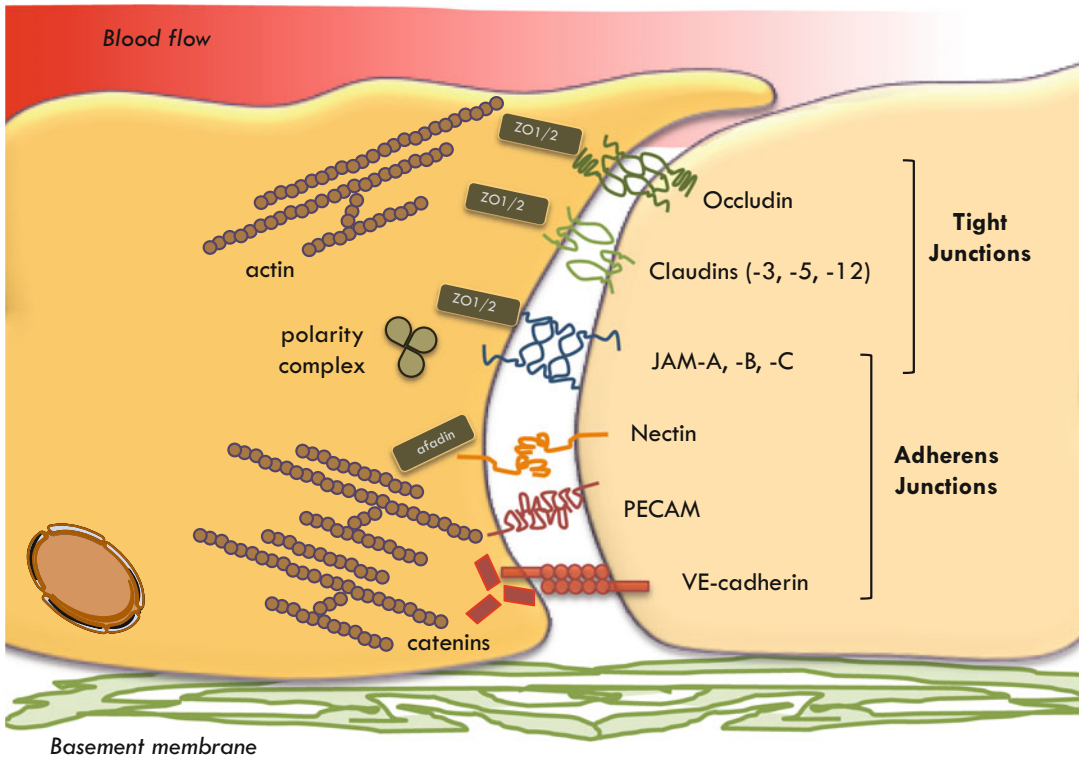

Fig. 5 Endothelial cell-cell contacts. Representation of tight and adherens junctions between two neighbor endothelial cells. Cell-cell adhesion molecules tethered adjacent cell, cell membrane, to the intracellular compartment and the actin cytoskeleton. (*JAM* junctional adhesion molecules, *ZO* zona occludens)

junction to the actin cytoskeleton and reinforces cell-cell contacts (Walsh et al. 2011). Conversely, acute shear stress triggers occludin tyrosine phosphorylation, prevents ZO recruitment, and consequently promotes endothelial permeability (Walsh et al. 2011). Moreover, the N-terminal part of occludin interacts with the E3 ubiquitin ligase Itch that can in turn drive occludin degradation upon VEGF challenge (Murakami et al. 2009).

Likewise, claudins are integral components of tight junctions (Matter and Balda 2003). They can be engaged in homophilic and heterophilic interactions with identical and different adhesion molecules, respectively. Interestingly, claudin-5 deficient mice do not show any embryonic vascular defects, at neither the morphological nor the functional level. However, such mice display higher permeability of the blood-brain barrier for small molecules, provoking to postnatal death (Nitta et al. 2003). Others claudins expressed by endothelial cells are claudins-3 and 12 (Schrader et al. 2012), but to date there are few information available on their functions. Of note, endothelial tight junction formation depends on the establishment of VE-cadherin-based adherens junctions (Taddei et al. 2008). In details, VE-cadherin mediates AKT-dependent phosphorylation of the transcriptional forkhead box factor (FoxO1), which results in the nuclear export of its phosphorylated form. When at the plasma membrane, VE-cadherin traps β-catenin away from a FoxO1/β-catenin repressor unleashed in turn the claudin-5 promoter (Taddei et al. 2008) and thereby allows expression of claudin-5. Similar mechanisms might occur at the occludin promoter (Leclair et al. 2016).

Junctional adhesion molecules (JAMs) belong to the immunoglobulin (Ig) transmembrane superfamily of cell-cell adhesion proteins expressed in epithelial and endothelial cells, as well as lymphatic cells, smooth muscle cells, and some blood cells (Bauer et al. 2014). JAM-B and JAM-C are predominantly expressed in endothelial cells, whereas JAM-A is present in endothelial and epithelial cells. In brain endothelial cells, only JAM-A and JAM-C are expressed (Aurrand-Lions et al. 2001). The intracellular domain of JAM-B and JAM-C can interact with partitioning defective protein 3 (PAR-3), which is instrumental in the establishment of endothelial cell polarity (Ebnet et al. 2003). Moreover, both JAM-B and JAM-C associate via their C-terminal part with ZO and PAR proteins through the PDZ domain (PDZ stands for post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and ZO-1). The biological functions of JAMs have been initially explored in the epithelium, where the role of JAM-A was established in the epithelial barrier function and to allow inflammatory responses (Laukoetter et al. 2007). Interestingly, JAM-C exhibits different cellular localization in micro- and macro-vascular endothelial cells. Indeed, in macrovascular cells, JAM-C constitutively accumulated at cell-cell junctions while recruited upon stimulation (such as VEGF or histamine) in microvascular endothelial cells (Orlova et al. 2006). Beside well-described JAM function in leukocyte diapedesis, the overall role of JAMs in the intact and damaged endothelium is not fully elucidated.

The Endothelial Adherens Junctions

Unlike epithelial cells where tight junctions and adherens junctions are distinctly structured along the apicobasal plan, both junctions are intertwined in endothelial cells. JAMs illustrated this interface between tight and adherens junctions (Fig. 5). In addition, weakening and strengthening of adherens junctions echo on the organization, composition, and localization of tight junctions (Gavard and Gutkind 2008).

Platelet endothelial adhesion molecule (PECAM or CD31) is a single-span transmembrane glycoprotein from the immunoglobulin superfamily, exclusively expressed in endothelial cells and blood circulating cells (monocytes, neutrophils, T and B lymphocytes). While its extracellular N-terminal part composed of six immunoglobulin domains is mainly involved in homophilic interactions (i.e., PECAM-PECAM), PECAM was reported to bind to glycosaminoglycans (GAG) from the extracellular matrix or to alternate membrane receptors such as $\alpha_V\beta_3$ integrin, CD38, and CD177 (Gandhi et al. 2008; Privratsky and Newman 2014). PECAM intracellular domain harbors several tyrosine and

serine/threonine phosphorylable sites, which can serve as docking sites for signaling molecules (Privratsky and Newman 2014). Interestingly, upon phosphorylation, PECAM recruits scaffolding molecules such as γ-catenin and thus associates with the actin cytoskeleton (Ilan et al. 2000). In this scenario, γ-catenin preferentially binds to phosphorylated PECAM in migratory cells (Ilan et al. 2000), while it associates with VE-cadherin in confluent endothelial cells and contributes to strengthen mature adherens junctions (Lampugnani et al. 1995).

PECAM operates in leukocyte transendothelial migration (diapedesis) during inflammatory processes. Unlike VE-cadherin knockout (see next section), PECAM1 gene invalidation does not impair developmental angiogenesis program (Carmeliet et al. 1999; Cao et al. 2009). Instead, the phenotype of mice depleted for PECAM1 unveils a diminution of neutrophil recruitment to inflammatory sites, a reduction of endothelial filopodia, and a lower incidence of subcutaneous tumor development (Cao et al. 2009). From a molecular standpoint, PECAM promotes heterotypic and homophilic interactions between monocytes and endothelial cells. Interestingly, the expression of PECAM (prodiapedesis) and VE-cadherin (anti-diapedesis) inversely correlates during transcellular passage of monocytes (Hashimoto et al. 2011). Indeed, in the course of monocyte transmigration, the levels of surface-exposed PECAM are increased, as opposed to VE-cadherin junctional localization (Hashimoto et al. 2011). The association of neutrophils and leukocytes to the endothelium induces Src-dependent destabilization of VE-cadherin-based cell-cell contacts and inversely correlates with PECAM bioavailability at the plasma membrane (Alcaide et al. 2012, 2008; Garnacho et al. 2008). PECAM may have broad impact on vascular barrier function, as its depletion quells neutrophil infiltration and perturbs angiogenesis (Solowiej et al. 2003). PECAM was also proposed to function as a sensor for mechanic shear stress generated by the blood flow (Tzima et al. 2005). Loss of PECAM expression in endothelial cells is associated with a defect in the activation of atherosclerosis-mediated pro-inflammatory pathways (Tzima et al. 2005; Conway and Schwartz 2012, 2013; Conway et al. 2013).

Nectins exist as four isoforms, among which Nectin-2 and Nectin-3 localized at endothelial cell junctions. Similarly to cadherins, they function as dimers that bridge neighboring cells together. Intracellularly, Nectins are bound to the afadin molecule, which associates in turn to the actin cytoskeleton and shuttles between junctions (Dejana 2004). Nectins are most likely cooperating with adherens junctions by impacting on the actin cytoskeleton organization at cell-cell junctions, but their exact contribution to vascular homeostasis and plasticity remains to be fully examined (Dejana 2004; Rehm et al. 2013).

VE-cadherin is an instrumental transmembrane adhesion molecule of the adherens junctions. This adhesion molecule actively and dynamically participates in cell-cell contact formation and remodeling and regulates the homeostasis of the endothelial barrier (Fig. 6). VE-cadherin known roles in the endothelial barrier is developed in the next section.

VE-Cadherin in the Endothelial Barrier

VE-cadherin is a type II classical cadherin, exclusively expressed in vascular and lymphatic endothelial cells. Classical cadherins are all defined by five repeated immunoglobulin-like cadherin motifs (EC) in their extracellular domain, while the presence of a large hydrophobic region in the first domain (EC1) differentiates between type I and type II cadherins (Yagi and Takeichi 2000). The intracellular domain is a highly conserved region, which bridges cadherins to the actin cytoskeleton through different catenins (α, β, γ, and p120) and other intracellular and plasma membrane components, most of them being specific of the endothelial compartment. VE-cadherin extracellular domain assembles as a hexamer from three VE-cadherins (Bibert et al. 2002; Hewat et al. 2007). This association allows the torsional flexibility of VE-cadherin bridges and enables the interaction between VE-cadherins harbored by neighboring cells (Bibert et al. 2002; Hewat et al. 2007). The transmembrane

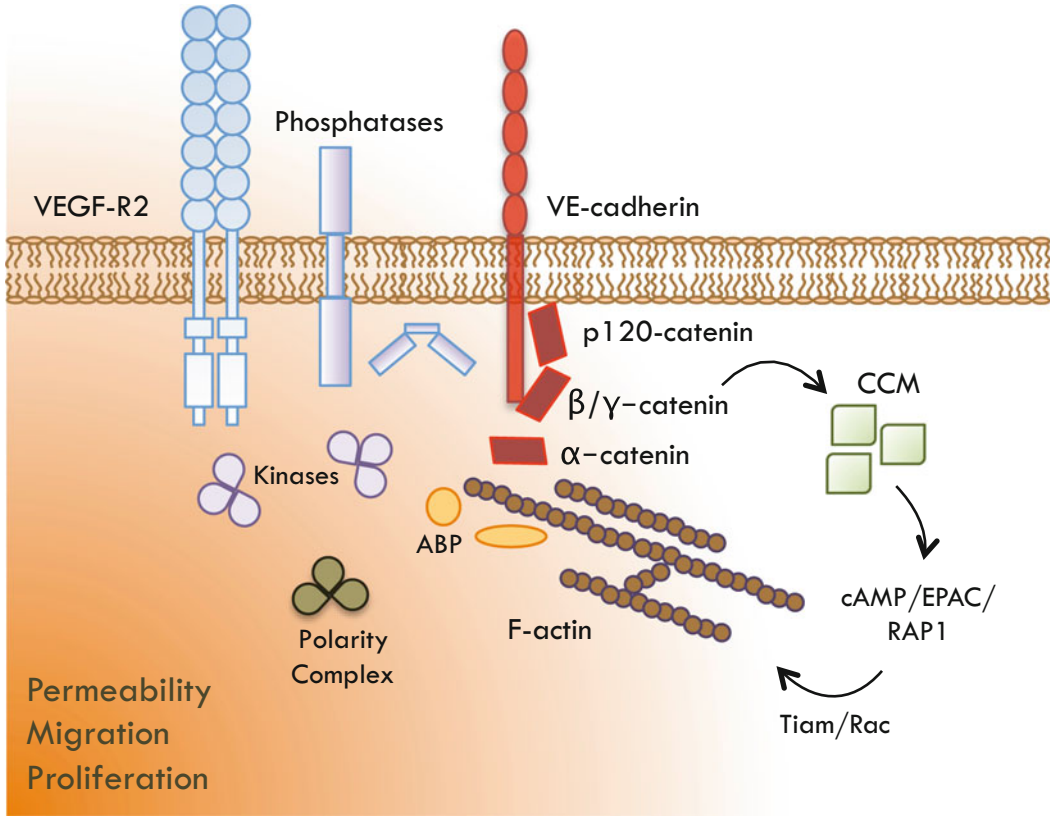

Fig. 6 VE-cadherin and partners at the endothelial junctions. Endothelial cells express the cell-cell adhesion molecule VE-cadherin. Cytosolic catenins (p120, α, β, and γ) bridge VE-cadherin to the actin cytoskeleton and actin-binding proteins (*ABP*). This adhesive complex interacts with the VEGF-R2 (vascular endothelial growth factor receptor 2, receptor and non-receptor phosphatases, cytosolic kinases, polarity complex, and CCM (cerebral cavernous malformation proteins)

domain entails VE-cadherin clustering at the plasma membrane. Catenins, namely, p120, α, β, and γ, are cytoplasmic proteins bound to cadherins that ensure a physical connection between the plasma membrane and the actin cytoskeleton.

The core domain of β-catenin consists of 12 repeated sequences (each of 42 amino acids) called armadillo repeats (arm) and is engaged into protein-protein interaction with the negatively charged C-terminal tail of cadherins. As part of the Wnt signaling pathway, β-catenin can shuttle to the nucleus and interacts with DNA binding sites, resulting in the activation of genes instrumental in embryogenesis and carcinogenesis (Clevers 2006). To reconcile its dual involvement in cell-cell adhesion and Wnt signaling, β-catenin coexists under different conformations, which most likely confers its distinct functions. Indeed, a β-catenin/α-catenin heterodimer is implicated in cadherin-dependent adhesive function, whereas β-catenin transcriptional activity resides exclusively in its monomeric closed conformation form. Additionally, the monomeric open conformation bears both adhesive and transcriptional functions (Gottardi and Gumbiner 2004). Interestingly, mice null for β-catenin were engineered by site-specific recombinase technology (CRE) under the control of the endothelial cell-specific promoter Tie2 (the tyrosine kinase receptor for Angiopoietin-1). These embryos exhibit severe perturbations in the vascular patterning of the head, vitelline membrane, umbilical cord, and placenta (Cattelino et al. 2003). Moreover,

β-catenin depletion significantly weakens endothelial cell integrity, hinders α-catenin expression, and promotes the accumulation of plakoglobin and desmoplakin at cell-cell contacts (Cattelino et al. 2003). Conversely, exon3 deletion mutant of β-catenin behaves as a gain of function (GOF) mutant, whose expression can be forced in the endothelial compartment of transgenic mice (Corada et al. 2010). This mutant cannot be phosphorylated and further degraded and therefore remains constantly active. Interestingly, this augments Wnt/β-catenin and Notch signaling activation in endothelial cells. Alteration of vascular remodeling and endothelial differentiation manifested by vessel branching defects and lack of arteriovenous specification further characterized the phenotype of mutant mice (Corada et al. 2010).

The p120-catenin belongs as well to the armadillo family and binds to the juxtamembrane domain of cadherins (Kowalczyk and Reynolds 2004). Mice in which the p120-catenin gene was silenced in the endothelial lineage die early in utero because of embryonic and extraembryonic vascular defects (Oas et al. 2010). Molecularly, both N-cadherin and VE-cadherin expression are dramatically reduced in vessels, explaining the phenotype of reduced microvessel density, low pericyte recruitment, and hemorrhages in knockout embryos (Oas et al. 2010). A highly conserved sequence of 10 amino acid (644–654 on the human sequence) bears the binding region to classical cadherins. In particular, mutation within the DEE motif results in VE-cadherin/p120-catenin uncoupling (Nanes et al. 2012). p120 is thought to mask putative internalization motifs on VE-cadherin and therefore contributes to maintain VE-cadherin at the plasma membrane (Nanes et al. 2012; Kowalczyk and Reynolds 2004). Additionally, p120-catenin has been described to orchestrate cell morphology, motility, and adhesion by modulating the activity of Rho, Rac, and Cdc42 small GTPases (Anastasiadis 2007). Indeed, p120-catenin can directly interact with Rho GTPase-activating protein (p190RhoGAP) via its C-terminal tail and subsequently tunes the Rho/Rac balance involved in cell-cell contact formation (Zebda et al. 2013). Furthermore, p120 binding to VE-cadherin modulates in a Rac-dependent manner the ability of endothelial cells to spread and thereby controls the adhesive contact area, while the adhesive strength rather relies on β-catenin association (Oas et al. 2013, 2010).

γ-catenin (or plakoglobin) can be recruited to VE-cadherin in place of β-catenin. In fact, VE-cadherin/plakoglobin association exists in mature confluent cells, while β-catenin preferentially interacts with VE-cadherin in nascent contacts (Lampugnani et al. 1995). Thus, VE-cadherin/β-catenin and VE-cadherin/plakoglobin interactions are inversely correlated. Different phases of endothelial cell-cell contact formation can be discriminated. The "initiation stage" corresponds to nascent junctions that are organized around VE-cadherin, β-catenin, and α-catenin. Next, the "extension stage" involves the reinforcement of cell-cell contact through plakoglobin recruitment. Finally, upon maturation, VE-cadherin is connected to the actin cytoskeleton through plakoglobin, and this complex can be biochemically isolated in triton-insoluble fractions (Lampugnani et al. 1995).

α-catenin actively participates in cadherin-mediated cell-cell contacts and connects the VE-cadherin/catenin complex to the actin cytoskeleton. Unlike other catenins, it is not an armadillo protein and operates as an actin-binding protein. Interestingly, α-catenin cannot simultaneously link actin and be part of the cadherin/β-catenin complex (Yamada et al. 2005). In fact, α-catenin coexists as a monomer or a dimer, while its conformation balances its binding affinity for the cadherin/β-catenin complex and the actin filaments. The monomeric α-catenin preferentially binds to cadherin/β-catenin, whereas the α-catenin dimer can only associate with actin filaments (Drees et al. 2005). This VE-cadherin/catenin complex connects cell-cell adhesion molecules to the actin cytoskeleton. In line with this, actomyosin contractility is enhanced upon junctional remodeling, where cytoskeletal pulling forces rely on the direct interaction of α-catenin with the actin-binding protein, vinculin. Here, vinculin prevents from the dismantlement of adherens junctions upon thrombin

challenge (Huveneers et al. 2012). Moreover, endothelial cell-cell contacts can be strengthened upon high intracellular concentration of cyclic (−3–5)monophosphate (cAMP) (Sakurai et al. 2006). Increase in cAMP concentration elicits a signaling pathway involving Epac (exchange protein activated by cAMP) and the small GTPase Rap1, to form circumferential actin bundles. α- and β-catenins from the VE-cadherin adhesion complex seem to play essential roles in the accumulation of VE-cadherin on the actin bundles (Noda et al. 2010). Thus, the cAMP/Epac/Rap1 signaling pathway regulates actin dynamics and reinforces cell-cell contact in endothelial cells. Besides, VE-cadherin plays a pivotal role in the regulation of small GTPases Rho and Rac activity (Lampugnani et al. 2002). Interestingly, the expression of VE-cadherin causes the reorganization of actin stress fibers through Rac activation and Rho inhibition. Furthermore, VE-cadherin requires the Rac1-specific guanine nucleotide exchange factor (GEF) Tiam to associate with the cytoskeleton (Lampugnani et al. 2002).

VE-cadherin can associate directly and indirectly with other molecules through its intracellular or extracellular domains to control endothelial cell proliferation, survival, polarity, migration, and the overall barrier function. In quiescent confluent endothelial cells, the expression of VE-cadherin adhesive complex at the cell surface allows VE-cadherin/VEGF-R2 association and thereby regulates VEGF-induced proliferation (Grazia Lampugnani et al. 2003). Indeed, this interaction promotes the activation of the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)/AKT survival pathway. In genetically modified VE-cadherin null endothelial cells or when VE-cadherin is excluded from junctions, VEGF-R2 phosphorylation and VEGF-R2-mediated proliferation are enhanced. Indeed, VEGF stimulation in VE-cadherin-depleted cells leads to rapid clathrin-dependent internalization of VEGF-R2 (Lampugnani et al. 2006). However, its endocytosis does not abort its signaling but rather elicits MAPK activation and enhances cell growth (Lampugnani et al. 2006). Thus, VE-cadherin junctional distribution hinders VEGF-R2 internalization and MAPK pathway activation. Early studies showed that β-catenin is required for VEGF-R2 and VE-cadherin functional interaction (Carmeliet et al. 1999). Likewise, intravenous delivery of VEGF in mice transiently disrupts the VE-cadherin/VEGF-R2 complex in heart endothelial cells (Weis et al. 2004b). Importantly, the inhibition of the proto-oncogene tyrosine-protein kinase (Src) can prevent VEGF-R2/VE-cadherin dissociation caused by VEGF. Next, the membrane-associated phosphatase (DEP-1) plays an important role in VEGF-mediated cell proliferation. Indeed, DEP-1 is localized at the endothelial cell-cell junctions in the proximity of VE-cadherin and VEGF-R2. Moreover, it has been reported that DEP-1 specifically interacts with VE-cadherin through β, γ, or p120-catenins (Holsinger et al. 2002). In DEP-1 knocked-down endothelial cells, VEGF-R2 phosphorylation and cell proliferation are boosted (Grazia Lampugnani et al. 2003). Interestingly, DEP-1 can also dephosphorylate the Src kinase; for example, DEP-1-dependent Y418 Src dephosphorylation limits its downstream signaling and participates in the inhibition of endothelial proliferation (Spring et al. 2012). Altogether, β-catenin- and DEP-1-dependent VEGF-R2/VE-cadherin coupling has a substantial impact on cell-cell contact growth inhibition in endothelial cells (Grazia Lampugnani et al. 2003; Lampugnani et al. 2006). In keeping with this idea, VE-cadherin can interact with cytosolic C-terminal Src kinase (Csk) and reduce Src kinase activity. In growing endothelial cells, VE-cadherin is strongly phosphorylated at tyrosine residues and Csk exclusively binds to the phosphorylated form of VE-cadherin on Y685 (Baumeister et al. 2005). The Csk/pY685-VE-cadherin interaction requires the Csk SH2 domain, while Csk co-immunoprecipitates with wild-type VE-cadherin, but not with a non-phosphorylable (Y685F) VE-cadherin mutant, even upon Src activation. Consequently, the association of Csk with pY685-VE-cadherin increases with cell density and modulates endothelial cell proliferation and permeability.

VE-cadherin has been described to associate with different phosphatases that stabilize endothelial cell-cell contacts. One of the most well-

documented interactions between VE-cadherin and phosphatases is illustrated by vascular endothelial protein tyrosine phosphatase (VE-PTP). VE-PTP is an endothelial-specific membrane protein, which impairs VE-cadherin tyrosine phosphorylation (Nawroth et al. 2002). Moreover, unlike most of the other VE-cadherin partners, VE-PTP association with VE-cadherin does involve neither its intracellular domain nor β-catenin, suggesting that this interaction is most likely mediated through VE-cadherin transmembrane and/or extracellular domains. The use of full-length and several deletion mutants of VE-PTP and VE-cadherin allows the mapping of this association and the identification of the 17th FNII repeat domain of VE-PTP and VE-cadherin fifth extracellular immunoglobulin domain (EC5). At the functional level, VE-cadherin is linked to VE-PTP in quiescent endothelial cells. However, this interaction can be abolished during lymphocyte and neutrophil infiltration and in response to angiogenic and inflammatory agents, such as VEGF or tumor necrosis factor (TNFα), respectively (Nottebaum et al. 2008). In this scenario, VE-cadherin/VE-PTP dissociation is accompanied with tyrosine phosphorylation of VE-cadherin, β-catenin, and γ-catenin (plakoglobin) resulting in the opening of VE-cadherin-based junctions. Interestingly, VE-PTP-dependent dephosphorylation of VE-cadherin orchestrates adhesion and permeability. This might rely rather on plakoglobin than β-catenin. Indeed, the absence of plakoglobin exacerbates the effects of the in vitro knockdown of VE-PTP on adhesive and barrier properties of endothelial cells. Thus, VE-cadherin/plakoglobin/VE-PTP association seems to be essential to stabilize endothelial cell-cell contacts (Nottebaum et al. 2008). Additionally, VE-PTP was indirectly implicated in VEGF-R2 dephosphorylation in the process of lumen polarization during zebra fish development (Hayashi et al. 2013). Simultaneous stimulation of endothelial cells with VEGF and Angiopoietin-1 (Ang1) leads to the accumulation of VE-PTP, Angiopoietin-1 receptor (Tie2), and VEGF-R2 at the endothelial junctions, causing further VEGF-R2 dephosphorylation. Overall, VE-PTP-dependent dephosphorylation of VEGF-R2 mediates Tie2-mediated action on endothelial cell lining and vessel maturation, while VEGF-R2 regulates VE-cadherin tyrosine phosphorylation, endothelial cell polarity, and lumen formation (Hayashi et al. 2013). The phosphatase SHP2 (Src homology two-domain-containing tyrosine phosphatase) associates with the VE-cadherin adhesive complex through β-catenin (Ukropec et al. 2000). Upon thrombin stimulation, β-catenin is phosphorylated and subsequently SHP2 dissociated from the VE-cadherin complex, leading to endothelial permeability increase (Ukropec et al. 2000). However, SHP2 can dephosphorylate β-catenin, when engaged in endothelial junctions (Timmerman et al. 2012). Conversely, β-catenin tyrosine phosphorylation levels are increased in SHP2-depleted cells. Moreover, SHP2 contributes to VE-cadherin-associated β-catenin dephosphorylation after thrombin stimulation, suggesting that SHP2 could play an important role in the recovery of disrupted endothelial adherens junctions. Protein phosphatase 2A (PP2A) was also found coupled to VE-cadherin in brain endothelial cells, while its activity governs barrier integrity. PP2A is a serine/threonine phosphatase, which is potentially able to dephosphorylate VE-cadherin at S665, and thereby contributes to maintain low brain endothelial cell permeability (Le Guelte et al. 2012; Gavard and Gutkind 2006). For instance, tumor-derived factors elicit a signaling pathway leading to the dissociation of the VE-cadherin/PP2A complex in endothelial cells. This causes VE-cadherin S665 phosphorylation and its further internalization (Le Guelte et al. 2012). Again, vascular permeability is augmented.

VE-cadherin orchestrates and maintains the endothelial barrier integrity and homeostasis. Of note, most of the tumor-derived factors released in the tumor microenvironment converge on modulating the VE-cadherin biology. Recovering VE-cadherin physiological behavior could emerge as a promising strategy to promote vascular normalization in anticancer therapies.

Endothelial Junctions in Tumor-Induced Angiogenesis

Dynamics of Endothelial Junctions in Migration and Sprouting

As mentioned in the previous section, the vascular sprout is a dynamic structure where endothelial cells compete for the tip position (Jakobsson et al. 2009, 2010). During elongation and migration of the growing vessel, endothelial cells exhibit a mixed pattern of tip and stalk phenotypes; a phenomenon coined as "salt-and-pepper" distribution. This lively process depends on individual cell behavior, with the coexistence of opposing molecular signaling pathways: in one hand the activation of the VEGF pathway and, on the other hand, the lateral inhibition through the Notch pathway under the control of DLL4 (Blanco and Gerhardt 2013) (please see above section).

VEGF signaling is tightly linked to VE-cadherin dynamics that orchestrate endothelial cell adhesion, behavior, and endothelium properties (Gavard 2009, 2013). Interestingly, there is substantial evidence that proliferative and migratory traits of endothelial cells rely on VE-cadherin (Grazia Lampugnani et al. 2003). The importance of VE-cadherin during development has been formally demonstrated with mice missing the VE-cadherin intracellular domain, while the early embryonic lethality of these embryos (E9.5) is associated with hemorrhagic vessels (Carmeliet et al. 1999). VEGF-R2 activation induced endothelial permeability by a mechanism involving VE-cadherin phosphorylation and subsequent endocytosis (Gavard and Gutkind 2006). In keeping with this idea, the differential VE-cadherin-dependent adhesion between endothelial cells in response to VEGF stimulation was recently shown to mechanically orchestrate tip and stalk cell rearrangement within the sprout (Bentley et al. 2014). VE-cadherin, actin rearrangement, and VEGF-R2/Notch balance are indeed involved. High VEGF-R2 activity in tip cells leads to VE-cadherin phosphorylation and its further internalization (Bentley et al. 2014). VE-cadherin internalization is known to elicit cell-cell junction weakening and transient elevation of endothelial permeability (Gavard and Gutkind 2006). This might ultimately governs the migratory phenotype of tip cells, especially protrusion formation. In this scenario, endothelial cells with weaker adhesion potential are oriented toward a tip cell phenotype and progress to the tip of the sprout (Bentley et al. 2014). In contrast, Notch induces downregulation of VEGF-R2 in stalk cells, low VE-cadherin phosphorylation, and Rac/Tiam activation. This collectively results in the inhibition of filopodial protrusions and favors their cell-cell adhesive phenotype. Likewise, low level of VEGF-R2 phosphorylation in stalk cells can be attributed to VE-PTP activity and depends on the presence of the Angiopoietin-1 receptor Tie2 at the endothelial cell-cell junctions (Hayashi et al. 2013). In this context, VE-PTP limits both VEGF-R2 signaling and VE-cadherin-mediated vascular permeability and thereby promotes neo-vessel stabilization.

Endothelial-to-mesenchymal transition (EndMT) is characterized by a series of morphological alteration including disruption of intercellular junctions, loss of cell polarity, accompanied with enhanced both proliferation and migration of endothelial cells that escape from the vasculature (Zeisberg et al. 2007b; Dejana et al. 2017). In pathological conditions, the acquisition of mesenchymal and stem-cell-like traits by endothelial cells contributes to fibrosis and accumulation of ectopic stromal cells and myofibroblasts (Zeisberg et al. 2007b; Dejana et al. 2017). Factors, including inflammation, shear stress, and TGF-β signaling (transforming growth factor), promote endothelial-to-mesenchymal transition and participate in tumor microenvironment heterogeneity in cancers (Zeisberg et al. 2007a; Xiao et al. 2015). This also suggests that anti-angiogenic treatments may also impact on the stromal cellular composition. Molecularly, the endothelial-specific deletion of cerebral cavernous malformation protein CCM1 leads to endothelial-to-mesenchymal transition and provokes vascular abnormalities in mice (Maddaluno et al. 2013). Interestingly, the role of CCM1 in

VE-cadherin-based adherens junction formation has been documented. CCM1 mutations were originally identified in patients affected by cerebral cavernous malformations (CCM), a disease characterized by cerebral blood leakage and abnormal vessel structure (Dejana et al. 2009). VE-cadherin indeed indirectly connects to CCM1 thanks to β-catenin and recruits Rap1, a GTPase that stabilizes endothelial adherens junction (Lampugnani et al. 2010). It has been further suggested that CCM1 via its direct interaction with the VE-cadherin/catenins complex triggers Rap1 and Tiam-dependent Rac activation, whereas CCM2 bound to the VE-cadherin adhesion complex through CCM1 inhibits RhoA (Lampugnani et al. 2010).

In parallel to the VEGF/VE-cadherin axis dominating endothelial junction remodeling and dynamics, tumor-secreted basic fibroblast growth factor (bFGF) is another known inducer of endothelial cell migration whose signaling have been associated to JAM-A. In resting endothelial cells, JAM-A is present at endothelial cell-cell contacts where it can complex with $\alpha_V\beta_3$ integrin, a mediator of endothelial cell adhesion on vitronectin (Naik and Naik 2006). Upon bFGF stimulation, JAM-A is partially delocalized from endothelial junctions and dissociates from $\alpha_V\beta_3$. In turn, this provokes the activation of the mitogen-activated protein kinase (MAPK) signaling and modulates endothelial cell adhesion, spreading, and migration (Naik et al. 2003). Accordingly, the stimulation of endothelial cells with bFGF fails to induce angiogenesis in JAM-A deficient mice (Cooke et al. 2006). Interestingly, bFGF opposes to endothelial permeability and VE-cadherin internalization and instead stabilizes adherens junction (Murakami et al. 2008).

JAMs may exert multiple functions in the course of tumor progression. For example, JAM-C inhibition using blocking antibodies was shown to abolish ex vivo angiogenesis in the aortic ring model of endothelial sprouting, while this treatment impairs tumor growth in vivo in a syngenic mouse model of lung carcinoma (Lamagna et al. 2005a, b). More recently, endothelial-specific JAM-C gene deletion has been associated with a reduced tumor growth in a mouse model of ovarian cancer, as compared to wild-type animals (Leinster et al. 2013). In this context, the overall tumor vessel density is not affected, but rather tumor vessel permeability is increased together with a reduction in pericyte coverage, suggesting that JAM-C function in tumor-induced angiogenesis is more likely related to vessel functionality through mediating cell-cell junction stability and/or pericyte anchorage.

JAM-B, also known as vascular endothelial, VE-JAM was recently reported to be a negative regulator of pro-angiogenic pathways interfering with VEGF/VEGF-R2 signaling (Meguenani et al. 2015). Interfering with JAM-B at endothelial junctions using a blocking antibody inhibited endothelial tube formation in vitro and reduced VEGF-induced aortic ring vessel outgrowth without altering pericyte coverage ex vivo. Moreover, blocking JAM-B in JAM-B-expressing murine endothelial cells and JAM-B-transfected HUVECs reduced ERK1/2 (extracellular regulated kinase) phosphorylation upon VEGF stimulation, further indicating that JAM-B-based adhesion in resting endothelial cells interferes with VEGF/ERK pathway activation, favoring in turn endothelial junction stability. Mice knockout for JAM-B are viable and do not display vascular abnormalities, while aortic rings from those mice stimulated with VEGF show an increased vessel branching, thus suggesting the existence of compensatory mechanisms regarding JAM-B functions in angiogenesis. Additionally, JAM-B anti-angiogenic function does not extend in vivo in inhibition of tumor vascularization. Indeed, treatment of mice with a JAM-B blocking antibody did not affected endothelium-derived tumor growth (hemangioma), and no effects on tumor vasculature and progression were observed in a model of pancreatic tumor. Similarly, in an ectopic model of Lewis lung carcinoma, tumor development was similar between wild-type animals and JAM-B deficient mice (Meguenani et al. 2015).

Endothelial Cell-Cell Junctions in Polarity

The overall organization of the vascular tubes with luminal and basolateral sides implies

polarity. The description of the endothelial polarity suffers from the comparison to the morphological and functional detailed knowledge of epithelial cell polarity. The molecular mechanisms governing the establishment of polarity involve cell-cell adherens and tight junctions in epithelial cells and are thus thought to be transposable to endothelial cells. However, the flattened endothelial morphology in blood vessels and the fact that tight and adherens junctions are intermingled might explain disparities between the two systems. In epithelial cells, cell polarity relies on JAM-A recruitment and stabilization at early cell-cell contacts through its association with Afadin and ZO-1 (Ebnet et al. 2000). The cell polarity complex composed of PAR-3, PAR-6, and aPKC is then recruited by the binding of PAR-3 to JAM-A intracellular PDZ binding domain. In an ectopic expression model, JAM-A expression leads to the recruitment of PAR-3 at cell-cell contacts (Suzuki and Ohno 2006; Ebnet et al. 2001). aPKC is activated by small Rho GTPase family members, Rac1 and Ccd42, and phosphorylates JAM-A on Ser285 to promote maturation of cell-cell junctions. Moreover, it was recently demonstrated that transient activation of Cdc42 by JAM-A during mitosis regulates cortical dynein localization to control planar spindle orientation (Iden et al. 2012; Tuncay et al. 2015; Ebnet 2013). These molecular mechanisms involving JAM-A polarity regulation are thought to be transposable to endothelial cells, as endothelial cells deficient for JAM-A exhibit spontaneous and random motility (Bazzoni et al. 2005). However, JAM-A deficient mice have a normal vascular system development (Bazzoni et al. 2005). In the context of endothelial polarity, JAM-C and JAM-B are also expressed at endothelial junctions and are able to recruit PAR-3 via their PDZ binding domain (Ebnet et al. 2003). Interestingly, JAM-C-dependent assembly of the cell polarity complex was shown to be critically required for round spermatid polarization and subsequent differentiation, further suggesting that endothelial JAMs might cooperate in endothelial polarity establishment and maintenance (Gliki et al. 2004).

It has been reported that VE-cadherin associates as well as with polarity proteins Par3 and Par6 that further participate in the formation of the apicobasal polarity complex in endothelial cells. However, unlike epithelial cells, this occurs independently of aPKC (Ebnet et al. 2003; Iden et al. 2006). VE-cadherin silencing causes irregular expression of apical and basal markers in endothelial cells (Lampugnani et al. 2010). Reminiscent phenotype is observed upon endothelial depletion of β1 integrin and can be partially rescued by Par3 (Zovein et al. 2010). In keeping with this idea, β1 integrin mediates endothelial sprouting while governing VE-cadherin localization and vessel maturation (Yamamoto et al. 2015). Additionally, VE-cadherin and cell-cell junctions were recently implicated in the control of lumen formation during embryonic development. The formation of the VE-cadherin/CCM complex allows lumen polarization, while the absence of any of these proteins causes severe alterations of the lumen (Lampugnani et al. 2010). Interestingly, mice bearing in the endothelial compartment a loss-of-function mutation in the CCM2 gene present aberrant vascular lumens (Whitehead et al. 2009). Moreover, CCM2 might regulate RhoA activity and vascular permeability (Whitehead et al. 2009). Similarly, it has been established in VE-cadherin null or partially depleted zebra fish embryos that VE-cadherin is crucial to vascular lumen formation (Montero-Balaguer et al. 2009). Indeed, vessel fusion is dramatically altered because of the weakening of cell-cell contacts between two newly formed vessels (Montero-Balaguer et al. 2009). Indeed, VE-cadherin plays an important role in vascular connection and lumen formation and stability, in association with membrane dynamics and actin cytoskeleton-based forces (Phng et al. 2015; Gebala et al. 2016). How and whether these pathways are pirated in tumor-induced angiogenesis will require in-depth investigation.

Remodeling of Endothelial Junctions in Tumor Vascular Permeability

The action of tumor cells and their secreted factors with the endothelium has also been extensively demonstrated to promote the loss of barrier

integrity, in many solid cancers (Le Guelte et al. 2011). For example, upon interaction between breast cancer cells and endothelial cells, VE-cadherin is phosphorylated on tyrosine residues and re-localized away from cell-cell contacts, contributing to increased permeability (Cai et al. 1999). VE-cadherin can also modulate endothelial permeability by recruiting the scaffolding molecule β-arrestin (Gavard and Gutkind 2006). Indeed, the phosphorylation of VE-cadherin upon VEGF stimulation can serve as a docking site for β-arrestins (Gavard and Gutkind 2006; Hebda et al. 2013). This connection causes VE-cadherin endocytosis and the elevation of endothelial permeability and can be turned down by anti-permeability agents, such as the maturation angiogenic factor Angiopoietin-1 (Gavard and Gutkind 2006; Gavard et al. 2008; Saharinen et al. 2008). Likewise, co-culture of ovarian cancer cells with endothelial cells demonstrated that tumor cell-secreted VEGF increases endothelial permeability in association with decreased VE-cadherin localization at the plasma membrane (Hu et al. 2006). Indeed, VEGF expression is broadly augmented in cancer cells, mainly upon hypoxia stress, although this heightened production is maintained upon ex vivo normoxic culture. Tumor-derived VEGF has been shown to be delivered in the milieu through extracellular vesicles and functionally to contribute to endothelial cell-cell destabilization, permeability, and angiogenesis (Skog et al. 2008; Treps et al. 2016; Feng et al. 2017). Thus, these data provide evidence for a critical role for VE-cadherin in cancer-associated vascular permeability.

Importantly, the non-receptor proto-oncogene kinase Src is instrumental in vascular leakage, upon challenge of VEGF and other tumor-emanating permeability mediators. Indeed, the integrity of both skin macrovascular and cerebral microvascular endothelial barriers was maintained in Src knockout animals upon acute VEGF challenge (Eliceiri et al. 1999; Paul et al. 2001). Src was indeed shown to take part in vascular permeability in tumors and influence tumor cell extravasation and metastasis (Weis et al. 2004a). Mechanistically, Src has been well established to modify adherens junction either directly via phosphorylation of VE-cadherin and its associated catenins (Lambeng et al. 2005; Weis et al. 2004a, b), or indirectly via the activation of intracellular pathways leading to VE-cadherin phosphorylation (Gavard and Gutkind 2006). Alternatively, Src kinase tunes the activity of the focal adhesion kinase (FAK), which is in turn recruited at cell-cell junctions, and thereby operates on vascular permeability and tumor dissemination (Chen et al. 2012; Jean et al. 2014). Some studies also document the impact of Src on tight junction stability at the plasma membrane via occludin phosphorylation (Elias et al. 2009; Takenaga et al. 2009).

The Wnt pathway, which is frequently aberrantly activated in cancers, is also a key regulator of the endothelial cell-cell junctions (Gavard and Mege 2005). β-catenin is the essential downstream effector of the canonical Wnt pathway and operates at the cell-cell junctions where it connects cadherins to the actin cytoskeleton and serves as an intermediate between cadherins and other intracellular signaling pathways (Carmeliet et al. 1999; Cattelino et al. 2003; Gavard and Mege 2005). Additionally, Fzd7, one of seven transmembrane domain receptor of the Wnt family, associates with VE-cadherin complex through its cysteine-rich extracellular domain (Ferreira Tojais et al. 2014). In vivo experiments show that the depletion of Fzd7 in endothelial cells promotes vascular leakage, as measured by Evans blue dye extravasation upon VEGF stimulation. Likewise, the silencing of Fzd7 gene in vitro causes the disorganization of tight junctions and the dissociation of the VE-cadherin/β-catenin complex. Fzd7 can further regulate the expression of VE-cadherin and β-catenin both in vitro and in vivo. In this scenario, the activation of the canonical Wnt/β-catenin axis rescues Fzd7 deficiency in terms of vascular permeability and VE-cadherin/β-catenin disruption (Ferreira Tojais et al. 2014). An additional interplay between VE-cadherin expression and tight junction organization resides in the β-catenin-dependent modulation of the FoxO pathway that negatively controls the transcription of occludin and claudin-5 (Taddei et al. 2008; Leclair et al. 2016).

Endothelial JAMs are important modulators of barrier integrity that are differentially expressed

throughout the vasculature, JAM-A being highly expressed in the blood-brain barrier where vessel permeability is reduced at the minimal (Aurrand-Lions et al. 2001). JAM-A and JAM-C were reported to maintain barrier integrity in several models. Indeed, impairing with JAM-A adhesive function with blocking antibodies results in corneal swelling due to impaired barrier function in rabbits (Mandell et al. 2006). Likewise, homozygous JAM-C mutation in humans was associated with the development of brain hemorrhages, suggesting an important role for JAM-C in the maintenance of the blood-brain barrier (Mochida et al. 2010). Contrarily to macrovascular endothelial cells where JAM-C is predominantly present at cell-cell contacts, its expression in quiescent microvascular cells is mostly cytoplasmic and is redirected to the junctions only upon challenge. In this context, JAM-C was shown to increase endothelial permeability through the modulation of VE-cadherin cell-cell contacts in a mechanism dependent of the small GTPase Rap1 (Orlova et al. 2006). Moreover, JAM-C overexpression in endothelial cells was reported to increase vascular permeability in thrombin-stimulated endothelial cells (Li et al. 2009).

While VEGF that was first identified as the vascular permeability factor (VPF) is the most studied factor, other cytokines found to be released in the tumor microenvironment can favor vascular permeability in a synergic manner, including the CXCL8 (IL-8) chemokine, TNFα, and more (Le Guelte et al. 2011). They frequently converge on the modulation of endothelial cell-cell junctions, notably regulating VE-cadherin-based adhesion.

Involvement of Endothelial Junctions in Tumor Vascular Aberrations

Inflammation is a hallmark of the tumor microenvironment playing critical roles in tumor initiation, progression, metastasis, and responses to therapy. While in developing tumors anti-tumorigenic and pro-tumorigenic inflammatory mechanisms coexist, inflammation and immune cells appear to be ultimately beneficial for tumors, notably by supplying chemokines and cytokines favoring cancer cell survival and proliferation, as well as angiogenesis (Grivennikov et al. 2010).

Immune cell recruitment to the site of inflammation is tightly regulated by endothelial junctional molecules, such as PECAM-1, MIC2 (also known as CD99, the Ewing's sarcoma marker), ICAM-2 (intercellular adhesion molecule 1), ESAM (endothelial cell adhesion molecule), and members of the JAM family that are partially re-localized in the activated endothelium to mediate leukocytes rolling, adhesion, as well as paracellular and transcellular transendothelial migration (Muller 2011). Endothelial JAM-A, JAM-B, and JAM-C are known regulators of this process (Arcangeli et al. 2013). JAM-A and JAM-C can notably be detoured from endothelial lateral borders under inflammatory stimulation with TNF-α, IFN-γ (Interferon) and oxidized LDL (low density lipoprotein) (Ozaki et al. 1999; Keiper et al. 2005). Briefly, partial JAM-A apical localization contributes to leukocyte adhesion on the endothelium through heterotypic interaction between JAM-A and lymphocyte function-associated antigen 1 (LFA-1) leukocyte integrin (Ostermann et al. 2002). Interactions between JAM-A and LFA-1 have been shown to destabilize endothelial JAM-A homodimers that therefore favor leukocyte transmigration (Wojcikiewicz et al. 2009). Impairing JAM-A function with a blocking antibody in vivo decreased inflammation and transendothelial migration (Woodfin et al. 2009; Martin-Padura et al. 1998), while the diapedesis of polymorphonuclear leukocytes is significantly reduced in JAM-A knockout mice (Cera et al. 2004). Endothelial JAM-B and JAM-C that preferentially form heterotypic JAM-B/JAM-C interactions at endothelial contacts are also actively involved in transendothelial migration, where JAM-B and JAM-C engage in both homophilic interaction with JAM-C found at the leukocyte membrane and heterophilic interactions with α4β1 and αMβ2 (Mac1) leukocyte integrins, respectively (Johnson-Leger et al. 2002; Cunningham et al. 2002; Lamagna et al. 2005b; Ludwig et al. 2009). JAM-C is essential to ensure unidirectional leukocyte transmigration from the blood to inflammatory sites. Indeed, JAM-C

pharmacological inhibition using blocking antibodies impairs leukocyte recruitment in several models of inflammation through an increased reverse transendothelial migration, rather than an inhibition of leukocyte transmigration, this process being recently described in vivo using real-time beam laser confocal microscopy and three-dimensional imaging in real time (Vonlaufen et al. 2006; Bradfield et al. 2007; Scheiermann et al. 2009; Woodfin et al. 2011). Importantly, in the inflammatory endothelium, endothelial cells express disintegrin metalloproteinase ADAM10 and ADAM17 that were shown to cleave JAM-A and JAM-C but also VE-cadherin, increasing endothelial permeability and facilitating leukocytes diapedesis (Schulz et al. 2008; Koenen et al. 2009; Rabquer et al. 2010). Additionally, processed soluble JAM-C mediates human microvascular endothelial cell migration and induces tube formation in vitro and angiogenesis in vivo in the tridimensional matrix-based plug and sponge granuloma models, this mechanism being potentially involved in tumor growth inhibition reported with JAM-C blocking antibodies and JAM-C endothelial depletion (Rabquer et al. 2010; Lamagna et al. 2005a, b; Leinster et al. 2013).

The elevated permeability found in tumor blood vessels interferes with the endothelial regulation of the inflammatory responses, further promoting endothelial instability and angiogenesis but also cancer cell metastasis. This latter dynamic process actively relies on JAM-mediated interactions between cancer cells and endothelial cells. First, JAM-C expressed on lung carcinoma cell lines mediates in vitro cancer cell adhesion to endothelial cells through JAM-C/JAM-C interaction and may thereby be involved in tumor metastatic processes (Santoso et al. 2005). Moreover, it was demonstrated that B16 melanoma cells metastasis to the lung was significantly decreased in JAM-C-deficient mice, as well as in mice with an endothelial-specific JAM-C depletion. Corroborating this, treatment of mice with soluble JAM-C prevented melanoma lung metastasis (Langer et al. 2011). Thus, JAM-C homophilic interaction contributes to melanoma cell transendothelial migration and lung metastasis. This involves endothelial JAM-B in a JAM-C/JAM-B heterophilic mode, as B16 melanoma cell metastasis was significantly reduced in JAM-B knockout mice (Arcangeli et al. 2012).

Several studies have unveiled the involvement of eNOS (endothelial nitric oxide synthase) in endothelial proliferation, in the context of inflammatory pathologies and central nervous system neoplasms (Argaw et al. 2012; Bulnes et al. 2010). Corroborating this, VEGF can no longer enhance vascular permeability in response to VEGF challenge in eNOS-deficient animals (Fukumura et al. 2001). Conversely, eNOS is over-expressed in vessels from brain tumors and might contribute to tumor edema (Bulnes et al. 2010). The loss of barrier integrity exacerbates this phenotype in a positive feedback loop, as the vascular leakage provokes erythrocyte invasion in the tissue and hemoglobin secretion. Hemoglobin drives in turn high eNOS expression together with reduced claudin-5, ZO-1, and JAM-A expression (Yang et al. 2013).

Several cancers, such as highly aggressive brain tumors (glioblastoma), hepatocarcinoma, and melanoma, share the ability to form de novo vascular networks composed of non-endothelial vascular channels that take part to blood perfusion of the tumor mass and facilitate the expansion of tumor cells (Kirschmann et al. 2012; Seftor et al. 2012). This event, also known as vascular/vasculogenic mimicry, is unique to tumors with a highly angiogenic, aggressive, and plastic phenotype. There is notably a strong correlation between VE-cadherin expression and aggressiveness of melanoma cells (Seftor et al. 2012). Cancer-derived endothelial-like cells express VE-cadherin and produce metalloproteases (MMP) that help vascular remodeling and promote vascular mimicry. In hepatocellular carcinoma cells, the overexpression of the transcription factor Twist1 is linked to vascular mimicry (Sun et al. 2010). Conversely, knockdown of Twist1 inversely correlated with invasiveness, migration, and vascular mimicry. Molecularly, high level of Twist1 in the nucleus coincides with the upregulation of VE-cadherin and MMP and the downregulation of E-cadherin in cells isolated from patients with hepatocellular carcinoma and found histologically positive for

vascular mimicry. To conclude, high level of Twist1 activity governs the plasticity of hepatocellular carcinoma cells. Vascular mimicry and epithelial-mesenchymal transition (EMT) operate using similar mechanisms and signaling pathways, but it still remains unclear whether the epithelial-mesenchymal transition is upstream vascular mimicry and how they are orchestrated. In addition to this vascular mimicry that concern a population of aggressive tumor cells, tumor stem-like cells that are mostly quiescent can also gain endothelial-like properties. These fake endothelial cells have been identified in aggressive brain tumors, where a fraction of cancer stem-like cells expresses VE-cadherin, and, together with normal endothelial cells, contribute to the tumor vasculature (Wang et al. 2010). Anywhere from 20 to 90% of tumor-associated endothelial cells have been scored to be of tumor origin (Ricci-Vitiani et al. 2010). They could also incorporate the tumor vasculature by mimicking pericyte, in a transdifferentiation process (Cheng et al. 2013).

Future Directions

In the course of tumor progression, new vessels are produced to sustain tumor cell growth. The tumor vasculature perverts the rules of harmonized, developmental program and displays a network of tortuous, leaky, and misconnected vessels. The increased vascular permeability was observed early in medicine, and considerable progresses have been achieved to delineate the signaling mechanisms underlying endothelial permeability. For instance, VEGF is one the best representative of the pro-permeability, pro--angiogenic factor involved in tumor-induced angiogenesis and modulating the endothelial barrier. We also learned that endothelial cell-cell junctions, and especially VE-cadherin-based contacts, are actively contributing to neovascularization and tumor growth, as well as tumor heterogeneity and plasticity. It can be envisioned that vessels with a normalized, functional endothelium barrier could restore vascular homeostasis and may favor chemotherapy delivery. One challenging question will be therefore to evaluate whether rescuing an intact endothelial barrier will be clinically valuable.

Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Benefits and Pitfalls of Tumor Vessel Normalization
- ▶ Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Impact of Endothelial Transcription Factors in Sprouting Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- ▶ The Role of VEGF in Controlling Vascular Permeability

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Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis

Inga Bekes and Christine Wulff

Contents

Introduction 122

Molecular Mechanism of Vascular Permeability 122
Transcellular Permeability 123
Paracellular Junctions/Permeability 123

Vascular Permeability in Health and Disease 126

Regulation of Vascular Permeability 126
Inflammatory Cytokines 127

The Impact of Vascular Permeability on Physiological and Pathological Angiogenesis 127

Molecular Mechanism of Physiological Angiogenesis 128
The Role of the Vascular Endothelial Growth Factor (VEGF) 128
Anti-angiogenic Therapy in Cancer via Suppression of the VEGF-VEGFR System 130

Summary 130

Cross-References 130

References 130

Abstract

The permeability of the vasculature is a property of the capillary wall to obstruct movement of fluid or solutes driven by physiological force. The vasculature is essential for the health of normal tissues, hemostasis, lipid transport, and immune surveillance and is also an influential characteristic of many diseases in which it is greatly increased. The control mechanism of vascular permeability is a complex process that needs to be tightly regulated in order to preserve not only the vascular homeostasis but also its integrity. Here, transcellular and paracellular pathways play an important role as well as direct and indirect influence of the vascular permeability by molecules or blood

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pressure. In pathologies the vasculature is often affected by the disease process. This may result in neoangiogenesis, where an excessive formation of new, unstable, and hyperpermeable vessels with poor blood flow takes place. In this scenario the vascular endothelial growth factor (VEGF) plays a key role.

Keywords
Vascular permeability · Transcellular permeability · Paracellular permeability · Junctions · VE-cadherin · N-cadherin · VEGF · Inflammatory cytokines · Angiogenesis

Introduction

All cells require a continuing supply of nutrients and a means of clearing waste. Vertebrates have solved these problems by developing a vascular system that extends into all organs and tissues. The main function of the vasculature is to serve as a blood conduit; this way sufficient oxygenation of the peripheral tissues, followed by return of the deoxygenated blood to the lungs, is ensured. Secondly, the vasculature is essential for the health of normal tissues, hemostasis, lipid transport, and immune surveillance and is also an influential characteristic of many diseases in which it is greatly increased. While the vascular system of higher organisms is often described as "closed," it needs to be sufficiently "open" (i.e., "permeable") to allow an exchange of small molecules (gases, nutrients, and waste products) with the surrounding tissues.

In the healthy individual, the vasculature is a more or less stable system: in this arrangement endothelial cell survival is continuously maintained. During physiological conditions – such as the development of the embryo, ovulation, regrowth of the endometrium, or in conjunction with injury or disease – there is a demand of new vessel formation. This growth of all new tissues, whether healthy or not, is accompanied by blood vessel formation. In this setting, the new vessels form de novo in a process called vasculogenesis, while angiogenesis implies vessel formation from the preexisting vasculature.

In most organs, the endothelial cells form a dynamic barrier between the blood and the tissue via which plasma and its solutes cross the vascular barrier. This was investigated over the last century by physiologists including Pappenheimer, Landis, Starling, Renkin, Michel, Curry, Rippe, and Bates (Pappenheimer 1953; Rippe and Haraldsson 1994; Michel and Curry 1999; Bates and Harper 2002; Curry 2005). They found out that capillaries are the vascular segment involved in molecular exchange in normal tissues and that gases, water, and other small molecules cross the capillary endothelial cell barrier freely. In contrast, the passage of larger molecules such as plasma proteins is tightly restricted.

In many diseases including cancer or chronic inflammatory conditions – on the other side – it can be observed that the vascular barrier disintegrates and leakage occurs and increases. This leakage of especially larger molecules and cells results not only in edema and inflammation but also in disease progression.

The following chapter will discuss the current knowledge about the mechanism of vascular permeability as well as its impact on angiogenesis.

Molecular Mechanism of Vascular Permeability

The transport of nutrients and small solutes is constant in physiological conditions, and it is essential for both the homeostasis of the vascular system and the organs. In most normal adult tissues, endothelial cells preserve basal vascular permeability at a low level, while they increase permeability in response to physiological situations like inflammation. Therefore, vascular permeability must be tightly controlled by a number of extracellular stimuli and mediators to maintain tissue homeostasis. Accordingly – with the disintegration of these conditions – debilitated regulation of endothelial permeability causes various diseases, including chronic inflammation, asthma, edema, sepsis, acute respiratory distress syndrome, anaphylaxis, tumor angiogenesis, and diabetic retinopathy.

Structurally the endothelium lining the vasculature constitutes a barrier which itself maintains the integrity between blood and interstitium and regulates extravasation of fluids and plasma proteins. Traditionally the term "vascular permeability" implies the basal vascular sieving of solute and small molecules. In this setting, molecules smaller than approximately 40 kDa can cross spontaneously the endothelial barrier, while larger molecules need active disruption of the vessel wall in order to extravasate to the surrounding tissues. Although such induced leakage preferentially takes place in postcapillary venules, capillaries and larger venules may also leak (Majno et al. 1969; Kohn et al. 1992; Roberts and Palade 1995).

Permeability is mediated by the strictly regulated opening and closing of cell-cell junctions. Here, the active transport occurs at least through two mechanisms called the *transcellular* or *paracellular* pathways, both for macromolecules and inflammatory cells. The transcellular permeability is probably governed by signaling pathways that are responsible for endocytosis and vesicular trafficking in cells (Mehta and Malik 2006). These pathways include vesicular transport systems, fenestrae, and biochemical transporters. On the other hand paracellular permeability is controlled by the dynamic opening and closing of endothelial junctions implying that a complex rearrangement of adhesion proteins with their related cytoskeleton must occur.

It is likely that the two described pathways are interconnected in some way, since many permeability-increasing agents expanse vesicular transport and also disrupt the integrity of endothelial cell-cell junctions (Feng et al. 1999; Dejana 2004; Weis and Cheresh 2005); however, whether this occurs in the same vessels and at the same time is still a matter of debate. It is possible that, in some areas of the vasculature – such as the microvasculature of the glands – the transcellular pathway is better developed, whereas in others, such as the postcapillary venules, the paracellular pathway is favored. This way it is reasonable to assume that the involvement of these different mechanisms may depend on factors like the type of vessel, the organ, the kinetics of the transport, and the nature of what is transported across the wall. It will be of interest once more knowledge is available on the vesicular transport systems in endothelial cells, to try to integrate both systems into a more comprehensive picture.

Transcellular Permeability

For transcellular permeability, the formation of vesiculo-vacuolar organelles (VVOs) or of fenestrae is required. Transcytosis is an important mechanism for delivery of macromolecules to tissues: during this process caveolae pinch off from the plasma membrane, form vesicular carriers, shuttle to the opposite side of the endothelial cell where vesicles fuse with the plasma membrane, and discharge their cargo into the perivascular space. The VVOs have been described and investigated primarily using electron microscopy analyses, which have shown that VVOs are prominent structures in both normal vessel endothelial cells and tumor-supplying cells (Caruso et al. 2001; Dvorak and Feng 2001). At the same time VVOs have been implicated as the primary pathway for macromolecular extravasation. However, up to date the origin of VVOs is not known precisely. Originally vesicles and vacuoles that form the VVOs were thought to derive from caveolae. A main protein in caveolae is caveolin-1. However, studies with caveolin-1 knockout mice showed a lack of caveolae with reduced permeability for macromolecules, while the vasculature still contained VVOs.

Paracellular Junctions/Permeability

The scientific findings about the molecular organization of the different types of endothelial cell-cell junctions have established a basis for understanding how these structures might crosstalk and interact reciprocally (Bazzoni 2004; Vestweber 2007; Wallez et al. 2007): endothelial cell junctions present a particularly complex network of adhesion proteins that are linked to intracellular cytoskeletal and signaling partners. These proteins are organized into

distinct structures called *tight junctions (TJs)* and *adherens junctions (AJs)*. In addition, several adhesion proteins (such as platelet endothelial cell adhesion molecule (PECAM1), MUC18, intercellular adhesion molecule 2 (ICAM2), CD34, endoglin, and others) cluster at cell-cell contacts that are distinct from TJs and AJs. Regarding the regulation of paracellular permeability, there are at least the above mentioned two different types of intercellular junctions involved: the AJs and TJs. These junctions are localized in the lateral cell membrane between neighboring endothelial cells sealing the space between these cells. The AJs and TJs consist of different families of transmembrane proteins that promote homophilic cell-cell interactions and transfer intracellular signals. Many reports support the concept that intercellular junctions are dynamically remodeled not only in embryogenic cells but also in resting cells (Dejana et al. 2009). Adhesive membrane proteins of AJs and TJs form adhesive complexes which act as zipper-like structures between the interacting cells (Nelson and Veshnock 1987; Yap et al. 1997; Chitaev and Troyanovsky 1998; Cavey et al. 2008). These proteins are localized hierarchically from the apical to the basal pole of the lateral membrane: in the most apical position, the TJ protein family is localized followed by occludin and the nectin family. Unlike the TJs, the AJs are localized more basal than apical, mainly consisting of the cadherin family.

An important emerging concept is that intercellular junctions are dynamic structures undergoing continuous remodeling not only during morphogenesis in the embryo or upon exposure of cells to agents that increase permeability but also in confluent and resting cells. Continuous recycling of adhesive proteins and signaling partners may occur at AJs and also at TJs. Cadherins, and in particular VE-cadherin, show a flow-like movement in a basal to apical direction which is accompanied by actin reorganization.

Adherens junctions (AJs) initiate cell-to-cell contacts and promote their maturation and maintenance. AJs compromise the cadherin family of adhesion proteins. Endothelial cells express relatively high levels of two important cadherins: a cell-type-specific cadherin called *VE-cadherin* and the *neuronal cadherin (N-cadherin)*. N-cadherin is also present in other cell types such as neural cells and smooth muscle cells (Bazzoni 2004). VE-cadherin can be found in essentially all types of vessels, whereas other non-cell-type-specific cadherins, such as T-cadherin and P-cadherin, are variably expressed in different types of endothelial cells (Ivanov et al. 2001).

VE-cadherin is an endothelial-specific transmembrane protein consisting of five immunoglobulin-like domains in the extracellular region, a single transmembrane domain, and a short intracellular region. Each domain repeats a single transmembrane domain and a short cytoplasmic region from which each molecular region accounts for a different function. While the extracellular region is responsible for homophilic interactions in trans, the transmembrane domain is involved in cis interactions and lateral clustering. In contrast, the cytoplasmic tail of the protein forms complexes with catenins, such as b-catenin, p120, and plakoglobin, and many other signaling and cytoskeletal partners. Many reports in the literature support the idea that adherens junctions might influence the tight junction organization by modulating their expression and assembly. For instance, in confluent endothelial monolayers, vascular endothelial cadherin clustering inhibits the transcriptional activity of forkhead box protein O1 (FoxO1), which is a repressor of claudin-5 expression – a key component of tight junctions.

The second most important cadherin expressed in endothelial cells is *N-cadherin*. Although N-cadherin is expressed at levels that are comparable to VE-cadherin, it presents a diffuse distribution on the endothelial cell membrane and is poorly clustered at intercellular junctions. In 1998 Navorro et al. found out that when VE-cadherin is present at junctions, it excludes N-cadherin from those sites (Navarro et al. 1998). It is therefore possible to assume that in stabilized endothelial monolayers N-cadherin does not play a role at endothelial cell-cell junctions. Instead, N-cadherin seems to act at heterotypic cell-cell contacts between endothelial cells and pericytes (mesenchymal cells that associate with the walls of small blood vessels).

Adherens junctions dissolve in response to a number of stimuli: these catalysts include the vascular endothelial growth factor (VEGF) and inflammatory cytokines such as histamine and bradykinin. This dissolution allows extravasation of macromolecules. It is well known that concerning the molecular mechanism of vascular permeability phosphorylation of VE-cadherin and leukocyte extravasation are essential events. However, the available data is conflicting, and up to date it is not yet exactly clear how the molecular events trigger vascular permeability.

There is scientific evidence that C-Src and Src family kinase are required for VEGF-, histamine-, and bradykinin-induced VE-cadherin phosphorylation and for vascular permeability (Esser et al. 1998; Weis et al. 2004; Wallez et al. 2007; Orsenigo et al. 2012; Hox et al. 2015). In addition, focal adhesion kinase (FAK) phosphorylates VE-cadherin downstream of VEGF in a Src-independent manner, inducing vascular permeability. Moreover several protein tyrosine phosphatases (PTP) act as regulators of junctional stability. For example, PTP1b overexpression reduces VEGF-induced VE-cadherin phosphorylation, leading to destabilization of the junction (Nakamura et al. 2008); density-enhanced phosphatase (DEP)-1 inhibition reduces Src activity and VEGF-induced permeability. In addition, VE-PTP (vascular endothelial-PTP) dissociation from VE-cadherin is required for vascular permeability in vivo and VE-PTP inhibition induces Y685 VE-cadherin phosphorylation (Broermann et al. 2011; Wessel et al. 2014). Phosphorylation of distinct serine and tyrosine residues on VE-cadherin induces molecular and leukocyte extravasation. In vitro, VEGF induces phosphorylation of serine (S)665, thereby modulating VE-cadherin endocytosis by recruitment of β-arrestin to the phosphorylated serine. In addition, phosphorylation of tyrosines (Y)658 and Y731 causes the dissociation of β-catenin and p120. Phosphorylation of Y658 and Y731 in vitro is triggered by binding of leukocytes to intercellular adhesion molecula 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in endothelial cells. In vivo, basal VE-cadherin phosphorylation of Y658 and Y685 is present in capillaries and venules of certain tissues (Orsenigo et al. 2012). However, Wessel et al. (2014) showed basal phosphorylation of Y731 and phosphorylation of Y685 in capillaries and venules only after induction with peroxyvanadate (Wessel et al. 2014). These seen differences may be dependent on the studied tissue and the specificity of the phosphoantibodies.

It is well investigated that VE-cadherin phosphorylation in capillaries and venules (not in arterioles and arteries) correlates with the sites of vascular permeability. Indeed, leakage takes place in postcapillary venules, occasionally in capillaries and larger venules, but not in arteries. Bradykinin and histamine induce the loss of Y658 and Y685 phosphorylation in the sites of vascular permeability, inducing VE-cadherin endocytosis (Orsenigo et al. 2012). Another study showed that histamine and VEGF induce vascular permeability through the phosphorylation of Y685. In the same study, IL-1β and TNF-α led to leukocyte extravasation accompanied by a decrease in the phosphorylation of Y731 (Wessel et al. 2014).

In physiological conditions, the dissolution of adherens junctions is transient, and the junctions will soon close again in part due to VE-cadherin recycling and reappearance on the cell surface. Some reports show that junctions can remain in their open state (Baluk et al. 1997). However, in pathological situations where vascular permeability is increased (also denoted vascular leak), the regulation of junction dynamics is lost and the junctions remain open, thus indicating chronic permeability.

Tight junctions (TJs) are located at the most-apical side of the lateral interendothelial membrane. They consist of three prevailing families of transmembrane proteins: claudins, occludin, and junctional adhesion molecules (JAMs). TJs are known to regulate the passage of ions and solutes through the paracellular route (Bazzoni 2004; González-Mariscal et al. 2008)**.** TJs may also act as a membrane "fence" to limit the free movement of lipids and proteins between the apical and the basolateral cell surfaces. The core components of TJs that promote cell-to-cell adhesion are members of the claudin family (Furuse and Tsukita 2006; Van Itallie and Anderson 2006).

The claudin family consists of more than 20 members, only a few of which are expressed by endothelial cells. Claudin 5 is more or less ubiquitous along the vascular system whereas non-cell-specific claudins are also found in endothelial cells; in responding to the different needs of the perfused organ, the combination of claudins may vary.

Multiple intracellular partners of TJ adhesive proteins have been described. Among the best investigated factors, the members of the zonula occludens-familiy (ZO) family (ZO1 and ZO2 in the endothelium) are found – a closely related subgroup of the membrane-associated guanylate kinase (MAGUK) family that localizes at TJs in most tissues including the endothelium. Other intracellular TJ proteins include signaling and actin-binding proteins.

In many vessels, endothelial cells make the vasculature uninterrupting. This is due to the fact that in certain organs the endothelial cells display specialized structures to facilitate rapid transport across the endothelium. Examples for fenestrated endothelium in vessels are endocrine glands, digestive tract mucosa, or the kidney peritubular capillaries. Here, the endothelial cells are equipped with so-called endothelial fenestrae, circular pores, covered by diaphragm. A key protein is the plasmalemmal vesicle-associated protein-1 (PV1). In addition there are naturally occurring fenestrae without diaphragm, i.e., in the kidney.

Another unique endothelial barrier system is the *blood-brain barrier (BBB)*. The BBB has the purpose to prevent the brain from exposure to the blood and the adverse consequence of edema. In addition to the presence of adherens junctions, the brain vasculature consists of high-resistance tight junctions and an abundant basement membrane. Perivascular components such as astrocytes, pericytes, and neurons participate functionally in creating the BBB. A potentially unique feature of the BBB is the transendothelial vesicular transport of a range of nutrients and metabolic waste products. Up to date, there is still limited information on to what extent the BBB can be transiently opened in response to growth factors and inflammatory cytokines (Hudson et al. 2014). Also comparative information on molecular mechanisms in the central nervous system and peripheral permeability is until now lacking.

Vascular Permeability in Health and Disease

In physiological conditions regulated vascular permeability occurs in a well-controlled manner. Here, vascular permeability to solutes and small molecules takes place constitutively and appears not to require an active process. It is likely that the constant sieving of solutes is important in maintaining the interstitial pressure in the tissue. It is also evident for maintaining the immune surveillance function of the lymphatics. Interstitial fluid collected by the lymphatics is carried via lymphatic capillaries to lymph nodes where foreign antigens will be exposed to the immune system. The extravasation of macromolecules serves diverse purposes, for example, to maintain the balanced blood and interstitial pressures, to act in immune surveillance, and to carry other molecules, such as hormones and lipid, across the vessel wall. Extravasated fibrinogen, processed to fibrin, may form a provisional matrix on which new blood vessels extend. Extravasation of inflammatory and immune cells serves specific purposes in different pathologies. The cells are necessary for the healing of an acute disease process but may also propagate a chronic disease. In pathologies excess leakage leads to tissue deterioration and to the progression in severity of diseases. In this setting, exaggerated and uncontrolled vascular permeability is associated with many diseases, among them cancer, myocardial infarction, ischemic stroke, and retinopathies.

However, a clear role for *physiological* vessel permeability is not yet established. Overall, studies on the regulation of vascular permeability often suffer from the lack of physiological interpretation.

Regulation of Vascular Permeability

The vascular permeability can be regulated directly or indirectly.

The direct influence is given by molecules that make the barrier to disintegrate; this can occur via a transvessel pore or a junction that needs to be opened. Indirectly the vascular permeability may be regulated by the blood pressure.

Molecular regulators of vascular permeability include angiogenetic growth factors like VEGF (which will be discussed later on in this chapter) and inflammatory cytokines like histamine and bradykinin.

Inflammatory Cytokines

The two best-studied *inflammatory cytokines* in vascular permeability are histamine and bradykinin. Histamine is produced by mast cells and binds to G-protein-coupled H1- and H2-histamine receptors (GPCRs) on endothelial cells (Marshall 1984). Bradykinin is cleaved from kininogen; it acts via GPCRs B1 and B2. It is quite well investigated that both inflammatory cytokines (histamine and bradykinin) mediate activation of the serine/threonine kinase Akt, which itself phosphorylates and thereby activates endothelial nitric oxide synthase (eNOS). Thereby, p-eNOS catalyzes the generation of NO. NO is a key regulator of the vascular tone; it mediates vasodilation by stimulating soluble guanylyl cyclase and increasing cyclic guanosine monophosphate (GMP) in smooth muscle cells (Forstermann and Sessa 2012). Akt is not the only kinase that can phosphorylate and activate eNOS, but it is the best-studied pathway. Another target effect of nitric oxide is S-nitrosylation of beta-catenin that will cause its dissociation from VE-cadherin and consequently the disassembly of adherens junctions. The eNOS-NO pathway is implicated also in VEGF-regulated vascular permeability since ablation of eNOS expression blocks the VEGF response (Fukumura et al. 2001).

The Impact of Vascular Permeability on Physiological and Pathological Angiogenesis

Angiogenesis (the growth of new blood vessels) is a complex multistep process that involves multiple cell types, numerous growth factors, and complex regulatory checks and balances. The families of proteins that appear to be most critical to blood vessel growth are the various members of the vascular endothelial growth factor (VEGF), angiopoietin (Ang), and ephrin (Eph) families.

Physiological angiogenesis is coordinated by these molecules to produce viable, patent, and mature vessels in which vascular permeability is low. These vessels are often of arterial or venular (rather than capillary) phenotype. Overexpression of special vascular growth factors, such as VEGF, becomes evident in *pathological* angiogenesis where a new, immature vasculature develops; in this condition the blood flow is often unregulated, and there is an inappropriate relationship between metabolic demand and flow.

Originally the mechanism of angiogenesis was described in 1946 when Abell showed that dyes, such as India ink blue, leak out of capillaries growing into a recent wound (Abell 1946). An ultrastructural study in 1963 showed that intravenous injection of large molecular weight tracers, such as colloidal carbon, resulted in deposits in the interstitium outside growing capillaries in wound healing models. Increased vascular permeability during angiogenesis is now recognized as a cardinal feature of pathological angiogenesis. Many diseases are associated with an uncontrolled sprouting angiogenesis process; among the best known diseases are particularly cancer, psoriasis, arthritis, and retinopathies. The novel vasculature is often characterized by weak, friable vessels that are inherently leaky and that often bleed. At the same time the tissue itself may become edematous as a result of this increase in permeability, and this effect is compounded by unregulated flow through these vessels seen in the clinical appearance such as cerebral edema in glioblastoma multiforme and in ascites and pleural effusions in liver metastasis and ovarian cancer (Xu et al. 2000; Yano et al. 2000; Bekes et al. 2016).

Physiological angiogenesis, on the other hand, can occur throughout adult life as well as during both pre- and postnatal development. However, in physiological angiogenesis increased vascular permeability appears to have a minor effect. Physiological angiogenesis has been shown to occur during muscle remodeling after exercise-induced training; during hair growth, fat deposition, and wound repair; as well as during the female reproductive cycle in the endometrium and the developing follicle. In pathological conditions the dysregulation of vessel growth contributes to disease progression.

Molecular Mechanism of Physiological Angiogenesis

Angiogenesis entails a sequence of steps including vessel branching (defined by the activation of quiescent endothelial cells), sprouting, anastomosis, regression, and finally maturation to a new stable quiescent status. In the quiescent state, endothelial cells form monolayers where cells are interconnected by junctional molecules. Alone, endothelial cells are unable to establish a mature and functional vasculature. Peri-endothelial mural cells, such as smooth muscle cells (SMCs) for large vessels and pericytes for small vessels, surround and support endothelial cells, suppressing their proliferation and transducing cell survival signals. Quiescent vessels become activated and start the branching process as a result of stimulation by angiogenic factors, such as VEGF-A, VEGF-C, Ang-2, FGFs, or chemokines. These factors are released from inflammatory cells and tumor cells, e.g., through hypoxia-inducible factor-1α (HIF-1α)-dependent regulation. Lumen formation depends on the establishment of apical basal polarity and cell-cell contacts. Adhesion receptors such as E-cadherin in adherens cell-cell junctions and integrins at cell-extracellular matrix attachment sites are responsible for apical-basal polarity of endothelial cells and for lumen formation. In addition, tight junctions are, as a rule, localized to the apical-lateral membrane. The anastomosis with another vessel branch, reestablishing the junctions at the point of cell-cell contact, allows for blood flow. Mural cells and endothelial cells deposit new basement membrane to ensure an optimal flow distribution and a functional vessel network. Now the new vessel is mature and endothelial cells reattain a quiescent status.

Microenvironmental feedback is essential for matching angiogenesis to the requirements of the tissue. Thus, tissues that are stressed due to an inadequate supply of oxygen and nutrients signal for induction of angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF); this signaling is suppressed when perfusion meets the needs of the tissue. However, the process of invasion of capillaries into a tissue and establishment of circulation cannot meet rapid fluctuations in vascular needs, and excessive cycles of vascular invasion and regression can be destructive. Thus, the vascular bed must include a sufficient reserve for matching fluctuations in tissue requirements, and it must have the ability to respond to acute changes by recruiting those reserves and to utilize proper angiogenesis only when its needs exceed the available reserves. Acute physiological needs for increased perfusion are met by local regulation of blood flow, mediated by neuronal control of vasodilation through perivascular contractile cells. Induction of local vascular dilation and hyperpermeability are affected not only by VEGF but also by NO.

The Role of the Vascular Endothelial Growth Factor (VEGF)

In the recent decades, the molecular basis of angiogenesis has been extensively studied, and a variety of signaling systems, such as the vascular endothelial growth factor (VEGF), angiopoietin-Tie, ephrin-Eph, and Delta-Notch, were found to play important roles in angiogenesis. Among them, VEGF, the strongest angiogenetic growth factor, is crucial not only for vascular maintenance and permeability regulation but also for physiological angiogenesis from early embryonic to adult stages and especially pathological angiogenesis, such as in cancer (Senger et al. 1983; Murakami 2012). Although it is more marked during disease states, there has also been evidence demonstrating that there is a regulated increase in vascular permeability to both solutes and water as capillaries grow and form new vessels in physiological systems (Spanel-Borowski and Mayerhofer 1987; Dejana et al. 2001).

The immediate response to stimulation by VEGF leads to vasodilation and increased vessel permeability. These changes occur within minutes after stimulation and thus may be useful as biomarkers for activation of endothelial VEGF receptors. The mammalian genome encodes five VEGF family members, VEGF-A (also known as VEGF), placenta growth factor

(PlGF), VEGF-B, VEGF-C, and VEGF-D, which regulate vasculogenesis, angiogenesis, and lymphangiogenesis. Particularly VEGF-A is crucial for blood vessel formation during early embryogenesis. Not only the VEGF-A homozygous knockout mice but also its heterozygous mice (VEGF-A fl/_) exhibit an embryonic lethal phenotype due to immature blood vessel formation, indicating that the local concentration of VEGF-A in the embryos has to be tightly regulated for proper angiogenesis. Several VEGF-A subtypes are generated by alternative splicing. Among these, VEGF-A165 has the highest biological activity with binding affinity for a coreceptor, neuropilin-1 (Nrp1). VEGF-A was first described as early as 1983 by Senger et al. who partially purified a factor secreted by hepatocarcinoma cell lines that increased dye extravasation into the skin of guinea pig (Senger et al. 1983). Further characterization resulted in a publication in 1986 describing the dose dependency and some details of the protein (Senger et al. 1986). However, the protein structure and amino acid sequence of this factor were not described until 1990 (Senger et al. 1990). VEGF was originally denoted vascular permeability factor (VPF) implying its essential role in regulation of the vascular barrier (Senger et al. 1983). VEGF exerts its action by binding to VEGFRs, a family of three tyrosine kinase receptors: VEGFR1, VEGFR2, and VEGFR3. VEGFRs share a similar structure characterized by immunoglobulin-like loops in the extracellular domain, a transmembrane domain, a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal tail (Koch et al. 2011). Preferentially VEGF2 has been implicated in the regulation of permeability and angiogenesis. VEGFs can be presented in cis to the VEGFRs, when the coreceptor is expressed together with the VEGFR in the endothelial cell, or in trans, when the coreceptor is expressed in a different endothelial cell or another cell type (Jakobsson et al. 2006; Koch et al. 2011). The binding of VEGFs to VEGFRs induces dimerization of the receptors, creating homo- or heterodimers (Lemmon and Schlessinger 2010; Nilsson et al. 2010). Dimerization is followed by conformational changes that allow the subsequent activation and autophosphorylation of the tyrosine kinase. A number of phosphorylation sites in VEGFR2 have been identified (Matsumoto et al. 2005). Several of these phosphorylation sites have been studied in loss-of-function analyses by phenylalanine knock-in, in vivo and/or in vitro. The most interesting site at this point appears to be the Y949 site in the VEGFR2 kinase insert. It serves as a binding site for an adaptor molecule, T cell-specific adaptor (TSAd), which binds to the cytoplasmic tyrosine kinase c-Src. Silencing or gene inactivation of TSAd makes endothelial junctions unresponsive to VEGF, resulting in loss of VEGF-induced vascular permeability (Sun et al. 2012). Several studies from the David Cheresh lab implicate c-Src in phosphorylation of the critical adherens junction protein VE-cadherin (Eliceiri et al. 1999; Weis et al. 2004). According to the model, c-Src-induced phosphorylation of VE-cadherin promotes dissolution of VE-cadherin contacts between cells, followed by internalization and degradation or recycling of VE-cadherin (Fukuhra et al. 2006). The other VEGFR2 phosphorylation sites induce signaling pathways that also contribute to vascular permeability regulation. These sites include Y1173 (Y1175 in the human VEGFR2), which binds phospholipase Cg, as well as the adaptor molecule Shb, and Y1212 (Y1214 in the human VEGFR2), which binds the adaptor Nck. Whether other growth factors for which there are receptors on endothelial cells, such as placenta growth factor (binding exclusively to VEGFR1) or fibroblast growth factors (FGFs, binding to FGFR1 and FGFR2), mediate acute or chronic vascular permeability has not yet been addressed in detail.

In "normal" physiological angiogenesis, there is an association between the VEGF expression and hypoxia, for instance, in exercise-induced angiogenesis in the skeletal muscle, in the corpus luteum, and in the endometrium. However, nonhypoxic angiogenesis can also be stimulated, for instance, by increasing shear stress in skeletal muscle or by hormonal control in the female reproductive system (Mukhopadhyay et al. 1998). In pathological states, the same growth factors are overexpressed (Damert et al. 1997),

but despite significant understanding of the mechanisms underlying regulation of transcription and translation of VEGF (Sandner et al. 1997; Wenger et al. 1997), the underlying mechanisms of permeability regulation by growth factors in pathology are still not well understood.

The actions of VEGF on permeability and angiogenesis have been extensively studied. However, the vasodilator actions of VEGF may underlie its actions in physiological and pathological angiogenesis to an extent not currently appreciated. Increasing blood flow to a tissue is a more controllable and direct mechanism for increasing tissue growth than stimulating angiogenesis or permeability, particularly in the short term. The fact that VEGF is uniquely able to stimulate angiogenesis directly, act as a potent vasodilator, and is able to increase vascular permeability means that its upregulation in all known endogenous physiological and pathological forms of angiogenesis is a fundamental switch in tissue perfusion.

Anti-angiogenic Therapy in Cancer via Suppression of the VEGF-VEGFR System

In general, the possibility of controlling vascular permeability has several therapeutic implications. An uncontrolled and lasting increase in permeability that is not balanced by the reabsorption of lymphatic fluid causes edema, which, in turn, increases ischemic tissue injury in conditions such as stroke or myocardial infarction. Furthermore, vascular leakage in tumors not only facilitates tumor cell penetration into the vessels and metastatic dissemination but also contributes to the accumulation of fluid in the stroma and the elevated interstitial pressure that are common to several solid tumors (Weis and Cheresh 2005). Elevated interstitial pressure is probably the cause of altered tumor perfusion, the development of necrotic areas, and impaired drug delivery. Conversely, increasing vascular permeability in a reversible manner might be beneficial because it might increase drug accessibility to different tissues in which fluid exchange between blood and tissues is limited, such as in the brain.

Summary

Vascular permeability and angiogenesis occur in physiological and pathological conditions. Whereas vascular permeability is the process where blood vessels exchange nutrients, solutes, and inflammatory cells with the surrounding tissues, angiogenesis is characterized by endothelial cell sprouting, migration, and anastomosis. Both processes – vascular permeability and angiogenesis – are tightly regulated physiological processes. However, uncontrolled, increased permeability and angiogenesis lead to pathological conditions such as the progression of several diseases. VEGF is one of the most important players in this scenario, being regulated by a multifactor system, and can be therapeutically influenced in controlling vascular permeability and angiogenesis in diseases.

Cross-References

- ▶ Endothelial Cell-Cell Junctions in Tumor Angiogenesis
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part II

Mechanisms of Tumor Lymphangiogenesis

Angiopoietins and TIE Receptors in Lymphangiogenesis and Tumor Metastasis

Yulong He

Contents

Introduction 136

Comparison of Developmental and Tumor Lymphangiogenesis 136
Origin of Lymphatic Endothelial Cells in Development Versus Tumor 136
Functional Comparison of Lymphatic Network in Embryos and Tumor 136

Angiopoietins and TIE Receptors in Developmental Lymphatic Remodeling and Maturation 138
Angiopoietins in Developmental Lymphangiogenesis 138
TIE Receptors in Lymphatic Network Formation 140
Regulation of Lymphatic Remodeling and Maturation 142

ANGPT-TIE Pathway in the Modulation of Tumor-Associated Lymphangiogenic Microenvironment 144
Angiopoietins in Tumor Lymphangiogenesis and Lymphatic Metastasis 144
Lymphatic Regulator-Mediated Modulation of Tumor Immune Response 146

Summary 148

Cross-References 148

References 148

Abstract

In contrast to the normal lymphatic network comprised of initial and collecting vessels, intratumor lymphatics are disorganized and lack vessel hierarchy due to the continuous lymphangiogenesis. Lymphatic vessels originate from veins during mammalian development, while tumor-associated lymphatics are largely formed by vessel cooption or sprouting from the preexisting lymphatics of adjacent tissues. Among the known lymphangiogenic regulators, angiopoietins and TIE receptors are crucial for the process of lymphatic remodeling to form a mature network. Accumulating evidence from animal and clinical studies has laid a solid foundation that tumor lymphangiogenesis contributes to tumor dissemination. It has been shown in animal tumor models that targeting the key

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lymphangiogenic signaling pathways, including ANGPT-TIE mediated signals, could efficiently block lymphatic tumor metastasis. Meanwhile, ANGPT-TIE pathway is also actively involved in modulating tumor immune microenvironment. Therefore, strategies to fine-tune the interaction of lymphatic EC-immune cells could be employed in the prevention of tumor progression.

Keywords
Angiopoietin · TIE receptors · Lymphatic development · Tumor lymphangiogenesis · Lymphatic metastasis · Tumor-immune microenvironment

Introduction

Lymphatic vessels contribute to tissue homeostasis by draining excess tissue fluid together with large substances and immune cells (Tammela and Alitalo 2010; Petrova and Koh 2018). The lymphatic route can also be employed by tumor cells during their metastatic dissemination to distant organs after evasion from immune surveillance (Alitalo 2011; Karaman and Detmar 2014; Stacker et al. 2014). Mechanisms underlying lymphatic formation, including cellular events and molecular players, are largely shared in development and in tumor (Li et al. 2012). However, due to the distinct tissue microenvironment in embryos and tumors, the finally formed lymphatic networks are quite different, including the lymphatic vessel hierarchy, structural integrity, and functionality.

Comparison of Developmental and Tumor Lymphangiogenesis

Origin of Lymphatic Endothelial Cells in Development Versus Tumor

The initiation of lymphangiogenesis differs in development and in tumor (Fig. 1a, b). Following the arterial-vein specification in mammalian development, venous endothelial cells (ECs) are the major source of lymphatic ECs with PROX1 as the key regulator (Wigle and Oliver 1999; Adams and Alitalo 2007; Yang and Oliver 2014; Potente and Makinen 2017). Non-venous origin of lymphatic ECs has been found to participate in mesentery, heart, and superficial dermal lymphatic vessel formation in mice (Klotz et al. 2015; Martinez-Corral et al. 2015, Stanczuk et al. 2015). Venous EC-independent route of LEC initiation was also demonstrated in other species including chicken embryos (Wilting et al. 2003; Mahadevan et al. 2014), Xenopus tadpoles (Ny et al. 2005), and zebrafish (Nicenboim et al. 2015). In comparison with this, tumor-associated lymphatic endothelial cells mainly originate from the preexisting lymphatic network in the surrounding tissues (He et al. 2004). It is uncertain whether there is any differentiation of lymphatic endothelial cells from venous ECs in tumor. One interesting observation is that intratumor lymphangiogenesis mainly occurs in regions undergoing necrosis (Fig. 1b), suggesting that tumor-associated macrophages may be able to trans-differentiate into lymphatic ECs in tumors as demonstrated in inflamed tissues (Maruyama et al. 2005).

Functional Comparison of Lymphatic Network in Embryos and Tumor

The formation of a mature lymphatic system involves the remodeling of primitive lymphatic plexus into structurally specialized network containing initial and collecting lymphatics in development. Although a functionally competent lymphatic system is crucial for maintaining tissue fluid homeostasis in the postnatal life, the primary lymphatic network without collecting vessels is functional for lymph draining during embryonic development. This has been demonstrated in several genetically modified mouse models. For example, there was no lymphedema observed in *Angpt2* deficient embryos or the downstream *Akt1* null mice although there was no collecting vessel formation (Zhou et al. 2010; Shen et al. 2014). However, severe tissue lymphedema occurred in mice without lymph sac formation or with abnormal formation of the primitive lymphatic network in mutants targeting *Vegfc*, *Vegfr3* or *Tie1*

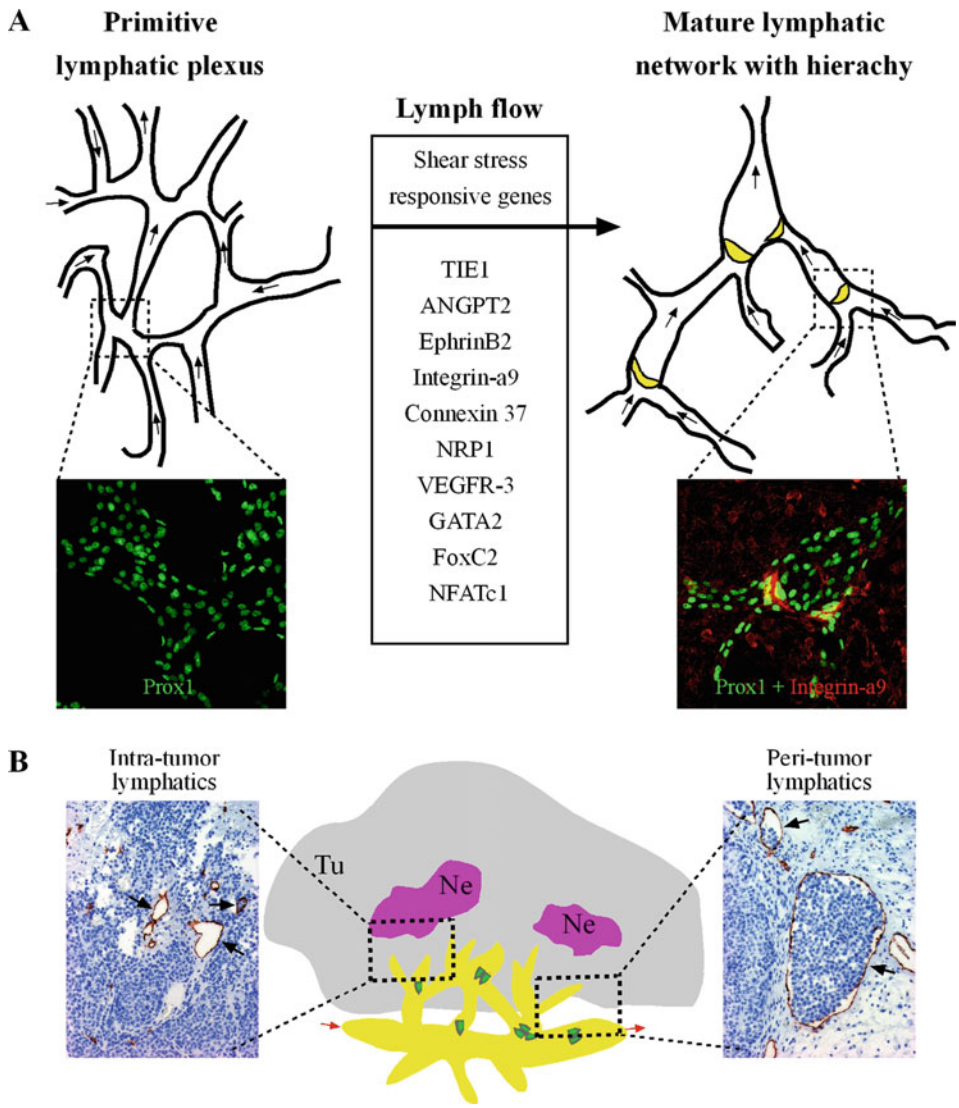

Fig. 1 Comparison of lymphatic network formation in development and tumor. (**a**). Lymphatic development involves the first formation of primitive lymphatic plexus followed by the process of lymphatic remodeling to form collecting vessels with intraluminal valves (green for PROX1 to indicate lymphatic ECs, and red for Integrin-α 9 to indicate lymphatic valves; and images are modified from Supplemental Figure II and Figure 3 in Arterioscler Thromb Vasc Biol. 2014;34:1221–1230, by permission of Wolters Kluwer Health Inc., through Copyright Clearance Center's RightsLink® service). This process is likely to be driven by lymph flow generated shear stress, which could induce a number of key lymphatic regulators as listed in the illustration. (**b**). Tumor-associated lymphatic vessels are formed by vessel cooption or sprouting from the pre-existing lymphatics of adjacent tissues. Intratumoral lymphatic vessel growth is often detected in necrotic areas, which is connected to the dilated peritumoral lymphatic network for tumor cell dissemination (red for LYVE1, and images are modified from Figure 5 in Cancer Res. 2005;65:4739–46, by permission from American Association for Cancer Research). Arrows point to the intra- and peritumoral lymphatics and some are already invaded by tumor cells (Tu, tumor, and NE, necrosis)

(Karkkainen et al. 2004; Zhang et al. 2010; Shen et al. 2014). Fluid flow generated shear stress has been shown to regulate the expression of various genes in endothelial cells including the key lymphatic regulators such as TIE1 and ANGPT2 as listed in Fig. 1a (Porat et al. 2004; Tressel et al.

2007; Sabine et al. 2012; Li et al. 2014; Baeyens et al. 2015; Kazenwadel et al. 2015; Sweet et al. 2015). Therefore, it is likely that lymph flow in the primitive lymphatic network plays a critical role in the process of remodeling to form a mature network.

In contrast, the formation and function of tumor-associated lymphatic network may largely be compromised by the specific tumor microenvironment. Tumor-associated lymphatic network is usually lack of vessel hierarchy due to the continuous lymphangiogenesis, which may to some extent resemble the primitive lymphatic plexus observed in development. Factors contributing to the lymphatic abnormality also include the hypoxic and acidic tumor microenvironment, mechanical stress generated by uncontrolled tumor cell proliferation, and high interstitial pressure resulting from the defective vascular wall integrity (Hanahan and Weinberg 2011; Li et al. 2012). The non-homogeneous distribution of lymphatic vessels in tumor tissues (Beasley et al. 2002; He et al. 2005) may partly account for the failure to detect functional lymphatics in the draining assay (Padera et al. 2002). However, lymph node metastasis occurs frequently in solid tumors (Alitalo et al. 2005; Achen and Stacker 2008; Karaman and Detmar 2014). Therefore, at least a proportion of tumor lymphatics are functional after connecting with collecting vessels mainly located at peritumoral regions (Karpanen et al. 2001; He et al. 2005).

Angiopoietins and TIE Receptors in Developmental Lymphatic Remodeling and Maturation

A range of factors have been identified to coordinate the complex processes of lymphatic development, including transcription factors, lymphangiogenic growth factors and membrane-bound receptors, intracellular signal mediators, extracellular matrix proteins, and cell junction molecules (Bertozzi et al. 2010; Schulte-Merker et al. 2011; Li et al. 2012; Bazigou and Makinen 2013; Yang and Oliver 2014; Zheng et al. 2014a; Aspelund et al. 2016; Vaahtomeri et al. 2017). Among the molecular regulators, ANGPTs and TIE receptors are crucial in the regulation of lymphatic cell-cell junction, cell survival, collecting lymphatic vessel formation, and valve morphogenesis (Fig. 2a, b) (Gale et al. 2002; Shimoda et al. 2007; Dellinger et al. 2008; D'Amico et al. 2010; Qu et al. 2010; Shen et al. 2014; Saharinen et al. 2017).

Angiopoietins in Developmental Lymphangiogenesis

ANGPT2 is a ligand for TIE2 and has important roles in both angiogenesis and lymphangiogenesis. In blood vessels, ANGPT2 was reported to antagonize ANGPT1 to destabilize the integrity of formed vasculature and to keep the sprouting ECs free from mural cell coverage. This allows vascular growth and remodeling in response to angiogenic factors such as vascular endothelial growth factor-A (VEGFA) (Maisonpierre et al. 1997; Gale et al. 2002). In *Angpt2* knockout mice, although blood vascular development during embryogenesis was normal, postnatal angiogenesis in retina was retarded and there was also the failure of hyaloid vessel regression (Gale et al. 2002). Furthermore, deletion of *Angpt2* did not affect the formation of lymph sacs and the capillary lymphatic network during embryonic development (Dellinger et al. 2008; Shen et al. 2014). However, ANGPT2 deficiency disrupted the formation of collecting lymphatic vessels with defective valve formation and abnormal recruitment of smooth muscle cells (SMCs) associated with lymphatic capillaries (Fig. 2b) (Gale et al. 2002; Dellinger et al. 2008; Shen et al. 2014). Mice null for *Angpt2* also displayed thinner lymphatic diameter and decreased LEC number in lymphatic vessels in comparison with that of control littermates (Shen et al. 2014). Consistently, transgenic overexpression of ANGPT2 in endothelial cells under the control of tetracycline was shown to increase the caliber of lymphatic vessels and also LEC number (Zheng et al. 2014b). Interestingly, the lymphatic phenotype of *Angpt2* null mice is similar to that of *Akt1* knockout mice (Zhou et al. 2010). In *Akt1* deficient mice, but not in *Akt2* or *Akt3* knockouts, a significant decrease of the diameter and endothelial cell number of lymphatic capillaries

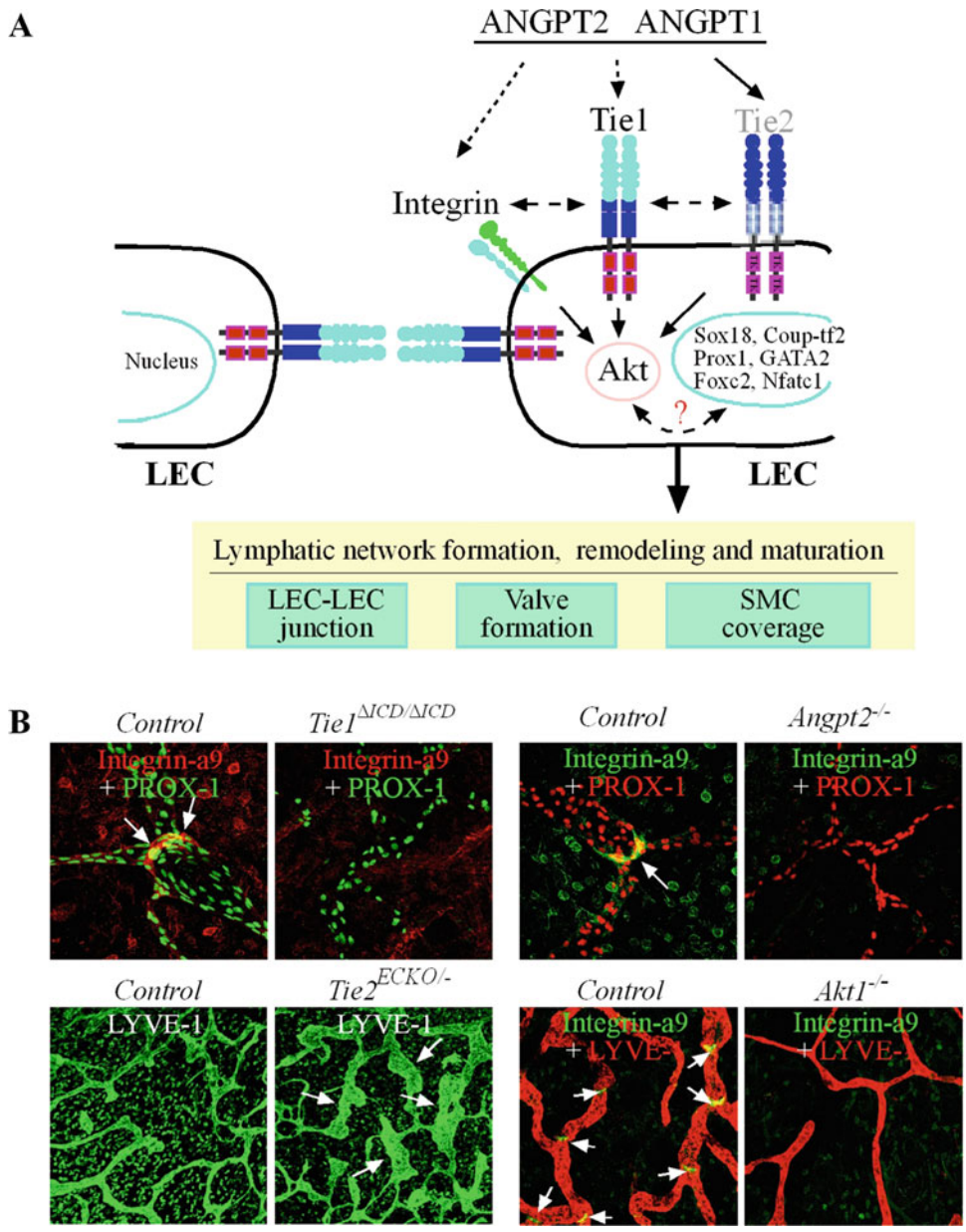

Fig. 2 Regulation of lymphatic development by ANGPT-TIE-AKT pathway. (**a**) Schematic illustration of angiopoietin and TIE receptors, together with other key lymphatic regulators, in lymphatic formation, remodeling, and maturation. AKT1 is a critical signal mediator downstream of TIE pathway and the detailed molecular circuits are yet to be elucidated. (**b**) Lack of collecting lymphatic vessels and valves was observed in the skin of *Tie1*$^{\Delta ICD/\Delta ICD}$ embryos (E18.5, green for PROX1 and red for Integrin-α9). In *Angpt2*$^{-/-}$ mice (E18.5), the diameter of lymphatic capillaries was less than that of control mice (red for PROX1 and green for Integrin-α9), and there were no collecting lymphatic vessels and valves detected in the skin of *Angpt2* mutants. A significant decrease of the diameter of lymphatic capillaries compared with that of control mice was also observed in *Akt1*$^{-/-}$ mice (red for LYVE1, and green for Integrin-α9). In contrast, lymphatic dilation was observed in the skin of *Tie2*$^{ECKO/-}$ mutant mice (green for LYVE1). (Panel B was modified with permission from Figure 3 and 7 in Arterioscler Thromb Vasc Biol. 2014;34:1221–1230 by Wolters Kluwer Health Inc., from Figure 3 in Am J Pathol 2010, 177:2124–2133 by Elsevier, and from Figure 1-figure supplement 3 in Elife. 2016 Dec 22;5. pii: e21032). Arrows point to dilated lymphatics in *Tie2*$^{ECKO/-}$ mice and lymphatic valves in other panels

was also observed, in addition to the abnormal collecting vessel formation as well as valve morphogenesis (Zhou et al. 2010). It is likely that AKT1 acts downstream of ANGPT2-mediated signals for LEC survival, lymphatic remodeling, and maturation during lymphatic development (Fig. 2a).

In contrast, the known biological function of endogenous ANGPT1 in lymphatic formation is still limited. Although local administration of recombinant ANGPT1 to mouse cornea or overexpression of ANGPT1 delivered via adenoviral vectors in ear skin was shown to stimulate lymphatic vessel growth (Morisada et al. 2005; Tammela et al. 2005), systemic treatment with ANGPT1 or other angiopoietins did not produce such an effect with cutaneous lymphatic vessels (Kim et al. 2007). Induction of lymphatic sprouting and filopodia formation by angiopoietins was observed at margins of healing wounds in ear skin at the initial period and also in mouse trachea (Kim et al. 2007). Genetic evidence to support a role of ANGPT1 in lymphatic formation is from this study where lymphatic defects in *Angpt2* deficient mice could be rescued when a cDNA encoding ANGPT1 was placed in the *Angpt2* locus (Gale et al. 2002). However, induced deletion of *Angpt1* during embryogenesis (E16.5) did not affect lymphatic growth in the corneal limbus. While simultaneous deletion of both *Angpt1* and *Angpt2* disrupted lymphatic formation in the corneal limbus, lymphatic vessels could still be detected in nonocular tissues such as ear skin (Thomson et al. 2014). The abnormal lymphatic patterning in *Angpt1*/*Angpt2* double knockout mice could be mainly due to the loss of ANGPT2 as demonstrated by other studies (Dellinger et al. 2008; Shen et al. 2014). It was previously thought that angiopoietins might function via their receptor TIE2 in lymphatic ECs. As to be detailed in the next section, the induced deletion of *Tie2* gene at postnatal stages did not affect the lymphatic network formation and maturation (Shen et al. 2014). Furthermore, Schlemm's canal (SC), formed postnatally, is a type of vessel with venous and lymphatic features. ANGPT1 and TIE2 were shown to be indispensable for SC development, while Angpt2 deficiency alone did not affect SC formation (Thomson et al. 2014; Kim et al. 2017). It is possible that ANGPT1 may exert a tissue-specific role in lymphatic system (Petrova and Koh 2018). At the molecular level, it was proposed that the biological consequences of TIE1/TIE2 interaction complex on cell surface depended on the presence of angiopoietin ligands, which may explain the context dependent function of ANGPT2 as an agonist or antagonist in vascular ECs (Seegar et al. 2010). However, as TIE2 is lowly expressed by lymphatic ECs, it is not known whether such TIE1/TIE2 complexes exist on LEC surface and have a role in lymphatic growth and maintenance.

TIE Receptors in Lymphatic Network Formation

TIE1 as a Critical Regulator of Collecting Lymphatic Vessels

TIE1 has high homology to TIE2, and lymphatic endothelial cells co-express TIE1 with PROX1 (Qu et al. 2010). High expression of TIE1 was detected in valve lymphatic ECs (Iljin et al. 2002; Shen et al. 2014). Mice null for *Tie1* exhibited edema and hemorrhage due to abnormal blood and lymphatic vascular development (Puri et al. 1995; Sato et al. 1995; Qu et al. 2010; Shen et al. 2014). Specifically, TIE1 deficiency was shown to result in abnormal lymphangiogenesis during embryogenesis (D'Amico et al. 2010; Qu et al. 2010). The primary lymphatic network became disorganized with a significant increase in the number of abnormal lymphatic connections (Shen et al. 2014). Furthermore, TIE1 deficiency led to the failure of lymphatic remodeling to form collecting vessels during embryogenesis (Fig. 2b) (Shen et al. 2014; Qu et al. 2015). The postnatal deletion of *Tie1* also disrupted lymphatic network formation with a significant decrease of intraluminal valves, suggesting an important role of TIE1 in lymphatic maturation and maintenance (Shen et al. 2014). It is worth pointing out that *Tie1* mutant model ($Tie1^{\triangle ICD/\triangle ICD}$) (Shen et al. 2014) is different from those by D'Amico et al. (2010) and Qu et al. (2010). The specific difference in genetic targeting between the models was detailed in the original articles. It was originally

aimed to generate a mutant mouse model expressing the truncated TIE1 lacking the intracellular domain (TIE1$^{\Delta ICD}$) for the characterization of TIE1 tyrosine kinase in vascular development. Unfortunately, the expression level of TIE1$^{\Delta ICD}$ was low in *Tie1*$^{\Delta ICD/\Delta ICD}$ mice compared with that of wildtype *Tie1* allele, which may be due to the nonsense-mediated mRNA decay (Amrani et al. 2006). However, it is possible that TIE1$^{\Delta ICD}$, in spite of its low expression, retains some functions of TIE1. This may account for the discrepancy, such as lymph sac formation, between the *Tie1*$^{\Delta ICD/\Delta ICD}$ mutants (Shen et al. 2014) and other genetic models targeting *Tie1* gene (D'Amico et al. 2010; Qu et al. 2010).

TIE2 in Lymphatic Versus Blood Vessel Formation

TIE2 (also named TEK) is expressed by endothelial cells and several other cell types and mediates a crucial pathway in vascular formation and maturation (De Palma et al. 2005; Augustin et al. 2009; Shen et al. 2014; Teichert et al. 2017). Angiopoietins are the ligands of TIE receptors, with ANGPT1 expressed by vascular mural cells and platelets while ANGPT2 mainly from endothelial cells (Davis et al. 1996; Li et al. 2001; Fiedler et al. 2004). TIE2 is activated by ANGPT1 with a tetrameric or higher order of multimeric structure (Cho et al. 2004). ANGPT1-TIE2 pathway-mediated signals are required for blood vascular endothelial cell (BEC) survival, migration, and the establishment of vascular wall integrity. Although mice deficient of TIE2 showed embryonic lethality with defective cardiovascular development (Dumont et al. 1994; Sato et al. 1995), the underlying mechanism was not defined. It has been shown recently that *Tie2* deletion induced by gene targeting leads to defective vein formation and maintenance during embryogenesis and the postnatal development. Further biochemical analysis revealed that TIE2 participated in the specification of venous EC identity via AKT-mediated regulation of COUP-TFII protein stability (Chu et al. 2016). Consistently, *Angpt1* deficiency produced similar vascular defects as observed in *Tie2* null mice (Suri et al. 1996). It was revealed that myocardial-specific *Angpt1* deletion disrupted the coronary vein formation and atrial chamber morphogenesis (Arita et al. 2014; Kim et al. 2018). The requirement of ANGPT1 in vascular development is time-dependent as *Angpt1* deletion at E13.5 or later did not produce any obvious vascular defects (Jeansson et al. 2011).

In the lymphatic system, TIE2 expression in lymphatic ECs was much lower compared with that in blood vascular ECs (Shen et al. 2014). This was also confirmed by *Tie2-GFP* transgenic mice, where no GFP positive lymphatic vessels were detected in ear skin examined (Dellinger et al. 2008). The expression of TIE2 in lymphatic vessels was suppressed in lymphatic ECs with high expression of PROX1 (Petrova et al. 2002; Kim et al. 2010). As *Tie2* null or *Angpt1* deficient mice died before the emergence of lymphatic vessels during embryogenesis, conditional gene knockout models targeting TIE pathway were employed for further studies. It was found that induced deletion of *Tie2* in neonate mice did not affect lymphatic growth (Shen et al. 2014). However, abnormal dilation of lymphatic vessels was observed when *Tie2* deletion was induced at earlier stages of embryogenesis (Fig. 2b) (Chu et al. 2016; Souma et al. 2018). As mutant mice with Tie2 insufficiency had abnormal blood vascular development with hemorrhage and edema (Chu et al. 2016), it is possible that the lymphatic defects may be secondary to the increase of blood vascular leakage. Further studies are needed to characterize the role and underlying mechanism of TIE2 in lymphatic development. In addition, lymphatic defects resulting from inactivating mutations have been reported with several factors including VEGFR3, GATA2, and FOXC2 (Fang et al. 2000; Karkkainen et al. 2000; Petrova et al. 2004; Kazenwadel et al. 2012; Brouillard et al. 2014). However, there is still no evidence linking *Tie2* gene mutation to any lymphatic malformation, although a number of activating mutations have been identified with *Tie2* gene in human patients with blood vascular abnormalities including cutaneomucosal venous malformations and ventricular septal defects (Vikkula et al. 1996; Wouters et al. 2010).

Regulation of Lymphatic Remodeling and Maturation

Lymphatic Endothelial Cell Junctions in Initial and Collecting Vessels

During lymphatic development in mammals, a primitive lymphatic plexus is first formed with a homogeneous tubular structure. Subsequent remodeling leads to the formation of a functionally specialized vascular network containing initial and collecting lymphatic vessels. Both types of lymphatic vessels are lined by a single layer of lymphatic ECs. The major structural differences lie in the lymphatic endothelial cell-cell junctions between them, in addition to the differential investment with basement membrane, mural cell coverage, as well as the existence of intraluminal valves (Tammela and Alitalo 2010; Schulte-Merker et al. 2011; Yang and Oliver 2014). By immunostaining for an adherens junction molecule, VE-Cadherin, it was found that endothelial cells of mature initial lymphatic vessels were joined by discontinuous button-like junctions while collecting lymphatic vessels contained continuous zipper-like junctions (Baluk et al. 2007). Interestingly, initial lymphatic ECs of primitive lymphatic plexus were first joined by continuous zipper-like junctions, which were transformed into button-like junctions at later stages of embryonic development and postnatally (Yao et al. 2012). Although genetic studies have revealed the essential requirement of several genes in the process of lymphatic remodeling and maturation, mechanisms underlying the establishment of distinct lymphatic vessel identity are still incompletely understood.

It has been shown that *Angpt2* gene deletion or ANGPT2 blockage by neutralizing antibody disrupted the button-like junction formation in initial lymphatic vessels due to the suppression of VE-Cadherin phosphorylation at the tyrosine residue 685 (Zheng et al. 2014b). Disorganization of primary lymphatic network was also observed in *Tie1* mutant mice at both embryonic and postnatal stages (Shen et al. 2014). In blood vascular endothelial cells, TIE1 has been shown to associate with trans-endothelial complexes including TIE2 and VE-PTP, which support endothelial junction integrity by associating with VE-cadherin, a key component in adherens junctions (AJs) (Saharinen et al. 2008; Frye et al. 2015). In addition, several integrins have been shown to interact with both TIE receptors and angiopoietins (Cascone et al. 2005; Felcht et al. 2012; Lee et al. 2013), which may coordinate their effects in lymphatic network formation and remodeling. It has also been shown recently that CELSR1, a planar cell polarity protein, suppressed the stabilization of lymphatic endothelial AJs by delaying VE-Cadherin recruitment during the rearrangement of valve forming lymphatic endothelial cells (Tatin et al. 2013). Furthermore, it has been reported recently that the increased VEGFA-VEGFR2 signaling, in the absence of NRP1 and VEGFR1, induced lacteal junction zippering and disrupted chylomicron absorption (Zhang et al. 2018). Further studies are required for elucidating whether there is any effect secondary to the increased blood vascular permeability resulting from excess VEGFA bioavailability after VEGFR1 deficiency. This could be answered by employing the genetic mouse models with specific *Vegfr2* gene knockout in lymphatic endothelial cells, in combination with *Vegfr1* gene deletion. So far, the available information on this topic is still fragmented, and a system approach is required to explore how the above-mentioned factors interact with each other in this process.

Lymphatic Valve Morphogenesis

Valve morphogenesis occurs in collecting lymphatic vessels, veins, and heart, which ensures the unidirectional fluid flow (Bazigou and Makinen 2013). Interestingly, some key factors identified in lymphatic valves are also expressed by venous valve endothelial cells (Bazigou et al. 2011), suggesting a similar regulatory mechanism underlying vascular valvulogenesis. Lymphatic valves are semilunar structures with its leaflet composed of a connective tissue core invested by lymphatic ECs on both sides and are positioned close to vessel bifurcations (Zhou et al. 2010). The process of valve morphogenesis involves extracellular matrix organization including fibronectin fibril assembly mediated via the interaction of

integrin-α9 (ITGA9) and Fibronectin-EIIIA (FN-EIIIA) (Bazigou et al. 2009). Valve-associated endothelial cells are from vessel wall via the process of cell rearrangement including lymphatic EC elongation, reorientation, and migration (Tatin et al. 2013). Valve lymphatic ECs express higher levels of PROX1, FOXC2, ITGA9, TIE1, and cell junction molecules such as connexins (Petrova et al. 2004; Kanady et al. 2011; Sabine et al. 2012; Shen et al. 2014). Genetic studies have revealed that valve morphogenesis is disrupted in mutant mice targeting the following genes, including *Tie1* or *Angpt2* (Dellinger et al. 2008; Shen et al. 2014; Qu et al. 2015), *Foxc2* (Petrova et al. 2004), *Efnb2* (Makinen et al. 2005), *Cx37* (Kanady et al. 2011; Sabine et al. 2012), *Itga9* and *Fn-EIIIA* (Bazigou et al. 2009), and *Akt1* (Zhou et al. 2010). It remains to be clarified whether the defects with valvulogenesis are primary or secondary to the failure of lymphatic remodeling to form collecting vessels. Conditional knockout models in combination with valve LEC expressing Cre transgenic mice, such as *Nfatc1-Cre* (Qu et al. 2015), are needed to better elucidate their specific roles in valve development and maintenance. In addition, it is still incompletely understood how these factors coordinate to control the process of lymphatic valve morphogenesis. It has been found recently that GATA2, a zinc finger transcription factor, was shown to regulate the expression of factors involved in lymphatic maturation, including PROX1, FOXC2 and NFATC1, ITGA9, and ANGPT2 (Kazenwadel et al. 2012, 2015). BMP9, acting via ALK-1, could also induce several genes involved in valve formation including FOXC2, CX37, Ephrin-B2 (EFNB2), and NRP1, but suppresses LYVE-1 expression (Levet et al. 2013). The findings suggest a synergistic effect of the above-mentioned factors in different aspects during lymphatic development.

SMC Coverage with Collecting Lymphatics

Besides the valve morphogenesis during the process of lymphatic remodeling and maturation, another important event is the formation of a continuous basement membrane and SMC coverage with the collecting vessel wall. However, valve regions of collecting lymphatics are free of mural cells so that intraluminal valves could open and close freely during the SMC-mediated contraction to move lymph forward. There is also no mural cell investment with initial lymphatic vessels lined by a single layer of lymphatic ECs, where overlapping endothelial flaps function as primary valves for fluid draining.

Several factors have been found to participate in the regulation of SMC investment with lymphatic vessels, including ANGPT2, TIE1, FOXC2, EFNB2, or SEMA3a. ANGPT1 is known to regulate EC-mural cell interaction in the process of blood vessel maturation while ANGPT2 blocks this event to allow vessel sprouting during angiogenesis (Zhang et al. 2003; Hammes et al. 2004; Feng et al. 2007). Deletion of *Angpt2* leads to the abnormal SMC coverage of lymphatic capillaries (Gale et al. 2002; Shimoda et al. 2007; Dellinger et al. 2008; Shen et al. 2014), suggesting that ANGPT2 plays a similar role in lymphatic development to create a mural-cell free lymphatic vessels. *Tie1* deficient mice also showed similar defects with mural cell coverage with lymphatic capillaries (Qu et al. 2015). There was an increased expression of endoglin in capillary lymphatic vessels of *Tie1* null mice, which may account for the abnormal recruitment SMCs (Li et al. 1999; Qu et al. 2015). Increase of SMC coverage with lymphatics was detected in *Foxc2* deficient mice (Petrova et al. 2004), and in *Efnb2* mutant mice lacking its C-terminal PDZ interaction site (Makinen et al. 2005). SMC coverage at lymphatic valve region was reported in *Sema3a* null mice or mice treated with neutralizing antibodies blocking SEMA3A binding to NRP1 (Bouvree et al. 2012; Jurisic et al. 2012). It seems that lymphatic ECs in valve regions are able to generate signals to exert an inhibitory role in mural cell recruitment. FOXC2 and NFATC1 could cooperate in the transcriptional control of several genes involved in vascular development such as downregulation of PDGF-B. This may account for the lack of mural cell recruitment in certain lymphatic regions (Petrova et al. 2004; Norrmen et al. 2009).

Interestingly, FOXC2 has been shown to regulate *Angpt2* expression by direct activation of its promoter (Xue et al. 2008). On the other hand, Reelin, an ECM glycoprotein secreted by lymphatic ECs, might mediate SMC-LEC interaction during lymphatic maturation. It was reported that reelin deficiency led to the reduction of SMC recruitment with dermal collecting lymphatic vessels (Lutter et al. 2012).

ANGPT-TIE Pathway in the Modulation of Tumor-Associated Lymphangiogenic Microenvironment

Angiopoietins in Tumor Lymphangiogenesis and Lymphatic Metastasis

Consistent with the observation made in developmental lymphangiogenesis, intratumor lymphatic vessel growth occurs after tumor angiogenesis (He et al. 2005). Tumor also actively remodel the preexisting lymphatic network, including lymphatic sprouting and vessel dilation, in adjacent tissues to facilitate its dissemination and the establishment of metastatic foci in lymph nodes and other organs (Fig. 3a, b). The molecular regulators identified in development are also essentially required for tumor-associated lymphangiogenesis, including VEGFR3, angiopoietins, and TIE receptors (Alitalo et al. 2005; Augustin et al. 2009; Saharinen et al. 2017). In animal tumor studies, lymphatic metastasis could be efficiently suppressed by blocking VEGFR3 and TIE signaling pathways. This has been demonstrated by using soluble receptors or peptide-Fc fusion protein for ligand-trapping (Karpanen et al. 2001; He et al. 2002; Krishnan et al. 2003; Karlan et al. 2012; Atkins et al. 2015), receptor activating and/or blocking antibodies (Roberts et al. 2006; Caunt et al. 2008; Tammela et al. 2008; Park et al. 2016), and small molecules tyrosine kinase inhibitors (Demetri et al. 2013; Garcia-Manero et al. 2015; Smith et al. 2015; Saharinen et al. 2017). Therapeutic targeting on angiopoietins and their receptors has been nicely reviewed by Dr. Kiss and Dr. Saharinen in this series.

Angiopoietins are expressed by tumor and tumor-associated stromal cells. In addition to its secretion from vascular mural cells and platelets, ANGPT1 expression was detected in tumor cells (Stratmann et al. 1998; Augustin et al. 2009; Holopainen et al. 2009). ANGPT1 could compensate for the loss of ANGPT2 in lymphatic development (Gale et al. 2002), suggesting that its function in lymphatic ECs is comparable to that of ANGPT2 when expressed in the proper environment. Transgenic expression of both ANGPT1 and ANGPT2 in pancreatic β cells of Rip1Tag2 mice showed an increase of peritumoral lymphangiogenesis (Fagiani et al. 2011). Consistently, ANGPT1 delivered via an adenoviral vector was shown to increase the rate of lymph node metastasis (Holopainen et al. 2009). The metastasis enhancing effect of ANGPT1 was abolished by the administration of soluble TIE2. Surprisingly, tumor-associated lymphangiogenesis was not inhibited by the soluble TIE2 (Holopainen et al. 2009). This is consistent with the observation that TIE2 is lowly expressed by lymphatic ECs and the postnatal deletion of *Tie2* did not affect the lymphatic vessel formation and maintenance (Shen et al. 2014). It is likely that the soluble TIE2Ig trapped ANGPT2 and ANGPT1, which were required for the lymphatic remodeling to form a functional network for tumor cell dissemination to the sentinel lymph nodes. Furthermore, TIE1 expression is increased in tumor vasculature and endothelial-specific deletion of *Tie1* led to the suppression of tumor angiogenesis and growth. Tie1 deletion in combination with soluble TIE2 treatment produced an additive inhibition of tumor progression (D'Amico et al. 2014). It is worth noting that although the restoration of tumor vascular perfusion is essential for therapeutic drugs targeting tumor cells, vascular normalization by ANGPT1 treatment could also promote both hematogenous and lympahtic tumor metastasis as described (Holopainen et al. 2009). There is an elegant review article on tumor vessel normalization by Dr. Koh and colleagues in this series.

In contrast, ANGPT2 is expressed in activated endothelial cells in tumors and plays a crucial role together with VEGFA in tumor-associated

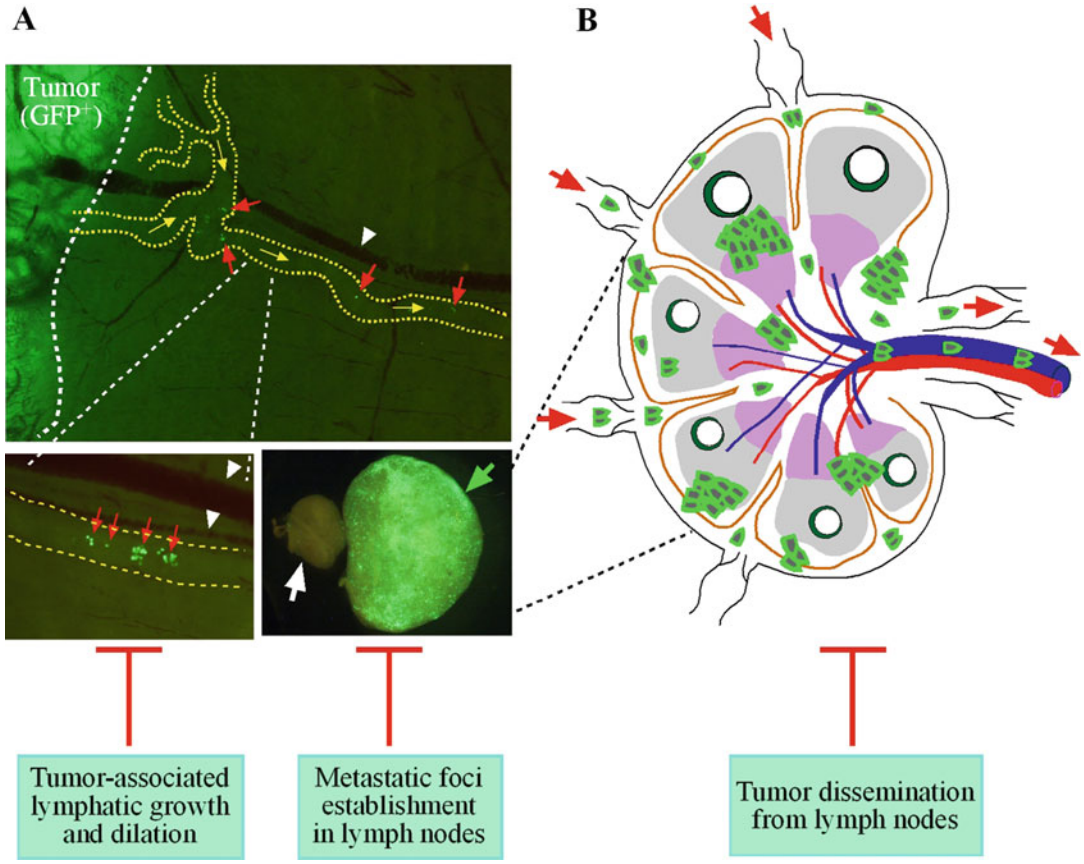

Fig. 3 Lymphatic regulators as targets for blocking lymphatic tumor metastasis. (**a**) Tumor cells (GFP^+) invaded into the lymphatic system are transported via the dilated collecting lymphatic vessels (dotted yellow lines) of adjacent normal tissues to the draining lymph nodes (yellow arrows indicate the flow direction; red arrow indicate GFP^+ tumor cells; and white arrowheads indicate blood vessels). Single tumor cell or tumor emboli (green, GFP^+) were detected in collecting vessels. (Images are modified with permission from Figure 2 and 4 in Cancer Res. 2005;65:4739–46). (**b**). Establishment of metastatic tumor foci in lymph nodes and schematic illustration of further tumor cell dissemination via efferent lymphatic vessels and blood vessels to distant organs. Candidate drugs targeting the key signaling pathways including ANGPTs and TIE receptors are in clinical development, including peptide-Fc fusion protein for ligand-trapping, blocking antibodies and small-molecule tyrosine kinase inhibitors. Green arrow points to the axillary lymph node with GFP^+ tumor cells, and white arrow to the contralateral axillary lymph node without tumor metastasis

vascular growth and metastasis (Holash et al. 1999; Oliner et al. 2004; Augustin et al. 2009). VEGFA was also shown to increase the endothelial ANGPT2 expression via the calcineurin and nuclear factor of activated T cells (NFAT) pathway. ANGPT2 upregulation was implicated in the preparation of premetastatic niche to facilitate the establishment of tumor metastasis (Minami et al. 2013). Circulating ANGPT2 levels was shown to increase in patients with pancreatic cancer, which correlated with lymph node metastasis (Schulz et al. 2011). ANGPT2 overexpression promoted tumor lymphangiogenesis and lymph node metastasis in mice with the subcutaneous pancreatic and lung tumor xenografts (Schulz et al. 2011; Holopainen et al. 2012). *Angpt2* deficiency was shown to suppress tumor angiogenesis at early stages of tumor progression and increased mural cell coverage with blood vessels in mouse models (Nasarre et al. 2009). Consistently, ANGPT2-blocking antibodies suppressed tumor-associated lymphangiogenesis and enhanced the integrity of

endothelial cell-cell junction (Holopainen et al. 2012). Furthermore, ANGPT2 was shown to promote glioma cell invasion (Hu et al. 2003, 2006) and breast cancer metastasis by upregulation and activation of matrix metalloprotease 2 (MMP-2) (Imanishi et al. 2007, 2011). The effect is mediated via α5β1 integrin pathway but independent of TIE-2 signaling (Imanishi et al. 2007). Similar mechanism may also account for the role of ANGPT2 in lymphatic formation as TIE2 expression is low in lymphatic ECs.

Lymphatic Regulator-Mediated Modulation of Tumor Immune Response

There is an active interaction between lymphatic ECs and immune cells during tumor progression. On one hand, tumor-infiltrating leukocytes modulate the tumor vascular network by stimulating angiogenesis and lymphangiogenesis, and create a protumor inflammatory microenvironment (Mantovani et al. 2008). In addition to neutrophils and tumor-specific T cells, mononuclear phagocytotic lineage, comprising of tumor-associated macrophages, dendritic cells, and monocytes, constitutes the major component of infiltrating leukocytes (Pollard 2004). Macrophages are the major source of lymphangiogenic factors such as VEGF-C (Kerjaschki 2005; Condeelis and Pollard 2006; Kataru et al. 2009), and VEGF-C expression was induced by TNFα via NF-κB pathway (Ristimaki et al. 1998; Baluk et al. 2009). Blockage of the macrophage recruitment reduced lymph node metastasis by suppressing VEGF-C expression in tumor (Fischer et al. 2007; Iwata et al. 2007). In addition to the intratumoral lymphangiogenesis, active lymphangiogenesis was detected in tumor draining lymph nodes before the arrival of metastatic tumor cells (Hirakawa et al. 2005; Van den Eynden et al. 2007; Rinderknecht and Detmar 2008; Ruddell et al. 2008). Besides the lymphangiogenic factors transported with lymph from tumor, immune cells in lymph nodes also actively participate in the regulation of lymph node-associated lymphatic vessel growth. Follicular B cells could produce lymphangiogenic factors such as VEGF-A to stimulate lymphangiogenesis in lymph nodes (Angeli et al. 2006; Shrestha et al. 2010), while T cells have been found to modulate lymphatic growth in a negative manner via secreting IFN-γ (Kataru et al. 2011).

On the other hand, the tumor-associated lymphatic system regulates immune responses by delivering antigen presenting cells (APCs) and lymph containing soluble antigens from tumor to the draining lymph nodes. After reaching the subcapsular sinus of lymph nodes, small lymph-borne antigens are delivered directly to B cell follicles and paracortical T cell zones via the reticular conduit system while large antigens were taken up and transported by macrophages (Roozendaal et al. 2009). Interestingly, the sinus lymphatic endothelium acts as a physical sieve depending on diaphragms formed by PLVAP (plasmalemma vesicle-associated protein) fibrils in transendothelial channels (Rantakari et al. 2015). Lymphatic ECs also actively participate in the regulation of immune cell entry and emigration from lymphatic vessels via the expression of chemokines and adhesion molecules (Forster et al. 2008; Card et al. 2014). VEGF-C was shown to upregulate chemokine expression in lymphatic ECs (e.g., CCL21), which are immobilized by glycosaminoglycans (e.g., podoplanin) on the luminal surface of lymphatic ECs to guide the migration of immune cells expressing CCR7 (Forster et al. 2008; Alitalo 2011). Lymphatic semaphorin-3A was shown to promote actomyosin contraction during the DC entry into lymphatic vessels (Takamatsu et al. 2010), and lymphatic ECs lining the ceiling of subcapsular sinus also expressed CCRL1, a scavenger receptor for CCL21/CCL19, to create a chemokine gradient for DC trafficking into the parenchyma (Ulvmar et al. 2014). Furthermore, it is known that tumor-associated macrophages have poor antigen-presenting capability and express immunoinhibitory factors to suppress T cell proliferation in comparison with macrophages derived from normal tissues (Forster et al. 2008). However, there exist distinct populations of dendritic cells (DCs) including the resident and migratory DCs in lymph nodes and the periphery tissues. It has

been shown that a subset of dendritic cells ($CD103^+/CD141^+$) expressing CCR7 in melanoma were critical for trafficking tumor antigens via afferent lymphatics to prime $CD8^+$ T cells in the draining lymph nodes. Increase of T cell infiltration in tumor showed survival benefits for patients (Roberts et al. 2016). Consistently, lymphatic absence or dysfunction was shown to impair antitumor immune responses (Kimura et al. 2015; Lund et al. 2016). Specifically, xenograft melanoma implanted intradermally displayed a markedly reduced leukocyte infiltration and failed to mount an antitumor immunity in response to dermal vaccine delivery in a transgenic mouse model lacking skin lymphatics. The finding was further verified in metastatic human cutaneous melanoma samples where tumor immune cell infiltrates correlated well with the expression level of lymphatic markers (Lund et al. 2016).

In addition to the involvement of lymphatic system in immune defense, it also promotes self-tolerance (Card et al. 2014). DCs constantly migrate via afferent lymphatic vessels to the draining lymph nodes, carrying self and foreign antigens from the periphery tissues (Forster et al. 2008). This is important for tolerance induction towards environmental antigens and may also be employed by tumor to evade the immune surveillance. VEGF-C was shown to promote immune tolerance in murine melanoma, and lymphatic ECs are involved in maintaining peripheral immune tolerance by inducing CD8 T-cell deletion (Cohen et al. 2010; Lund et al. 2012). As innate immune cells including macrophages and DCs express VEGFR-3, it is also likely that VEGF-C may have a direct role in the restriction of their inflammatory activation (D'Alessio et al. 2014; Zhang et al. 2014). Interestingly, in spite of the immunosuppressive tumor microenvironment, it was also reported that VEGF-C induced lymphangiogenesis could enhance the antitumor immunotherapy resulting from the increased naïve T cell infiltration dependent on CCL21 in the antigen-expressing melanoma (Fankhauser et al. 2017). Furthermore, lymphatic ECs in lymph nodes were found to function as tolerogenic APCs by expressing major histocompatibility complex (MHC) class I and II molecules as well as immunoregulatory factors (Card et al. 2014). Lymphatic ECs rely on DCs to present peripheral tissue antigens to CD4 T cells to induce anergy (Rouhani et al. 2015). Expression of programmed death-ligand 1 (PD-L1) by lymphatic ECs transmitted an inhibitory signal to suppress the proliferation of antigen-specific T cells via its receptor PD-1 (Tewalt et al. 2012).

Interestingly, ANGPT-TIE pathway plays an important role in the regulation of tumor immune microenvironment. There is a subset of TIE2-expressing macrophages (TEMs) identified in tumor, which interact with vascular ECs to promote tumor progression dependent on ANGPT2-TIE2 pathway (Mazzieri et al. 2011; Matsubara et al. 2013). Overexpression of ANGPT2 promoted tumor-infiltrating macrophages and neutrophils while ANGPT1 suppressed this event (Fagiani et al. 2011). Consistently, myeloid cell-specific deletion of *Tie2* or *Angpt2* deficiency, or the administration of ANGPT2 blocking antibodies, led to the suppression of tumor growth and relapse after chemotherapy or anti-angiogenic therapy in animal tumor studies (Nasarre et al. 2009; Brown et al. 2010; Mazzieri et al. 2011; Chen et al. 2016). Endothelial-derived ANGPT2 was elevated in mice with the bevacizumab-resistant murine glioblastoma model. The combined inhibition of VEGF and ANGPT2 was shown to extend survival of tumor-bearing mice, accompanied by the favorably altered immune microenvironment, including the suppression of M2-polarized macrophages as well as an increase of intratumoral T cell infiltration (Scholz et al. 2016). ANGPT2 also stimulated IL-10 release by TEMs from tumor to suppress T cell proliferation while promoting regulatory T cell (T_{reg}) expansion (Coffelt et al. 2011). Inhibition of ANGPT2 with simultaneous TIE2 activation was shown to reduce T_{reg} cells in tumor (Park et al. 2016). Modulation of T_{reg} cell-mediated immune suppression by lymphatic EC-derived cytokines such as angiopoietins could be another important mechanism contributing to the immune tolerance to tumor-derived antigens.

Summary

Tumor cells disseminate to sentinel lymph nodes via intratumoral lymphatic vessels connecting to the lymphatic network in the adjacent normal tissues. It was frequently observed that there was a dramatic increase of lymphatic vessel diameter at peritumoral areas to facilitate tumor dissemination as single cell or emboli. Lymph node metastasis is an early event in solid tumors and analysis of sentinel lymph node biopsy from cancer patients is routinely practiced for prognostic evaluation in clinic. One long-lasting question is that whether lymph node metastasis contributes to systemic tumor spread to other organs. Two recent articles provided evidence that metastatic tumor cells could spread further via blood vessels from lymph nodes (Brown et al. 2018; Pereira et al. 2018). As anti-lymphangiogenesis treatment had limited effect on tumor progression after dissemination, it is necessary to make early detection of lymphangiogenic event in tumor and/or the draining lymph nodes before tumor cells metastasize. On the other hand, insufficient lymphatic drainage may account for a low level of immune cell infiltration in primary tumors and poor response to immunotherapy. It seems contradictory to enhance the efficacy of immunotherapy by improving the vascular perfusion including lymphatic draining function and to simultaneously suppress the metastatic tumor spread via the tumor-associated vascular network. Further studies are needed to develop combined therapies to fine-tune the interaction of vascular EC-immune cells to block tumor progression.

Cross-References

- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Benefits and Pitfalls of Tumor Vessel Normalization

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Significance and Molecular Regulation of Lymphangiogenesis in Cancer

Mihaela Skobe and Bronislaw Pytowski

Contents

Introduction 158

Normal Lymphatic Structure, Function, and Molecular Regulation 158
Functions of the Lymphatic Vasculature 158
Structural Features of the Lymphatic System 159
Molecular Regulation of Lymphangiogenesis: VEGF-C and VEGF-D 160
Embryonic Lymphangiogenesis 162
Other Regulators of Lymphangiogenesis 162

Tumor Lymphangiogenesis and Lymphatic Metastasis 163
Tumor Lymphangiogenesis 163
Lymphogenous and Hematogenous Pathways of Tumor Metastasis 164
Mechanisms of Lymph Node Metastasis 165
Lymphangiogenesis in the Lymph Nodes 167
Lymph Node Metastasis Is an Important Prognostic Indicator 168
Historical Perspective on Lymph Node Metastasis 168
Lymph Node Metastasis as a Source of Distant Metastases 169
Lymphangiogenesis in Target Organs for Metastasis 170

Clinical Implications of Lymphangiogenesis 171
Prognostic Significance of Lymphangiogenesis in Human Tumors 171
Therapeutic Targeting of Lymphangiogenesis 172

Conclusions 173

Cross-References 173

References 173

Abstract

Lymphatic dissemination of tumor cells involves invasion into tumor-associated lymphatic vessels, seeding of metastases in the lymph nodes, and, ultimately, delivery into the blood circulation and to distant organs. Tumor lymphangiogenesis is induced by factors released by tumor or stromal cells, such as macrophages, and facilitates metastasis by

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providing pathways for cancer cell spread. Vascular endothelial growth factors VEGF-C and VEGF-D are the most specific lymphangiogenic factors that mediate signals for lymphatic endothelial cell growth and migration by binding to and activating VEGFR-3 receptors. Extensive preclinical data in mouse tumor models with specific inhibitors of lymphangiogenic signaling pathways provided the impetus for clinical trials of such agents in patients. In clinical practice, the presence of tumor cells in sentinel lymph nodes is an adverse prognostic factor for patients with solid tumors and constitutes a major consideration in tumor staging. Lymphovascular invasion, lymphatic vessel densities, and the expression of lymphangiogenic factors are also strongly correlated with poor prognosis. Although lymphatic and blood vascular endothelium share many molecular features, they are structurally and functionally distinct and play very different roles in tumors. Here, we discuss the distinct functions and significance of the lymphatic vascular system in cancer.

Keywords
Tumor lymphangiogenesis · VEGF-C · VEGF-D · VEGFR-3 · Lymphatic vessels · Lymph node · Metastasis

Introduction

This chapter discusses tumor lymphangiogenesis, a process by which solid tumors induce the formation of new lymphatic vessels into peritumoral and tumor tissue from pre-existing lymphatic vessels. Tumor lymphatic vessels are involved in draining the tumor interstitial space of fluid, while also providing conduits for the traffic of immune cells from the tumor to draining lymph nodes. Lymphangiogenesis has also been implicated in tumor progression, primarily by facilitating the dissemination of tumor cells. As few nonspecialists are familiar with the unique biology of the lymphatic system, the beginning of this chapter provides a general introduction to its structure, function, and development as a foundation for the subsequent discussion of tumor lymphangiogenesis.

Normal Lymphatic Structure, Function, and Molecular Regulation

Functions of the Lymphatic Vasculature

Lymphatic vessels carry out several important functions, which broadly fall into two different categories: transport and regulatory functions. Lymphatics transport fluid, macromolecules, and immune cells from tissues back into the blood circulation. The endothelial lining of blood vessels must provide sufficient barrier functions to prevent the significant loss of plasma into tissues. However, blood vessel walls, particularly in capillaries, must also maintain sufficient plasticity to permit an increase in permeability in response to injury or infection, during regeneration of damaged vessels and angiogenesis. Furthermore, the endothelial lining of blood capillary walls must be sufficiently permeant to allow the bidirectional transport of gases, nutrients, and waste products. These opposing requirements necessitate a compromise between the barrier and transport functions of blood endothelium. The hydrostatic fluid pressure of blood varies depending on the type of blood vessel, but, even at the capillary level, significantly exceeds that of tissue interstitial fluid. As a consequence, the circulation in all vertebrates must be able to accommodate a degree of continuous, low-level leakage of plasma and tissue-derived proteins that result in the formation of interstitial fluid (Moore and Bertram 2018; Wiig and Swartz 2012). Lymphatic vessels mediate the return of excess interstitial fluid into the blood in the form of lymph and thus play a central role in maintaining tissue fluid and pressure homeostasis. Lymphatics also perform the important function of returning solutes and macromolecules that have leaked into the tissues back into the blood circulation. In humans, 8–12 L of protein-rich fluid that would otherwise accumulate in tissues is transported by the lymphatic

system daily (Scallan et al. 2016; Wiig and Swartz 2012). In addition, a unique system of lymphatic capillaries called the lacteals plays a vital role in the absorption and transport of dietary lipids. Triglycerides, absorbed into the lumen of the small intestine and packaged into chylomicrons, are transported by lacteals in the form of a substance called chyle to lymph nodes in the mesentery, and eventually into the blood circulation (Dixon 2010).

Another key role of the lymphatic vasculature is to transport soluble antigens and antigen-presenting dendritic cells from the tissue periphery to secondary lymphoid organs, where they interact with naïve T and B lymphocytes to allow the initiation of adaptive immune responses. Distinct T-cell subsets also traffic through the lymphatics and lymphatic endothelial cells (LECs) directly interact with T cells and dendritic cells to modulate their function. Furthermore, lymphatic endothelial cells help regulate innate and adaptive immune responses through the expression of cytokines, inhibitory receptors, and adhesion molecules.

While the vital role of blood circulation is apparent even to nonscientists, the importance of efficient lymphatic functioning is only revealed when the system is compromised by genetic errors, infectious agents, trauma, or surgery. Dysfunction of lymphatics in the peripheral tissues and extremities manifests itself as tissue swelling, known as lymphedema (Rockson 2001; Rockson et al. 2019). Lymphedema commonly leads to disability by inducing irreversible tissue fibrosis, chronic inflammation, and susceptibility to infections and represents a significant clinical problem. Dysfunctional lymphatics in internal organs compromise their function, leading to serious, often fatal, medical conditions.

Structural Features of the Lymphatic System

Lymphatic vessels or lymphatic-like structures have been identified in almost all organs, including, most recently, in the brain and eye (Aspelund et al. 2014, 2015; Louveau et al. 2015; Park et al. 2014; Petrova and Koh 2018). Lymphatics possess structural features that are distinct from those of blood vessels (Fig. 1) and exhibit unique characteristics depending on their location along the lymphatic vascular tree. The uptake of interstitial fluid occurs in lymphatic capillaries, which are

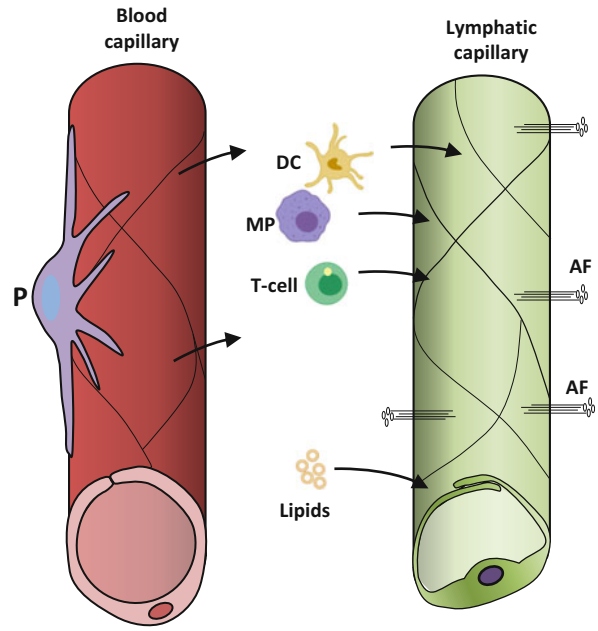

Fig. 1 Lymphatic capillary structure and function. Lymphatic capillaries have thin endothelium, overlapping junctions, irregular-shaped lumen and lack pericytes. Anchoring filaments (AF) connect LECs directly to the interstitial collagens. Lymphatic capillaries are uniquely adapted for the uptake of fluid, macromolecules, lipids, and cells from the interstitium. *DC* dendritic cell, *MP* macrophage, *P* pericyte

blind-ended initial lymphatic vessels typically found in close proximity to blood capillaries. Lymphatic capillaries generally possess a wider and more irregular lumen than blood capillaries, and their endothelium is extremely thin. Diameters of lymphatic capillaries vary depending on the tissue and range from 20 to 300 microns. In contrast to blood capillaries, lymphatic capillaries have an incomplete basement membrane and are not invested by pericytes (Skobe and Detmar 2000). Lymphatic capillaries are also characterized by oak leaf-shaped endothelial cells that partially overlap and form flaps at sites of fluid entry (Leak 1971; Schmid-Schonbein 2003). Endothelial cells of lymphatic capillaries have unique junctions composed of VE-cadherin and tight junction-associated proteins that connect two overlapping cells in a discontinuous pattern. Discontinuous junctions in initial lymphatics are referred to as "buttons" in contrast to conventional, continuous junctions in blood capillaries, i.e., "zippers" (Baluk et al. 2007; Leak 1971).

Transient changes in pressure gradients across lymphatic vessel walls are thought to drive lymph formation (Breslin 2014; Moore and Bertram 2018; Wiig and Swartz 2012). An increase in interstitial fluid pressure causes the overlapping junctions to transiently open, thereby allowing the passage of fluid and particles into the vessel. As fluid enters the lumen, pressure differences across the vessel wall decrease, and the junctions begin to close, preventing retrograde flow back into the interstitium (Ikomi and Schmid-Schonbein 1996; Schmid-Schonbein 1990a). Lymphatic capillary function is critically dependent on its connections to the extracellular matrix. LECs are attached to interstitial collagen by anchoring filaments composed of elastic fibers (Gerli et al. 1990; Leak and Burke 1966), which allow lymphatics to directly sense biomechanical changes in the interstitium (Moore and Bertram 2018; Wiig and Swartz 2012). Lymphatic capillaries are frequently observed with closed or partially open lumina because intralymphatic fluid pressure is generally lower than the interstitial fluid pressure in the surrounding tissue (Aukland and Reed 1993; Schmid-Schonbein 1990b; Wiig and Swartz 2012).

Lymph is transported as a result of intrinsic and extrinsic pumping mechanisms (Moore and Bertram 2018; Scallan et al. 2016). From the initial lymphatics, lymph moves into collecting vessels, which are invested by smooth muscle and actively transport lymph. Intrinsic pumping involves the peristaltic contraction of smooth muscle that propagates along the lymphatic vessel wall, coordinated with the action of bicuspid luminal valves that prevent backflow. The segment of a collecting lymphatic vessel between two intraluminal valves is called a lymphangion. Contraction waves are coordinated over the length of a lymphangion, and lymph is transported in pulses from one lymphangion to the next. The driving force for extrinsic pumping includes the contraction of neighboring skeletal muscles or rhythmical pulsing of the adjacent artery. Together, these forces propel lymph along the coalescing branches of the lymphatic tree and into two great lymphatic ducts, the thoracic and right lymphatic duct, which exhibit an autonomous pumping motion and empty lymph into the blood circulation through the left and right subclavian veins in the neck. Since blood fluid pressure is greater than that of exiting lymph, specialized structures called lympho-venous valves at the lympho-venous junctions prevent the retrograde flow of blood into the ducts (Moore and Bertram 2018; Scallan et al. 2016; Zawieja 2009).

Molecular Regulation of Lymphangiogenesis: VEGF-C and VEGF-D

Physiological lymphangiogenesis, which occurs primarily during embryogenesis and postnatal development, is a tightly controlled process regulated by a number of sequential and cooperative molecular signals. Lymphangiogenesis in adults is largely restricted to wound healing and immune activation. However, lymphangiogenesis is also a major component of pathological processes such as chronic inflammation and cancer. Pathological lymphangiogenesis is mediated by highly perturbed signaling networks, leading

to the formation of lymphatic vessels with compromised organization and functional features.

The principal mediator of lymphangiogenesis is vascular endothelial growth factor C (VEGF-C). VEGF-C is the dominant ligand for the receptor tyrosine kinase (RTK) vascular endothelial growth factor receptor 3 (VEGFR-3), the only RTK whose expression in normal postnatal tissues is largely restricted to the lymphatic endothelium (Jeltsch et al. 1997; Joukov et al. 1996; Kaipainen et al. 1995). LECs can also express VEGFR-2, an RTK that is expressed by activated blood endothelium, and that, upon activation by the ligand VEGF-A, is a critical regulator of blood angiogenesis. The role of VEGFR-2, activated by either VEGF-A or VEGF-C in lymphangiogenesis, has been controversial (Tammela and Alitalo 2010). VEGF-C is expressed in all species possessing lymphatic vessels. The specificity and affinity of the binding of VEGF-C is dependent on tightly regulated proteolytic processing (Joukov et al. 1997). VEGF-C is secreted as a precursor protein in the form of an antiparallel dimer that must be processed in a highly conserved manner at both the N- and C-termini to acquire full function. Pro-peptides at the N- and C-termini are sequentially removed to yield active forms of VEGF-C. Partially processed VEGF-C homodimers are capable of activating VEGFR-3, but not VEGFR-2, and therefore specifically signal for lymphangiogenesis. Full proteolytic processing of VEGF-C enhances its affinity for VEGFR-3 and enables mature VEGF-C to also bind to VEGFR-2. Mature VEGF-C, therefore, has the ability to drive the growth of both lymphatic and blood vessels (Joukov et al. 1997; Sáinz-Jaspeado and Claesson-Welsh 2018; Zheng et al. 2014).

VEGF-D is a closely related ligand, whose processing and consequent receptor specificity parallels that of VEGF-C (Achen and Stacker 2012). The biological role of this cytokine has been difficult to elucidate, as in gene knockout experiments' VEGF-D is dispensable for the development of the lymphatic system (Haiko et al. 2008). The binding of VEGF-C or VEGF-D to VEGFR-3 induces receptor dimerization and leads to the phosphorylation of critical tyrosine residues on the cytoplasmic domain that, in turn, trigger downstream signaling events (Fig. 2). One key downstream event following

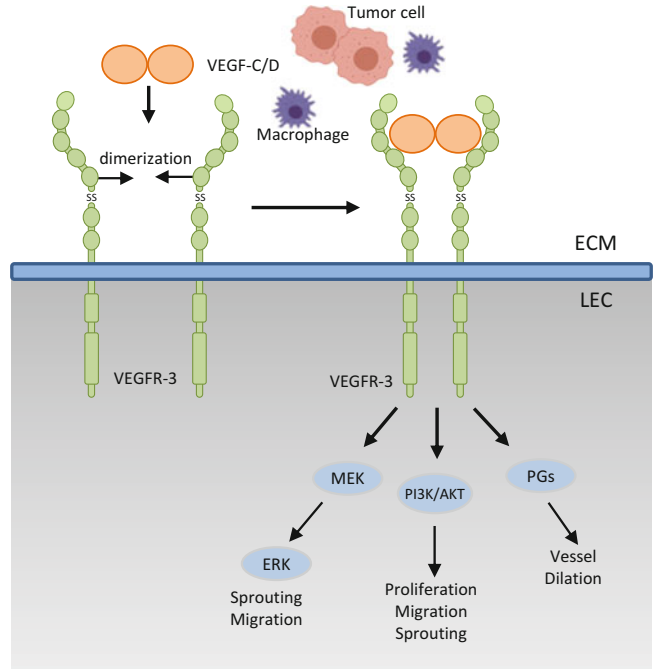

Fig. 2 VEGFR-3 signaling in lymphangiogenesis. VEGF-C and VEGF-D derived from tumor cells or inflammatory cells, mainly macrophages, activate VEGFR-3 and initiate signaling cascade leading to lymphatic endothelial proliferation, migration, and vessel dilation

VEGFR-3 activation is the phosphorylation of the serine/threonine kinases AKT and ERK, which mediate migration, survival, and proliferation of LECs (Davydova et al. 2016; Karaman et al. 2018).

Embryonic Lymphangiogenesis

Lymphatic vasculature develops primarily from veins during embryonic lymphangiogenesis (Makinen et al. 2007; Yang and Oliver 2014). Endothelial cells in the veins of the embryo express large amounts of VEGFR-3, in contrast to adult blood endothelium that does not express this RTK. During embryonic days 9.5–10.5 in mice, or approximately days 45–50 in humans, VEGFR-3-positive endothelial cells (ECs) of the cardinal vein begin to express the lymphatic vessel hyaluronan receptor-1 (LYVE-1), heralding the start of developmental lymphangiogenesis. The process is initiated when the expression of the transcription factor SOX18 is induced in the VEGFR-3/LYVE-1 positive ECs of the cardinal vein. SOX18 induces the expression of the transcription factor Prox1, a critical factor in determining lymphatic endothelial identity. The sprouting of lymphatic capillaries from the cardinal vein is initiated in response to VEGF-C produced by mesenchymal cells (Srinivasan et al. 2007). The crucial role of Prox-1 in this process is evidenced in embryos of Prox1-deficient mice that are not viable and completely lack lymphatic vasculature (Wigle and Oliver 1999). Concomitantly with the appearance of the first lymphatic endothelial precursor cells, VEGFR-3 expression is downregulated in embryonic blood vessels. The final step in developmental lymphangiogenesis is a separation of the blood and lymphatic vascular systems. This process is initiated when podoplanin, a mucin-type transmembrane glycoprotein expressed by newly differentiated LECs, binds to the C-type lectin receptor 2 (CLEC-2) on platelets, leading to platelet aggregation that blocks any remaining lympho-venous connections (Tammela and Alitalo 2010; Welsh et al. 2016). The lymphatic system subsequently undergoes several maturation steps, including the formation of a differentiated network of capillaries and collecting lymphatic vessels containing intraluminal valves and smooth muscle cells (Mauri et al. 2018; Tammela and Alitalo 2010; Ulvmar and Makinen 2016).

Other Regulators of Lymphangiogenesis

While VEGFR-3 signaling is indispensable for lymphangiogenesis, other cytokine/receptor systems also influence this process. Key among them are the angiopoietin (Ang) and fibroblast growth factor (FGF) families of ligands and their cognate receptors (Saharinen et al. 2017a, b; Sáinz-Jaspeado and Claesson-Welsh 2018; Zheng et al. 2014). In humans, the Ang family has three members: Ang1, Ang2, and Ang4. Mice express a related gene, Ang3, in lieu of Ang4. Angiopoietins function by activating a receptor tyrosine kinase denoted Tie2 (Tek) that is principally expressed on endothelial cells of blood and lymphatic vessels. Genetic experiments in mice have elucidated critical and complex roles of the Ang/Tie system in the development and maturation of lymphatic vessels. Blocking Ang2 or Tie2 disrupts the integrity of LECs, inducing leakage of lymphatic vessels. This has been linked to the observation that transmembrane form of Ang2 can bind Tie2 on adjacent endothelial cells and that the formation of this complex is crucial for lymphatic junctional stability. LECs also express two members of the FGF receptor tyrosine kinase family, FGFRs 1 and 3. Activation of these RTKs in LECs induces signaling through PKB/AKT and ERK1/ERK2 pathways that mediate proliferation, migration, and survival. There appears to be considerable redundancy in the pro-lymphangiogenic RTK signaling since the same pathways are triggered in LECs by the activation of the VEGF-C/VEGFR-3 system (Tammela and Alitalo 2010; Zheng et al. 2014). In addition, hepatocyte growth factor (HGF) is a lymphangiogenic factor that exerts its action directly and indirectly (Cao et al. 2006; Gibot et al. 2016; Kajiya et al. 2009). The HGF

receptor c-Met is constitutively expressed by LECs in the skin, where HGF promotes lymphangiogenesis directly by activating c-Met signaling. HGF strongly stimulates LEC proliferation and tubulogenesis, but is less effective in stimulating LEC migration (Gibot et al. 2016). The effects of HGF on lymphangiogenesis may be different in various tissues, since, in the model of corneal inflammation, c-Met is not expressed by LECs and HGF seems to stimulate lymphangiogenesis indirectly (Cao et al. 2006).

Tumor Lymphangiogenesis and Lymphatic Metastasis

Tumor Lymphangiogenesis

VEGF-C and VEGF-D are the two most specific lymphangiogenic factors and play a central role in tumor lymphangiogenesis and metastasis (Karaman and Detmar 2014; Podgrabinska and Skobe 2014; Stacker et al. 2014). VEGF-C and VEGF-D are primarily released by cancer cells, but may also be produced by stromal cells, in particular, by macrophages and fibroblasts. The initial discovery that lymphangiogenesis occurs in tumors was made in 2001, when three groups concurrently reported that the overexpression of VEGF-C or VEGF-D in experimental tumor models leads to intra- and peritumoral lymphangiogenesis and that the induction of lymphangiogenesis by the tumor facilitates metastatic spread (Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001). It is generally assumed that lymphangiogenesis promotes metastasis by facilitating tumor cell access to lymphatic vessels. In addition, VEGF-C and VEGF-D drive the remodeling of collecting lymphatic vessels that lead to the lymph nodes. The enlargement of collecting lymphatics and remodeling of smooth muscle cells result in an increased flow rate, which may promote metastasis by enhancing the delivery of tumor cells to the lymph nodes (Harrell et al. 2007; Hoshida et al. 2006; Karnezis et al. 2012).

Numerous studies using murine tumor models have shown that the inhibition of lymphangiogenesis by the neutralization of either VEGF-C or VEGFR-3 reduces lymph node metastases (Brakenhielm et al. 2007; Burton et al. 2008; Chen et al. 2005; He et al. 2005; Kawakami et al. 2005; Krishnan et al. 2003; Lin et al. 2005; Roberts et al. 2006). Importantly, VEGFR-3 inhibition does not reduce primary tumor growth, indicating that the consequence of tumor lymphangiogenesis is primarily an increase in tumor dissemination. Consistent with these findings, overexpression of VEGF-C or VEGF-D in epithelial cancers promotes metastasis, but does not change primary tumor growth rate. VEGF-C also facilitates metastatic spread to distant sites and, consequently, blocking VEGF-C or VEGFR-3 inhibits distant metastases in the majority of experimental models (Brakenhielm et al. 2007; Burton et al. 2008; Chen et al. 2005; Krishnan et al. 2003; Lin et al. 2005; Podgrabinska and Skobe 2014; Roberts et al. 2006).

Despite similarities in structure and receptor specificity, there are differences in the function of VEGF-C and VEGF-D in tumors that are just beginning to be elucidated (Davydova et al. 2016). For example, VEGF-C promotes the expression of COX-2 in the endothelial cells of collecting lymphatic vessels, whereas VEGF-D does not. COX-2 is an enzyme involved in the biosynthesis of prostaglandins and contributes to the dilation of collecting lymphatic vessels and metastatic spread. Similarly, although VEGFR-2 and VEGFR-3 are both expressed by LECs, the function of VEGFR-2 and VEGFR-3 in tumor metastasis is strikingly different. Studies in mouse models of cancer have demonstrated that while blocking VEGFR-3 significantly inhibits lymph node metastasis, the blocking of VEGFR-2 does not (Roberts et al. 2006).

There are several additional pleiotropic growth factors that mediate tumor lymphangiogenesis, including FGF2, HGF, IL-1, and TNFα. Because these factors bind to various receptors on non-vascular cell types, and are not selective for lymphatic endothelium, it is difficult to discern whether their action on lymphatics is direct or indirect, through the upregulation of VEGF-C. TNFα and IL-1, for example, promote lymphangiogenesis by recruiting inflammatory

cells that secrete VEGF-C and VEGF-D (Kataru et al. 2009; Kim et al. 2014). HGF and FGF2 seem less effective as sole drivers of tumor lymphangiogenesis, but they may exert an impact by cooperating with VEGF-C or VEGF-D. HGF has been shown to exert synergistic and FGF2 additive effects on lymphangiogenesis in the presence of VEGF-C (Cao et al. 2012; Gibot et al. 2016).

Many tumors induce lymphangiogenesis at the tumor periphery and promote the enlargement of the lymphatic vessel lumen (Podgrabinska and Skobe 2014; Sleeman et al. 2009). These enlarged, peritumoral lymphatics are considered a major site of tumor cell entry. Intratumoral lymphangiogenesis is induced in some, but not all, tumor types, and intratumoral lymphatics are typically seen in hot spots rather than uniformly distributed throughout the entire tumor (Fig. 3). While hot spots may be found in various locations within the tumor, there may be large tumor areas completely devoid of lymphatics. In contrast, blood vessels are typically present throughout the tumor, although their densities vary. This difference in the spatial organization of lymphatic and blood vessels in tumors relates to the differences in their function, which is drastically distinct despite the fact that the endothelial biology of these two vascular systems is shared on many levels. Angiogenesis is a requirement for tumors to grow, and therefore blood vessels are found in all tumors. Because lymphatics are not essential for tumor growth, they are not ubiquitously found in tumors. Furthermore, although tumor lymphangiogenesis profoundly increases metastatic spread, it is not required for metastasis as tumor cells can also disseminate using pre-existing lymphatic vessels.

Lymphogenous and Hematogenous Pathways of Tumor Metastasis

Metastasis, the escape of tumor cells from the primary tumor and the seeding of new tumor lesions in distant organs, is the primary cause of death in cancer patients. The metastatic process involves a sequence of key steps that need to be completed for the successful formation of metastases (Fidler 2003; Gupta and Massague 2006; Lambert et al. 2017). Among these steps are the entry and egress of cancer cells to and from the vasculature. Tumor cells may leave the primary site by entering either lymphatic vessels (i.e., lymphogenous spread) or the blood vasculature (i.e., hematogenous spread) (Fig. 4). Hematogenous metastasis is initiated by the intravasation of tumor cells into postcapillary

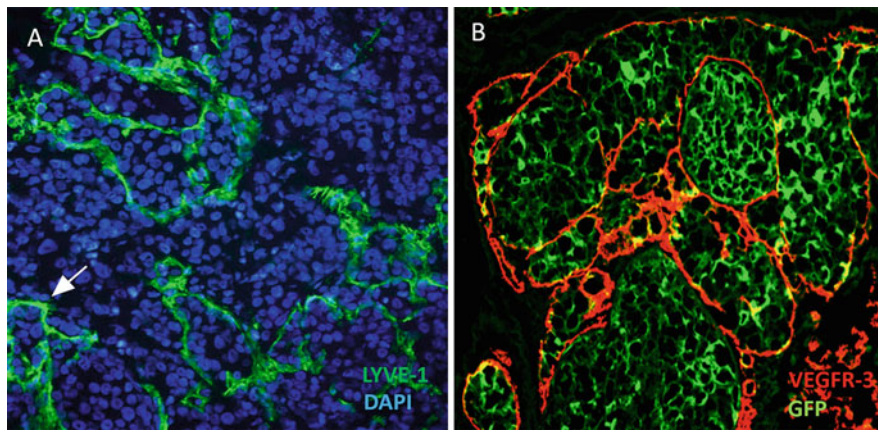

Fig. 3 Lymphangiogenesis in the primary tumor and in pulmonary metastases. (**a**) Immunostaining for lymphatic marker LYVE-1 (green, lymphatics) showing large lymphatics containing tumor cells in VEGF-C expressing primary tumors in a mouse xenograft model. (**b**) Spontaneous pulmonary metastasis from the same tumor. Immunostaining for lymphatic endothelial receptor VEGFR-3 (red, lymphatics) and GFP (green, tumor cell). Note that metastases are present exclusively within the lymphatic vessels. Arrow, lymphatic endothelium

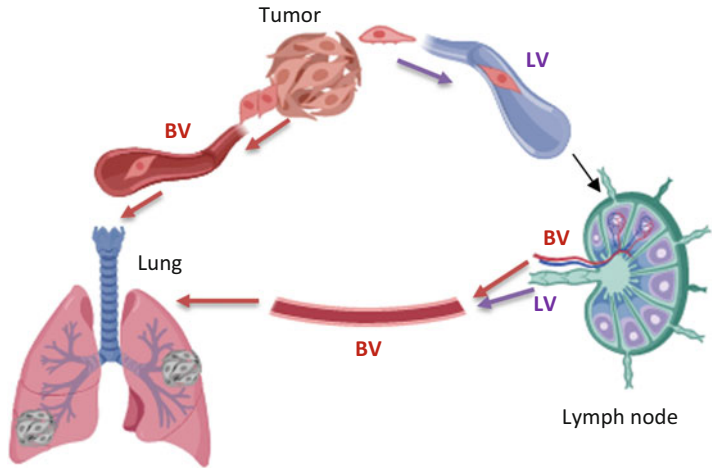

Fig. 4 Lymphatic and hematogenous pathways of metastasis. Tumor cells leave the primary tumor by entering into the lymphatic or blood vasculature. Lymphatic vessels deliver tumor cells first into the regional lymph nodes. Tumor cells can subsequently leave the lymph nodes through the blood or lymphatic vessels. Regardless of the initial path taken, lymphatic or blood, tumor cells ultimately reach distant organ by the blood vessels. *BV* blood vessel, *LV* lymphatic vessel

venules. Tumor cells are then transported via venous blood to a target organ that is a site of distant metastasis. Lymphogenous metastasis begins with the invasion of tumor cells into lymphatic capillaries and their subsequent transport into larger, collecting lymphatic vessels (Podgrabinska and Skobe 2014; Sleeman et al. 2009). Collecting lymphatics deliver the tumor cells into the draining lymph node through several afferent lymphatic vessels. Specifically, they converge onto the lymph node subcapsular sinus, which is lined by lymphatic endothelial cells. Tumor cells typically arrest and proliferate in the lymph node and may further disseminate by either lymph or venous blood. A single efferent lymphatic vessel transports the cells into the next lymph node in the regional cluster. The efferent lymph containing tumor cells eventually reaches great lymphatic ducts that deliver lymph contents into the venous blood. Therefore, cancer cells that first enter into the lymphatic vasculature at the primary tumor site are eventually delivered into the blood circulation and reach distant organs. It is important to recognize that circulating tumor cells detected in the blood may have originated from tumor cells that initially left the primary tumor by either the lymph or the blood. Thus, lymphogenous and hematogenous pathways of metastasis are intertwined and not mutually exclusive.

Traditionally, metastasis has been viewed as a unidirectional process, whereby tumor cells leave a primary tumor and seed metastases in regional lymph nodes or distant sites. Recent data, however, indicates that metastasis is multidirectional. This novel view, the self-seeding paradigm, implies that tumor cells may leave distant sites and reseed established metastases, as well return to their tumor of origin (Comen et al. 2011). Tumor cells in distant organ may further disseminate via lymph to form secondary metastases in lymph nodes or reenter into the blood to recirculate. Either scenario may lead to the establishment of novel metastatic foci. Migratory patterns of tumor cells, therefore, resemble the trafficking of leukocytes and hematopoietic stem cells through both the lymph and blood compartments.

Mechanisms of Lymph Node Metastasis

The invasion of tumor cells into lymphatic vessels is the first step on the path towards the lymph

node. Tumor cells can be guided into lymphatic vessels by subverting the process through which lymphatic endothelium guides leukocytes into these vessels (Ben-Baruch 2008; Das and Skobe 2008). For example, chemokine CCL21, a ligand for the chemokine receptor CCR7, is constitutively expressed by lymphatic capillaries (Kerjaschki et al. 2004; Podgrabinska et al. 2002; Shields et al. 2007a). CCL21 is immobilized by binding to sulfated proteoglycans within the extracellular matrix and forms steep gradients within the interstitium (Haessler et al. 2011; Schumann et al. 2010; Weber et al. 2013). These gradients induce a directed migration of dendritic cells towards lymphatics and may also attract tumor cells expressing the CCR7 receptor (Houshmand and Zlotnik 2003; Muller et al. 2001). CCR7 overexpression in melanoma has indeed been shown to increase lymph node metastasis in mouse tumor models (Takeuchi et al. 2004; Wiley et al. 2001; Zlotnik et al. 2011), and the correlation between CCR7 expression on tumor cells and lymph node metastasis has been demonstrated in various human tumors (Cabioglu et al. 2005; Ishigami et al. 2007; Mashino et al. 2002).

CXCL12 is another chemokine that has been shown to facilitate lymph node metastasis of tumor cells that express its receptor CXCR4 (Muller et al. 2001; Uchida et al. 2007; Zlotnik et al. 2011). A large body of literature provides evidence for the importance of CXCL12 in directing the homing of $CXCR4^+$ cancer cells to the bone and lung (McAllister and Weinberg 2014; Zlotnik et al. 2011). CXCL12 is upregulated on lymphatic vessels in the primary tumor and has been shown to promote the recruitment of $CXCR4^+$ melanoma cells into the proximity of lymphatic endothelium. Several studies have demonstrated a correlation between CXCR4 expression by tumor cells and lymph node metastases. However, direct evidence for the role of CXCL12 in directing tumor cells into the lymphatic capillaries has not been demonstrated.

In addition to producing chemokines that recruit tumor cells positioned in the vicinity of lymphatics into the lymphatic vessels, LECs can also help generate chemokine gradients around tumor cells that help direct them towards lymphatic vessels from greater distances. By draining fluids from the tumor tissues, lymphatics generate interstitial flow at velocities of 0.1–0.8 micron/s. This slow flow creates steep gradients of the CCL21 around the tumor cell that is secreting this chemokine. The same tumor cell expressing the corresponding chemokine receptor migrates along this chemokine gradient and is thereby directed towards the lymphatics. This mechanism, where interstitial flow creates and amplifies autocrine chemokine gradients to direct cells towards lymphatics, is termed autologous chemotaxis (Shields et al. 2007b). These findings underscore the importance of the biophysical microenvironment, created by the normal lymphatic function of transporting fluids, for homing of tumor cells to lymphatics.

The cellular mechanism of tumor cell intravasation into lymphatic vessels remains elusive. Although it has been assumed that tumor cells enter through intercellular lymphatic junctions, there is no direct evidence to support that concept. Furthermore, there has been a long-standing misconception that lymphatic capillaries are highly permeant and thus more easily penetrated by tumor cells compared to blood capillaries. On the contrary, studies indicate that the entry of cells into the lymphatic vessels is a process tightly controlled by LECs themselves and by signals in the microenvironment.

Conventional wisdom suggests that tumor cells are delivered into the sentinel lymph nodes passively, with the flow of lymph. This has indeed been demonstrated for cell transport within large, collecting lymphatic vessels (Dadiani et al. 2006; Hayashi et al. 2007), where flow velocities of up to several mm/min have been recorded (Dadiani et al. 2006; Swartz 2001). Lymph flow velocities in lymphatic capillaries, however, are much slower, ranging from 60 to 180 μm/min (Berk et al. 1996; Swartz et al. 1996). Interestingly, dendritic cells have been shown to crawl along the luminal side of LECs in lymphatic capillaries (Tal et al. 2011), opening up the possibility that tumor cells may exhibit a similar behavior.

Afferent collecting vessels deliver lymph content into the LN. The subcapsular sinus (SCS) of the LN, which is lined by LECs, is the first port of entry into the LN and first site of lymph node metastasis (Carr 1983; Carr et al. 1985; Dadiani et al. 2006; Das et al. 2013; Dewar et al. 2004). The presence of tumor cells induces the dilation of the SCS, which begins at the junction with the afferent lymphatic vessel (Fig. 5). Sinus dilation precedes the arrival of tumor cells (Das et al. 2013) and may be a prerequisite for the entry of tumor cells into the SCS. Indeed, when tumor cells are injected directly into the lymphatic system of a mouse in the absence of a primary tumor, the tumor cells arrest at the junction of the afferent lymphatic vessel and the LN (Hayashi et al. 2007). Scanning electron microscopy analysis revealed that the SCS is divided vertically and horizontally into smaller compartments, resulting in passages 5–15 microns wide (Das et al. 2013; Ohtani and Ohtani 2008). Since the diameter of a single circulating tumor cell is at least 15 microns (Vona et al. 2000), it has been concluded that the small dimensions of the sinus prevent the passive flow of tumor emboli into the SCS (Das et al. 2013). The chemokine CCL1 has been shown to be important for the entry of tumor cells into the LN. CCL1 is produced by the LECs of the subcapsular sinus and facilitates the entry of $CCR8^+$ tumor cells into the sinus and across the lymphatic endothelium into the LN cortex. Conversely, blocking CCR8, which is expressed in a large subset of melanomas, leads to the arrest of tumor cells at the junction of the afferent lymphatic vessel and the LN (Das et al. 2013). These studies demonstrate that the LECs of the SCS regulate the entry of tumor cells into the lymph node and thereby identify entry into the lymph node as another rate-limiting step in the metastatic process.

Lymphangiogenesis in the Lymph Nodes

The activation of the lymphatic vasculature is not restricted to the microenvironment of the primary tumor. Lymphangiogenesis has also been observed within sentinel LNs (SLNs) in many cancer types, including breast cancer and melanoma. It has also been documented within metastases in the sentinel and more distal lymph nodes (Kerjaschki et al. 2011). Interestingly, lymphatic remodeling and expansion in sentinel lymph nodes has been shown to precede lymph node metastasis (Harrell et al. 2007; Hirakawa et al. 2005, 2007; Ruddell et al.

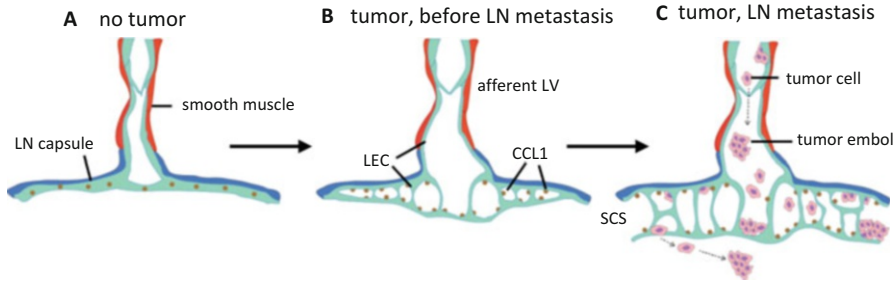

Fig. 5 Steps of tumor cell entry into the lymph node. (**a**) Afferent lymphatic vessel (LV) delivers lymph into the subcapsular sinus (SCS) of the lymph node (LN). In the normal setting, without a tumor present, SCS appears closed. (**b**) In the presence of a tumor, afferent lymphatic vessel leading into the sentinel lymph node becomes dilated, and SCS begins to open starting at the junction of the afferent LV and the LN. (**c**) In the collecting lymphatic vessels, tumor cells are carried passively with the flow of lymph towards the LN, both as single cells and clusters (emboli). Active cell migration is required for tumor cell entry into the sinus, which is wide open, and subsequently across the floor of the sinus into the lymph node cortex. For tumor cells expressing chemokine receptor CCR8, entry into the SCS and LN cortex is regulated by the CCL1 chemokine expressed constitutively by the LECs of the SCS. Tumor cells can also attach to the luminal side of the lymphatic vessel and to the LECs of the sinus and continue to grow and form intravascular lesions.

2008; Van den Eynden et al. 2006, 2007). Expanded lymphatic networks in the LNs have been suggested to represent a pre-metastatic niche that promotes the colonization of LNs by metastatic cells. However, because the selective inhibition of lymph node lymphangiogenesis is difficult to achieve experimentally, this conclusion is largely based on correlative studies. The extent to which LN lymphangiogenesis plays a role in cancer metastasis remains to be elucidated.

The mechanisms mediating lymphatic vessel expansion within tumor-draining LNs are not completely understood. Lymph node lymphangiogenesis may be, in part, driven by VEGF-C produced by cells of the immune system in the LNs, primarily B cells (Jones et al. 2018). Lymphangiogenic factors produced by the primary tumor may be transported into the SLNs via lymph and act directly on pre-existing lymphatic sinuses (Hirakawa et al. 2005, 2007). Once metastases have formed in the LNs, tumor cells may represent a major source of lymphangiogenic factors. Of note, LN lymphangiogenesis is not unique to cancer, as it also occurs during inflammation and is a component of a normal immune response (Angeli et al. 2006; Kim et al. 2014).

Lymph Node Metastasis Is an Important Prognostic Indicator

Clinical data has unequivocally established that lymph node metastases correlate with poor outcome. The status of regional lymph nodes is, therefore, an important parameter used for determining the stage of disease progression and treatment options. The first lymph node to receive lymph from the primary tumor is defined as a sentinel lymph node (SLN). Sentinel lymph node biopsy procedure involves the removal and examination of SLNs for the presence of tumor cells and is a standard of care for most cancers. SLNs are typically identified by preoperative lymphoscintigraphy and by intraoperative staining after blue dye injection at the primary tumor (Cochran et al. 2000; Morton et al. 1992). The SLN biopsy is performed based on the assumption that if sentinel lymph nodes are free of metastases, other lymph nodes will also be unaffected and their removal is not medically indicated. Indeed, if the SLNs do not contain cancer cells, other regional lymph nodes are almost certainly free of metastases, and the risk of having distant metastasis is low, indicating an excellent prognosis. Alternatively, if the biopsy shows the presence of metastases in the SLN, additional lymph nodes may be removed for diagnostic purposes. The number of lymph nodes involved and the presence of micro- versus macro-metastases influence decisions about the choice of postsurgical (adjuvant) therapy. Sentinel lymph node biopsy is the standard of care for melanoma, breast, prostate, and colon cancer, among others, and the presence of tumor cells in the SLN continues to be one of the most important prognostic indicators of patient survival.

Lymphovascular invasion (LVI), detected histologically by the presence of tumor cells within lymphatic vessels in the tumor microenvironment, is another important parameter in assessing the risk for tumor metastasis. LVI in a primary tumor indicates a significantly increased risk of lymph node involvement, distant metastases, and disease relapse and thus leads to an unfavorable prognosis in melanoma, breast, gastric, bladder, prostate, and many other cancer types (Dicken et al. 2006; Hoda et al. 2006; Lee et al. 2006; Lotan et al. 2005; May et al. 2007; Straume and Akslen 1996). In lymph node-negative cancers, LVI is an independent adverse prognostic factor for metastases to regional and distant sites.

Historical Perspective on Lymph Node Metastasis

French surgeon Henry LeDran (1684–1770) was the first to recognize the importance of cancer dissemination to the lymph nodes. He noted that cancer begins as a local disease that first spreads to lymph nodes and subsequently to distant organs. LeDran also observed that a surgical cure was much more likely when lymph nodes did not contain cancer cells. His theory offered the hope

that there may be a cure for the disease if surgery is performed sufficiently early (Rayter 2003). Building on this premise, William Halsted, an American surgeon, believed that the removal of the whole breast and associated lymph nodes would prevent malignant spread of breast cancer. He introduced radical mastectomy and lymphadenectomy as standard surgical treatments for breast cancer. This approach was later abandoned because the majority of patients relapsed despite the extremely aggressive surgery and suffered from significant morbidity. Lymphadenectomy – the partial or complete removal of regional lymph nodes – has continued to be a standard surgical practice for cancer management. Whether a complete regional lymphadenectomy in patients with metastatic disease in lymph nodes provides benefits in terms of patient survival, however, remains controversial. Many clinical studies have shown that patients with melanoma, gastric, colon, and prostate cancer who undergo extensive lymph node dissections have higher survival rates (Morton et al. 2006; Wu et al. 2006), yet other studies contradict these results (Bembenek et al. 2007; Hartgrink et al. 2004). Furthermore, lymphadenectomy is associated with a significantly increased risk of developing lymphedema, which is a chronic and disabling morbidity. Today, complete lymph node dissection is rarely performed.

Lymph Node Metastasis as a Source of Distant Metastases

While the presence of lymph node metastases is undoubtedly a strong negative prognostic indicator, the reason behind this association is unclear and remains a subject of significant controversy. This has led to the proposal of two alternative models. One model argues that lymph nodes are the first and critical site of metastasis, and the site from which tumor cells will further spread to distant organs. An alternative model posits that distant metastases arise independently of lymph node metastases and that the presence of metastases in lymph nodes only indicates that tumors have acquired the ability to metastasize. Indeed, distant organ metastases are occasionally detected despite the absence of tumor cells in the lymph nodes, although this clinical scenario is relatively rare.

Many studies have examined the evolutionary relationship between the primary tumor, lymph node, and distant metastases by phylogenetic analysis. If distant metastases are genetically more closely related to the clones in the primary tumor than to clones in the lymph nodes, distant metastases most likely originated directly from the primary tumor and independently of lymph node metastases. Conversely, if distant metastases are derived directly from lymph node metastases, they would be genetically more closely related to lymph node metastases than to the primary tumor. This would support a linear model, where tumor cells from the primary tumor first spread into the lymph nodes and subsequently to distant sites.

In this context, a recent study of colorectal cancer examined the origin of liver metastases through phylogenetic analysis (Naxerova et al. 2017). The authors found that in 35% of cases, lymph node and liver metastases shared a common clonal origin, indicating that liver metastases were derived from lymph node metastases. However, in 65% of the cases, lymph node and liver metastases arose from independent clones in the primary tumor, indicating that the seeding of lymph node and distant metastases developed in parallel and independently of each other (Naxerova et al. 2017). This study demonstrated that lymph node and liver metastases may have a common origin as well as arise independently from each other, thus reconciling the two seemingly opposing concepts. The relative contribution of lymph node metastases to the formation of distant metastases may be different in different types of cancer and for the specific distant organ. For example, liver is the most frequent distant organ site for metastasis of colorectal cancer. Since venous blood from the intestines reaches the liver directly through the portal vein, it is possible that liver metastases are preferentially seeded hematogenously (Naxerova et al. 2017). In contrast, cancer cells that egress from lymph nodes ultimately enter into the subclavian vein,

and the first capillary beds these cells encounter are in the lung. It is therefore plausible that lung metastases are more frequently seeded from the lymph nodes.

Recent studies of human cancers provide further evidence that tumor cells derived from metastatic foci in lymph nodes indeed contribute to distant organ metastases (Greaves and Maley 2012; Hunter et al. 2018; Marusyk et al. 2014; Nowell 1976). Furthermore, studies in mouse models have also shown that metastatic tumor cells can spread to distant organs from sentinel lymph nodes. These studies specifically demonstrate that tumor cells may exit lymph nodes via blood vasculature (Brown et al. 2018; Pereira et al. 2018).

Together, these studies suggest that the two models of metastatic dissemination likely represent extremes on a biological continuum. Even within the same patient, hematogenous spread may be a preferred pathway to certain organs (e.g., liver), whereas lymph nodes may be important hubs for spread to another organ (e.g., lung). The relative frequency and importance of the different pathways of metastasis in different tumor types will need to be established through additional large-scale studies of patient-matched primary tumors and metastases (Hunter et al. 2018).

Lymphangiogenesis in Target Organs for Metastasis

From a therapeutic perspective, it is critically important to understand what role lymphatics play in the formation and progression of distant metastases. Lymphangiogenesis can be induced in a distant organ that is a site of metastasis, such as the lung, and, in some patients, metastatic disease is characterized by the extensive involvement of lung lymphatics with cancer. This type of metastasis, referred to as pulmonary lymphangitic carcinomatosis, denotes the growth of metastases within pulmonary lymphatic vessels. Lymphangitic carcinomatosis has been observed primarily in patients with epithelial cancers, including breast, lung, gastric, pancreatic, and prostate cancer (Goldsmith et al. 1967; Janower and Blennerhassett 1971; Thomas and Lenox 2008; Tomashefski and Dail 2008). It is invariably associated with extremely poor prognosis, and most patients succumb to the disease within several months of diagnosis.

Lymphangitic carcinomatosis has a diffuse presentation and is very difficult to diagnose in patients using current imaging techniques. Approximately half of the cases of histologically proven pulmonary lymphangitic carcinomatosis present with normal radiographs (Janower and Blennerhassett 1971; Trapnell 1964). Because of these imaging limitations in patients and because an immunohistological evaluation of lung metastases is not commonly performed, the true incidence of lymphangitic carcinomatosis may be greatly underestimated. Indeed, imaging studies reported the incidence of this type of pulmonary metastases to be as low as 6%, whereas studies by pathologists reported it to be as high as 56% (Tomashefski and Dail 2008).

Data from one mouse model of spontaneous metastasis revealed that the overexpression of VEGF-C in tumor cells induced lymphangiogenesis in the lung and changed the pattern of metastases to pulmonary lymphangitic carcinomatosis (Das et al. 2010). The expansion of the pulmonary lymphatic network was accompanied by a dramatic increase in the size of metastases, which grew within the constraint of lymphatic vessel walls. VEGF-C was necessary for the manifestation of lymphangitic carcinomatosis, but not sufficient, since its overexpression alone did not induce lymphangitic carcinomatosis in all cancer cells tested. In agreement with these findings, another study using a mouse model with inducible VEGF-C expression in the lung found that increased pulmonary lymphangiogenesis promoted growth of metastases in the lung and dissemination to other organs (Ma et al. 2018). Together, with clinical observations, these experimental data demonstrate an unappreciated role of lymphatics in facilitating lung colonization.

Recent studies using the VEGFR-3 luciferase reporter mouse, which enables noninvasive whole-body imaging of lymphovascular niches,

revealed systemic lymphangiogenesis in lymph nodes and distant organs in tumor-bearing mice (Olmeda et al. 2017). Systemic induction of lymphangiogenesis preceded organ colonization, consistent with the role of lymphangiogenesis in the creation of pre-metastatic niches. Tumor cells at the primary site were the main source of the factors inducing systemic lymphangiogenesis, and this ability was attributed mainly to the pleiotropic factor midkine. Notably, different tumors showed preference for inducing lymphangiogenesis in different organs, suggesting that organotropism may also be influenced by the remodeling of distant vascular microenvironments by the tumor. Importantly, the metastatic capability of melanoma correlated with systemic lymphangiogenesis. Together, these findings provide evidence for the importance of systemic lymphangiogenesis in facilitating tumor metastasis.

Clinical Implications of Lymphangiogenesis

Prognostic Significance of Lymphangiogenesis in Human Tumors

Prognostic biomarkers are typically used to establish a statistical correlation between the levels of a particular marker in a patient's blood or tumor and the probability of disease progression, relapse, or overall survival, irrespective of treatment. Prognostic relevance of tumor lymphangiogenesis, as evidenced by an increase in lymphatic vessel density, has been investigated retrospectively in many types of human solid tumors. The availability of specific antibodies that recognize several lymphatic markers in immunohistological assays has made it possible to identify and quantify lymphatic vessels in tissues (Van der Auwera et al. 2006).

Podoplanin, also known as gp38, is a mucin-type glycosylated transmembrane protein that has been widely used as a marker for the identification of lymphatic vessels in many human tissues. Podoplanin is specifically expressed by lymphatic and not by blood vascular endothelium, but its expression is not restricted to the lymphatic vasculature. It is expressed by many other cell types, most notably on kidney podocytes, fibroblast-type reticular stromal cells in the lymph node, and by some tumor cells (Breiteneder-Geleff et al. 1997, 1999; Wicki et al. 2006). Another widely used lymphatic marker is LYVE-1 (lymphatic vessel endothelial hyaluronan receptor-1), a transmembrane glycoprotein ubiquitously expressed by lymphatic endothelium (Banerji et al. 1999; Jackson 2004). LYVE1 is also expressed by specialized blood endothelial cells such as liver sinusoids and by a subset of macrophages, and it can be downregulated by inflammation (Johnson et al. 2007; Lim et al. 2018b; Mouta Carreira et al. 2001). PROX1 (the prospero homeobox protein 1) is a nuclear transcription factor that defines lymphatic endothelial identity and is not expressed by blood endothelial cells (Escobedo and Oliver 2016; Wigle and Oliver 1999). The above markers accurately differentiate lymphatics from the blood vasculature, but because of their expression by certain non-endothelial cells, antibodies to lymphatic endothelial markers are typically combined with the pan-vascular marker CD31 to ensure endothelial identity.

Numerous clinical studies have reaffirmed the positive correlation between VEGF-C or VEGF-D, lymphangiogenesis, and adverse patient outcome. Lymphatic vessel density has emerged as a promising indicator of patient prognosis, showing high concordance with the incidence of regional and distant metastases, as well as poor survival in breast, lung, head and neck, colorectal, gastric, and endometrial cancers, among others (Beasley et al. 2002; Furudoi et al. 2002; Kyzas et al. 2005; Mohammed et al. 2007; Nakamura et al. 2005; Shields et al. 2004; Stacker et al. 2014; Van der Auwera et al. 2006; Zhang et al. 2017). The quantification of intratumoral and peritumoral lymphatic vessels in primary human malignant melanomas of the skin revealed that the extent of lymphangiogenesis was the most significant predictor of the presence of SLN metastases at the time of surgery and held higher

significance than tumor thickness (Dadras et al. 2005; Shields et al. 2004). Thus, the quantification of lymphangiogenesis as part of a routine pathological evaluation of tumor tissue has the potential of providing an important early prognostic marker that would be particularly beneficial for patients presenting with primary tumors, but without lymph node involvement.

Although VEGF-C and VEGF-D have both been correlated with adverse prognosis, these factors may exhibit different expression patterns in various human tumors types. For example, VEGF-C is highly expressed in head and neck cancer, whereas VEGF-D is not (Beasley et al. 2002; Pornchai et al. 2001). Conversely, VEGF-D, but not VEGF-C, was reported to be an independent predictor of poor outcome in epithelial ovarian cancer (Yokoyama et al. 2003). In breast cancer, both VEGF-C and VEGF-D are expressed and both are negative prognostic indicators (Nakamura et al. 2005; Wang et al. 2012). Overall, VEGF-C appears to be the more dominant lymphangiogenic and pro-metastatic factor as more studies reported a correlation of VEGF-C to poor prognosis.

Therapeutic Targeting of Lymphangiogenesis

Recognition of the importance of the lymphatic system in the pathology of cancer has raised interest in the possibility of developing anti-lymphangiogenic therapies. Preclinical studies strongly suggest a therapeutic utility of blocking lymphangiogenesis to down-modulate the rate of tumor spread. Conceptually, it is possible to delineate distinct clinical scenarios where such therapies may be useful: prevention of metastasis, slowing down the spread of existing metastases, and treatment of metastatic lesions within the lymphatic bed. These distinct, if related, scenarios are discussed below.

A subclass of tyrosine kinase inhibitors (TKIs) that are in clinical use as anticancer agents has been shown to inhibit the VEGFR-3 kinase. Typically, these compounds also inhibit other closely related RTKs such as VEGFR-2 and the PDGF receptors, and it is impossible to determine what clinical benefits, as well as toxicities, seen in cancer patients treated with RTK inhibitors stem from the anti-lymphangiogenic activity of these molecules. A more promising approach involved the development of targeted biologics. VEGF-C and VEGF-D emerged as initial targets based on the wealth of preclinical studies showing that the targeted inhibition of these molecules potently inhibited tumor lymphangiogenesis and lymphatic metastasis. In mouse tumor models, this initially involved the use of either monoclonal antibodies (mAb) to VEGF-C and VEGF-D or a trap macromolecule such as soluble VEGFR-3 (sVEGFR-3). Enhanced tumor lymphangiogenesis and accelerated metastasis induced by overexpression of VEGF-C or VEGF-D in human tumor xenografts were reversed by treatment with sVEGFR-3 or by the use of neutralizing mAbs to these growth factors. Further studies showed that metastasis could also be significantly reduced by downregulating VEGF-C either by co-expression of sVEGFR-3 as a transgene in human tumor cell lines, by injection of sVEGFR-3 cDNA in a viral vector, or by the use of small interfering RNAs(siRNA) (Stacker et al. 2014).

In the clinic, this approach was attempted with VGX-100, a selective anti-VEGF-C antibody. In the phase 1 trial (NCT01514123), VGX-100 was combined with the anti-VEGF-A antibody bevacizumab in the hope of a synergistic effect of a simultaneous blockade of VEGFR-2 and VEGFR-3 activation. Although treatment with VGX-100, either alone or in combination with bevacizumab, was well tolerated, major responses in patients with solid tumors were not observed (Falchook et al. 2014). Further development of VGX-100 in cancer has not been reported.

A more specific approach to selectively target VEGFR-3 required the development of antagonist mAbs to this RTK. In a preclinical model, treatment with an anti-murine VEGFR-3 antibody potently inhibited lymphangiogenesis in a wound regeneration model (Pytowski et al. 2005) and markedly reduced tumor lymphangiogenesis and lymphatic metastasis (Burton et al. 2008; Roberts et al. 2006). Based on these encouraging data, a fully human antagonist mAb to human VEGFR-3 was developed (LY3022856/IMC-3C5) (Persaud

et al. 2004). A phase 1 trial of this antibody was conducted in patients with advanced solid tumors (NCT01288989). The mAb was found to have an acceptable safety profile and limited activity in a subgroup of patients with colorectal cancer (Saif et al. 2016). However, clinical development of IMC-3C5 was discontinued.

Certain types of cancer exhibit high lymphatic vessel densities and prominent lymphovascular invasion and may be particularly amenable for treatment with anti-lymphangiogenic agents. Inflammatory breast cancer (IBC) is the most aggressive subtype of breast cancer, characterized by rapid, diffuse growth, extensive lymph node involvement, and frequent distant metastases. Skin edema and erythema are typically observed and related to extensive lymphovascular emboli in the dermal lymphatics. Intralymphatic tumor emboli are found in virtually all cases of IBC, and elevated levels of VEGF-C have been reported (Lim et al. 2018a). IBC has been hard to approach experimentally largely because of the lack of good animal models. However, it is tempting to speculate that anti-lymphangiogenic therapy may benefit IBC patients, but this remains unclear at this time.

Another tumor characterized by lymphangiogenesis, extensive intralymphatic emboli, and poor prognosis is cutaneous melanoma. Melanoma can also form cutaneous metastases, so-called "in-transit" metastases, which are clusters of tumor cells growing within the skin lymphatic vessels. The blockade of lymphangiogenesis in melanoma with VEGFR-3 antagonists has been attempted in murine tumor models with some success (Alitalo and Detmar 2011). Head and neck cancer also shows prominent lymphatic remodeling and lymphovascular invasion and may be particularly appropriate for future clinical trials of anti-lymphangiogenic therapy (Beasley et al. 2002; Kyzas et al. 2005).

Conclusions

A rapidly growing understanding of the biology of the lymphatic system and its role in cancer has catalyzed efforts to develop novel anti-lymphangiogenic therapies aimed at reducing metastatic tumor spread. While the specific targeting of lymphatic vessels in rodent tumor models of metastasis has shown promise, the critical difference between such models and the reality of human cancer imposes a formidable challenge to the design of clinical studies that, to date, have not progressed beyond phase 1 testing. Such hurdles notwithstanding, one can envision the use of anti-lymphangiogenic biologics, most likely in conjunction with chemotherapy or with other targeted agents, as part of neoadjuvant or adjuvant therapy, especially in patients whose tumors are not amenable to complete resection. Alternatively, an exciting possibility lies in combining pro- or anti-lymphangiogenic therapy with immunotherapy. Lymphatics play important roles in regulation of immune response and in preclinical models exhibit immunosuppressive as well as immune-activating functions. The answer to which combination approaches may be beneficial must await further research and clinical testing.

Cross-References

- ▶ Angiopoietins and TIE Receptors in Lymphangiogenesis and Tumor Metastasis
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part III

Mechanisms of Anti-angiogenic Therapy

Mechanisms of Anti-angiogenic Therapy

Roser Pons-Cursach and Oriol Casanovas

Contents

Introduction 184

Inhibition of Angiogenic Signaling 185
Vascular Endothelial Growth Factor Inhibitors 186
Tyrosine Kinase Inhibitors 186

Inhibition of Vascular Progenitor Cells 187
EPCs in Tumor Angiogenesis 187
EPCs and Tumor Microenvironment 187
Therapeutic Strategies 189

Inhibition of Extracellular Matrix Remodeling 190
Extracellular Matrix in Angiogenesis 190
Targeting Angiogenic Microenvironment 193

Regression of Tumor Blood Vessels 195
Vascular-Disrupting Agents 195
Types of Vascular-Disrupting Agents 196
Therapeutic Approaches of VDAs 197

Normalization of Tumor Vasculature and Microenvironment 198
Abnormalities of Tumor Vessels 199
Tumor Vessel Normalization 200
Therapeutic Implications of Vascular Normalization 201

Conclusion 202

Cross-References 202

References 202

Abstract

Angiogenesis inhibition is a promising approach to fight cancer. This strategy offers some advantages in comparison with conventional drugs, such as the inhibition of single vessels that can induce the death of many tumor cells. Moreover, this therapy can be used in the treatment of a wide range of solid

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tumors and may produce less resistance. Since angiogenesis is a complex process, it can be inhibited at different levels. The most established therapy is the inhibition of angiogenic signaling. Vascular endothelial growth factor (VEGF) pathway is the most important signaling pathway in the angiogenesis process, and for this reason, many inhibitors have been developed to block the action of VEGF or its receptors, VEGFRs. Another approach is the inhibition of endothelial progenitor cells (EPCs), mobilized from the bone marrow to the tumor site in hypoxia conditions, which contribute to the formation of new blood vessels and the pre-metastatic niche. The interaction between extracellular matrix and endothelial cells is very important during angiogenesis, so the inhibition of this interaction produces anti-angiogenic effects. An alternative strategy is based in the regression of preexisting tumor vasculature, which presents abnormalities in the structure and function in comparison with normal vessels. In this case, vascular-disrupting agents (VDAs) can cease the blood flow within minutes and lead to the formation of central necrosis. Finally, tumor vessel normalization produced after anti-angiogenic therapies may reduce the metastatic dissemination and improve delivery of drugs to the tumor.

Keywords

Angiogenesis · Anti-angiogenic drugs · VEGF · Endothelial progenitor cells (EPCs) · Integrins · Extracellular matrix · Basement membrane · Endothelial cells · Pericytes · Vascular-disrupting agents (VDAs) · Vascular normalization

Introduction

The treatment of solid tumors with chemotherapeutic drugs is based on the expectation that drugs at low doses will preferentially kill rapidly dividing tumor cells, rather than normal cells. However, chemotherapeutic drugs are not selective for tumor cells and produce toxicities in normal tissues with high proliferation, such as the bone marrow, gastrointestinal tract, and hair follicles. Moreover, the efficiency of these drugs is reduced due to the lack of accumulation of drug at the tumor site produced by the high pressure and the irregular vasculature of the tumor (Bosslet et al. 1998).

It has been recognized for decades that most tumors are highly vascular. This concept was introduced by *Ide and Algire* (Algire and Chalkley 1945; Ide et al. 1939) and confirmed by *Folkman's group* in the 1960s (Folkman et al. 1963). These studies proposed that when tumors acquire a size of 1–2 mm, the inadequacy of nutrient supply and metabolic waste clearance by vessels produce hypoxia and acidosis. At this moment, tumors initiate molecular signals in order to induce angiogenesis and continue growing. Therefore, angiogenesis is an essential process for tumor development. Thus, vascular targeting was proposed as a new approach to fight the limitations of conventional drugs. This promising strategy leads the tumor cell death by the lack of nutrients and oxygen. Importantly, anti-vascular therapies have some advantages in comparison with conventional drugs. Firstly, removing one blood vessel triggers the cell death of all tumor cells supplied by this vessel. Secondly, anti-vascular targeting can be used in the treatment of a wide range of solid tumors. Finally, anti-vascular treatments may produce less therapy resistance because endothelial cells and pericytes are genetically more stable than tumor cells.

Given many mechanisms are implicated in the formation of new blood vessels, tumor vasculature can be inhibited at different levels (Fig. 1). The inhibition of endothelial cells, pericytes, or extracellular matrix (ECM) remodeling produces tumor blood vessel reduction. Different classes of compounds are currently used for targeting the different anti-angiogenic mechanisms mainly small molecules and antibodies. In pharmaceutical biotechnology, antibodies are the binding molecule class, most of them used for tumor diagnosis and therapy. Nevertheless, antibodies present some disadvantages, such as the requirement for an expensive mammalian cell production system,

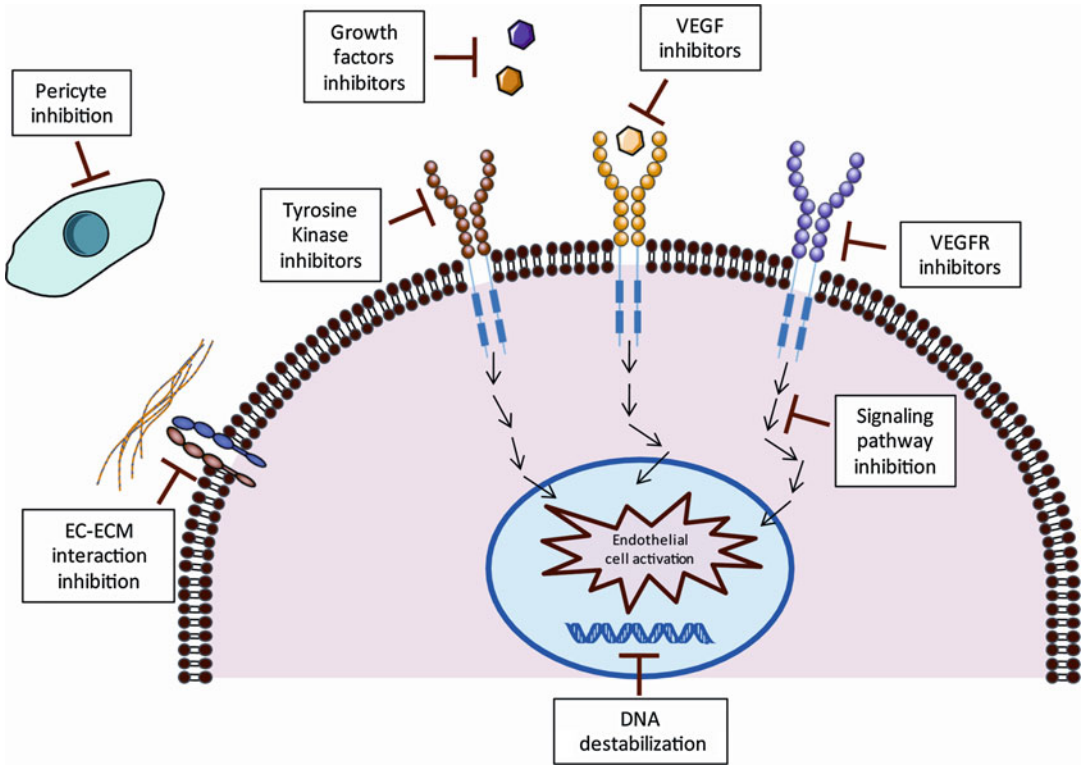

Fig. 1 *Strategies of angiogenesis inhibition*. Angiogenesis process is a complex mechanism and many molecules are implicated, so it can be inhibited at different levels. Angiogenic signaling can be blocked by VEGF and other growth factor inhibitors and by VEGFR or other tyrosine kinase receptor inhibitors. The interactions between endothelial cells and extracellular matrix can also be interfered by integrin inhibitors. Finally, the inhibition of pericytes produces anti-angiogenic effects

low expression yields, dependence on disulfide bonds for stability, and the tendency to aggregate. For this reason, other classes of molecules are being investigated for tumor-targeting applications, such as small globular proteins, peptides, and aptamers (Hey et al. 2005).

In the recent years, many progresses have been made to understand the mechanism of action of anti-angiogenic drugs. Many of those approaches have been obtained evaluating the effects of the anti-angiogenic inhibitors on tumor blood vessels in preclinical and clinical studies. Importantly, not all the angiogenesis inhibitors have the same cellular actions. Angiogenesis inhibitors have multiple effects and not all have the same therapeutic relevance. For example, vascular endothelial growth factor (VEGF) inhibitors influence endothelial cell survival, migration, growth, plasma leakage, blood flow, and recruitment of leukocytes and stem cells (Kamba and McDonald 2007).

The effects of angiogenesis inhibitors on tumor blood vessels can be classified into three categories: (1) inhibition of tumor blood vessels, (2) regression of tumor blood vessels, and (3) normalization of tumor blood vessels.

Inhibition of Angiogenic Signaling

Commonly, endothelial cells are activated by tyrosine kinase (TK) receptors and consequently their corresponding signaling pathway. Growth factors stimulate the endothelial cells to form new blood vessels. Therefore, growth factors, their receptors, and subsequent signaling

cascades are promising targets in angiogenesis inhibition.

The most important growth factor implicated in angiogenesis is VEGF, and many inhibitors have been developed to block the action of this molecule (Ferrara 1999). Interestingly, different approaches have been developed to inhibit VEGF or its signaling pathway, such as the neutralization of the ligand by anti-VEGF antibodies, soluble receptors, or oligonucleotide aptamers, the inhibition of VEGF receptors by antibodies or small-molecule inhibitors of TK phosphorylation, and the inhibition of the intracellular signaling pathway directly. Finally, the inhibition of pro-angiogenic signaling tilts the balance in favor of endothelial cell apoptosis and regression.

Importantly, many drugs have been developed to block growth factors and tyrosine kinase receptors, and some of them have been approved by the Food and Drug Administration (FDA) for the treatment of some tumor types or are currently in clinical development (Krause and Van Etten 2005). Clinical trials have been focused in the study of the two main angiogenesis pathways: VEGF pathway and platelet-derived growth factor (PDGF) pathway.

Vascular Endothelial Growth Factor Inhibitors

VEGF pathway is the most important process in angiogenesis regulation. The VEGF produced by tumor and stroma cells interacts with their TK receptors expressed by endothelial cells promoting proliferation, migration, and invasion, leading to angiogenesis. VEGF is a homodimeric protein and five different isoforms have been described. Equally, VEGF receptor (VEGFR) is divided in three different isoforms with different roles in angiogenesis (Hicklin and Ellis 2005).

VEGF pathway can be blocked inhibiting the VEGF ligand or inhibiting the VEGFR. The most important anti-VEGF drug used in clinics is the monoclonal antibody bevacizumab. Bevacizumab is a recombinant humanized monoclonal antibody against VEGF-A. The first evidence of targeting VEGF-A inhibit tumor growth was observed in a mouse model in 1993 using a monoclonal antibody anti-VEGF-A (Kim et al. 1993). Moreover, bevacizumab was the first clinically available angiogenesis inhibitor in the United States. This drug was approved for the treatment of certain lung cancers, renal cancers, ovarian cancers, and glioblastoma multiforme of the brain (Shih and Lindley 2006).

Tyrosine Kinase Inhibitors

TK inhibitors are useful in the treatment of cancer because they present dual effect; they block tumor cell proliferation and the pro-angiogenic signaling pathway. Given different TK receptors are expressed in both tumor and endothelial cells and TK inhibitors often target more than one type of receptors, the inhibition of TK can affect both types of cells (Krause and Van Etten 2005). A wide range of TK inhibitors have been developed and approved for the treatment of several cancers. The efficacy of TK inhibitors can vary depending on the expression levels of the different types of growth factors and TK receptors; therefore, different types of tumors may respond differently to these drugs. Several approaches have been proposed to target growth factors and their receptors, such as compounds that bind to ATP-binding site of the TK receptors and block receptor activation, or antibodies that bind to the growth factors or their receptors, preventing binding and subsequent receptor activation (Hartmann et al. 2009). The most important anti-VEGFR and PDGF TK inhibitors are sorafenib, sunitinib, and pazopanib.

Sorafenib is a synthetic compound which inhibits the angiogenesis process and also the growth signaling. Sorafenib has a dual inhibition: it inhibits rapid accelerated fibrosarcoma kinase (RAF kinase), a critical component of the RAF/MAPK/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway that control cell division and proliferation, as well as VEGFR-2 and PDGF receptor-beta (PDGFRB) signaling pathway that blocks angiogenesis process (Kelly et al. 2010). Sorafenib was approved

in the treatment of advanced renal cell carcinoma, hepatocellular carcinoma, and radiation-resistant advanced thyroid carcinoma.

Sunitinib is another TK inhibitor which blocks angiogenesis and cell proliferation. The therapeutic effect is produced by the inhibition of VEGFR-2, PDGFRB, and c-kit. Sunitinib was approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST) (Gan et al. 2009).

In the same way, pazopanib inhibits tumor angiogenesis, blocking VEGFR-1, VEGFR-2, and VEGFR-3, c-kit, and PDGFR. Pazopanib has been approved for the treatment of renal cell carcinoma and soft tissue sarcoma (Verweij and Sleijfer 2013).

Inhibition of Vascular Progenitor Cells

Two processes contribute to the formation of new blood vessels: vasculogenesis and angiogenesis (Risau 1997). In vasculogenesis, new vessels are originated by the differentiation of mesenchymal cells (angioblasts) into endothelial cells, while, in angiogenesis, endothelial cells from the blood vessels proliferate and sprout to constitute the new vascular structures. However, the discoveries about the idea that circulating vascular progenitors are involved in angiogenesis have changed this dogma (Moore 2002). Moreover, hematopoietic cells can contribute to the maintenance and initiation of these processes, being essential for neoangiogenesis.

Endothelial progenitor cells (EPCs) are derived from the bone marrow and contribute to many processes, such as wound healing, myocardial ischemia, neovascularization, and tumor growth. EPCs are defined by the co-expression of the markers CD34, CD309, and CD133. In physiological conditions, EPCs are quiescent, but in response to a vascular injury, they acquire the ability to circulate in the peripheral blood, proliferate, and differentiate into mature endothelial cells. In this process, the damaged site releases growth factors and cytokines that promote the migration of EPCs to the local endothelium, contributing to neovasculature (Peichev et al. 2000).

Therefore, acknowledging that EPCs contribute to tumor angiogenesis provides the basis for new therapies and monitoring strategies for several types of malignancies.

EPCs in Tumor Angiogenesis

In normal conditions, vascular injury or hypoxia mobilizes EPCs from the bone marrow by the secretion of paracrine factors, such as VEGF and stromal cell-derived factor-1 (SDF-1). In pathological conditions, such as tumors, the chronic state of hypoxia or inflammation produces constitutively activation of EPCs (Fig. 2a). EPCs can contribute to the formation of new tumor blood vessels by secreting pro-angiogenic growth factors and also due to their ability to form new vessels. Moreover, EPCs contribute maintaining the anti-inflammatory state. EPCs are mobilized from the bone marrow in different types of malignancies, such as hepatocellular carcinoma and lung, pancreatic, and breast cancer (Ono et al. 2014). Several studies have demonstrated that EPC levels are higher in tumor tissue and peripheral blood of cancer patients than in healthy donors. Thus, circulating EPCs may be used as predictors of the malignancy grade of some tumors. In fact, studies have observed that EPC levels decrease in patients who respond to cancer treatments. Interestingly, circulating EPCs have been proposed as predictive biomarkers for gastric patients treated with chemotherapy (Ahn et al. 2010).

EPCs and Tumor Microenvironment

Tumor microenvironment interacts direct or indirectly with different cell types, such as cancer cells, EPCs, inflammatory immune cells, and endothelial cells. Hypoxia, characteristic of tumors, induces the formation of new blood vessels supplying oxygen to tumor mass. In hypoxia, the transcriptional activation of hypoxia-inducible factor (HIF) activates the transcription of genes required for tumor progression, such as VEGF, PDGF, SDF-1, and C-X-C chemokine receptor

type 4 (CXCR4) (Tang et al. 2016). Studies have demonstrated that the loss of function of HIF inhibits EPC proliferation and differentiation, reducing their ability to form new vessels. The avoidance of the immune response and the maintenance of the chronic state of inflammation are some of the hallmarks of cancer described by *Hanahan and Weinberg* (Hanahan and Weinberg 2011). During the inflammation process, damaged or hypoxic tissue releases cytokines, producing a molecular gradient which guides EPCs to the inflamed tissue (Fig. 2b). Moreover, high plasma levels of VEGF, secreted by both tumor and stroma cells, promote the mobilization of EPCs from the bone marrow and their proliferation (Lyden et al. 2001). Interestingly, the remodeling of the basement membrane (BM) in the first stages of neoplastic transformation can produce the mobilization of EPCs by VEGF and angiopoietin-1 (Ang-1). The depletion of VEGF and Ang-2 activity inhibits tumor growth and EPC recruitment.

Moreover, possible roles of EPCs in the induction of tumor invasion have been proposed. Endothelial cells and immune cells secrete paracrine factors that promote mobilization of EPCs

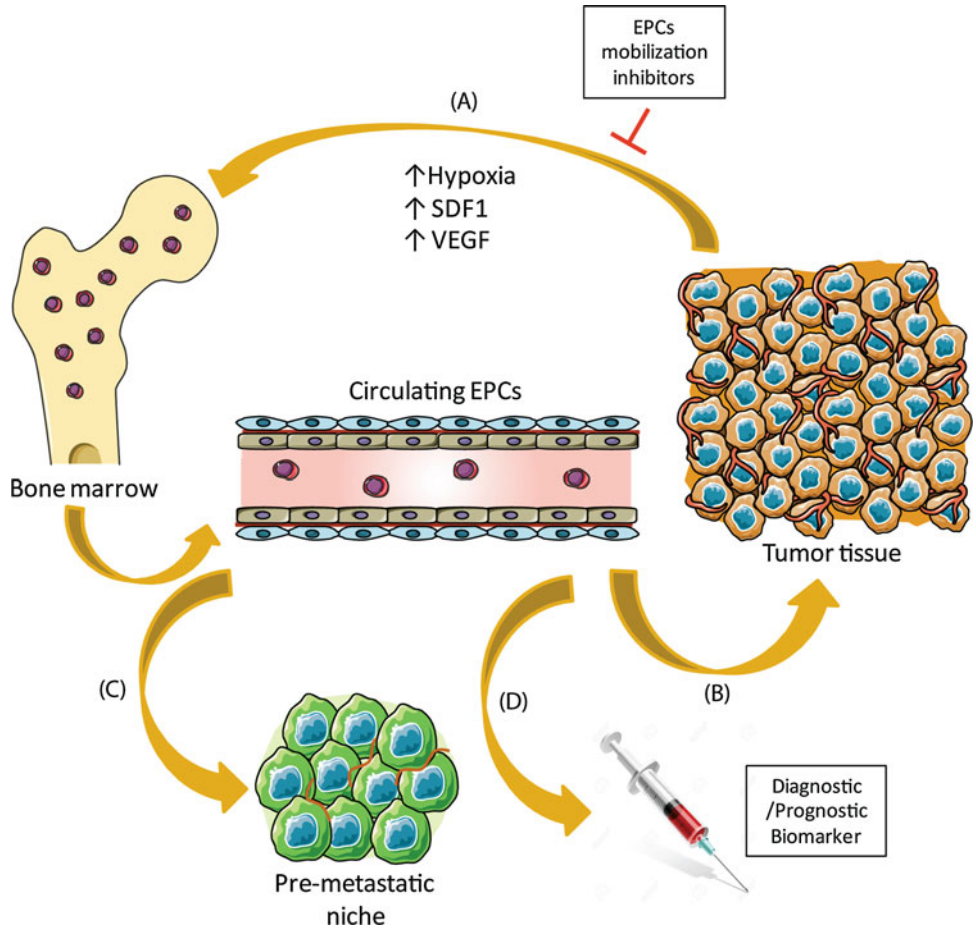

Fig. 2 *Inhibition of endothelial progenitor cell recruitment in tumor angiogenesis*. (**a**) Chronic hypoxia present in tumors mobilizes EPCs from the bone marrow to the circulation by the secretion of paracrine factors, such as VEGF and SDF-1. Therefore, the inhibition of VEGF or SDF-1 prevents the mobilization of EPCs to the tumor site. (**b**) Circulating EPCs home to hypoxic sites and contribute to the generation of new blood vessels. (**c**) However, circulating EPCs can also contribute to pre-metastatic niche formation before the arrival of malignant metastatic cells. (**d**) Levels of circulating EPCs have been proposed as diagnostic and prognostic biomarker

to form new blood vessels. HIF-1, upregulated in tumor hypoxia, induces the release of SDF-1 from tumor cells, endothelial cells, and stroma fibroblasts (Mohle et al. 1998). Additionally, EPCs highly express CXCR4, SDF-1 receptor. And thus, the gradient of SDF-1, produced in hypoxia, attracts the EPCs to the tumor tissue contributing to the generation of new blood vessels. Moreover, EPCs can also secrete SDF-1 and VEGF-1 during tumor progression. The interaction between SDF-1, secreted by EPCs, and CXCR4 expressed in tumor cells may contribute to the extravasation and development of a pre-metastatic niche before the arrival of malignant metastatic cells (Jin et al. 2012) (Fig. 2c). SDF-1 produced by the immune system may attract EPCs to distant sites and induce SDF-1 production spontaneously, promoting the spread of tumor cells to other sites. Moreover, the ability of EPCs to activate metalloproteinase-9 (MMP-9) could induce tumor cell migration and invasion. Thus, EPCs could facilitate the pre-metastatic niche formation by the secretion of SDF-1.

Therapeutic Strategies

EPCs in Cancer Diagnosis and Prognosis

Given that EPCs are mobilized from the bone marrow to tumor tissues and circulation, different studies have been proposed that they may be useful as diagnostic and prognostic factor (Fig. 2d). Numerous laboratories have studied whether variation in the number of EPCs in peripheral blood could represent a tool to predict a pathological state. Importantly, the number of circulating EPCs can vary between patients and healthy people and between patients affected by the same pathology. Moreover, the number of circulating EPCs could be correlated with clinical outcomes in some cases.

In cancer patients, EPC levels are higher than in healthy people, due to their mobilization from the bone marrow to contribute in new vessel formation. Therefore, circulating EPC levels could be useful as a diagnostic and prognostic tool to monitor the clinical state of patients. For example, EPC levels correlate with poor overall survival in non-small cell lung cancer (NSCLC). Moreover, NSCLC patients with partial or complete remission after anticancer therapy present lower levels of circulating EPCs than patients with stable or progressive disease, suggesting that EPC levels correlate with the efficacy of treatment (Dome et al. 2006). In addition, EPCs have been proposed as biomarkers to monitor the progression of the disease and discriminate between good and bad response to therapies. Several studies have demonstrated that EPCs may be used as a good marker to evaluate the response of colorectal cancer patients to anti-angiogenic drugs (Matsusaka et al. 2011). Additionally, chemotherapy reduces the number of circulating EPCs in breast cancer patients with high levels of EPCs, although it induces the mobilization of EPCs from the bone marrow at the same time. Therefore, the combination of anti-angiogenic therapies and chemotherapy could be useful to avoid the possible pro-angiogenic effects of EPC mobilization after chemotherapy. Another approach is the possible use of EPCs in the evaluation of tumor stage. In late-stage gastric cancer patients, the number of EPCs in cancer tissue and adjacent tissue was lower than in early-stage patients. Furthermore, levels of other molecules, such as VEGF and hematopoietic progenitor cells, are higher in cancer patients, and the combination of all of them may be considered to monitor the progression of the disease (Nowak et al. 2010). Nevertheless, further studies are needed to confirm the use of EPCs as a diagnostic and prognostic factor in cancer.

EPCs in Anti-Angiogenic Treatments

Given that EPC mobilization contributes to tumor angiogenesis and metastasis, blocking this process would inhibit the formation of new blood vessels and the metastatic niche (Moccia et al. 2015). One strategy could consist in blocking the molecules involved in the homing of EPCs to tumor vasculature or the factors responsible for their recruitment from the bone marrow (Fig. 2a). Given that SDF-1 is the most important regulator of EPC mobilization and CXCR4 disruption is essential for the mobilization of EPCs to the

circulation, different agonists and antagonists of CXCR4 have been proposed, such as small peptide antagonists and agonists, non-peptide CXCR4 antagonists, antibodies to CXCR4, and modified antagonists for SDF-1 (Burger and Peled 2009). All of these compounds act preventing the gradient of chemokines that allows the homing of EPCs to the tumor site.

Another important factor involved in the EPC mobilization is VEGF. Most anti-angiogenic therapies are designed to inhibit the interaction between VEGF and VEGFR using neutralizing antibodies, soluble receptors, and small-molecule inhibitors. Preclinical studies demonstrated that VEGF inhibition negatively modulates EPC-mediated vasculogenesis (Kerbel and Folkman 2002). Nevertheless, some clinical studies have suggested the possible role of EPCs in the acquisition of tumor resistance to vascular-disrupting agents (VDAs) (Taylor et al. 2012). For this reason, combinational treatments of anti-VEGF or VDAs with EPC-targeting drugs should be evaluated.

Inhibition of Extracellular Matrix Remodeling

The communication between cells and their microenvironment is very important in the control of development and homeostasis. Cell-ECM interactions regulate many processes, such as morphogenesis, differentiation, and organ architecture. Bidirectional communication between components of the ECM and cells led the cell to sense their microenvironment, transfer this molecular information from outside the cells to inside, and finally initiate cellular response. Additionally, the transfer of biochemical information from cells to the ECM is also important, because cells can response altering their local microenvironment by inside-out communication. Integrins are the most important cell adhesion receptors that mediate this bidirectional communication system (Stupack and Cheresh 2004).

Given the importance of cell-ECM interactions in the control of cell behavior, it is not surprising that cell-ECM interactions play a critical role in regulating angiogenesis, tumor growth, and metastasis. Consequently, affecting cell-ECM interactions may produce anti-angiogenic effects and could be used as anticancer drugs (Yang et al. 2003).

Importantly, targeting the link between endothelial cells and ECM-inhibiting integrins may be more effective (Serini et al. 2006). Finally, another approach is the inhibition of the interaction between endothelial cells and pericytes or adjacent tumor cells by the inhibition of N-cadherin-mediated junctions.

Extracellular Matrix in Angiogenesis

Composition and Structural Organization of Vascular Extracellular Matrix

ECM composition and organization is essential in the regulation of angiogenesis. This was evidenced in mice with alterations in the expression and functions of ECM molecules such as collagen, fibronectin, and laminin. It has been observed that mice with mutations in these proteins exhibit vascular abnormalities (Hirsch et al. 2000).

The ECM of vessels is constituted by BM. BMs are mainly composed by two multidomain glycoproteins, collagen type IV and laminin, which are interconnected with proteoglycans such as nidogen and perlecan (Kalluri 2003). Other minor components such as collagen type VIII, XV, and XVIII also constitute the BM. The composition and structural integrity of the BM can vary between tissue compartment and during developmental and pathological processes. Additionally, BMs provide critical binding sites for other ECM proteins, integrin receptors, growth factors, and cell surface proteoglycans. The interactions between cells and these BM components regulate many signaling pathways including phosphoinositide 3-kinase (PI3K)-AKT8 virus oncogene cellular homolog (AKT), mitogen-activated protein kinase (MAPK)-ERK, and Jun amino-terminal kinase (JNK) cascades, which are implicated in angiogenesis processes, such as cell adhesion, migration, invasion, proliferation, survival, and differentiation of endothelial cells (Chen et al. 2004). BMs also include the surrounding interstitial matrix composed by distinct

collagens such as type I, II, and III as well as fibronectin, fibrinogen, thrombospondin, vitronectin, and elastin. This interstitial matrix presents multiple protein-binding sites creating an elaborate network. Moreover, interstitial matrix can function as a reservoir of regulatory molecules including angiogenic growth factors, cytokines, motility factors, proteolytic enzymes, and protease inhibitors (Mott and Werb 2004). For example, two binding domains to fibronectin were identified in VEGF molecule, whose function is to enhance the activity of VEGF. Therefore, the interaction between ECM components, integrins, and growth factors controls the development of new blood vessels and may constitute a good target for anti-angiogenic therapies.

Cell Adhesion Receptors

In general, angiogenesis is organized in different steps and in all of them, cell adhesion has an important role (D'Amore and Thompson 1987). Firstly, in the initiation phase, growth factors and cytokines are released, initiating signaling transduction pathways that lead in endothelial cell activation. At this point, endothelial cells acquire an invasive phenotype, which produce cell-cell dissociation, extracellular matrix remodeling by the protease secretion, and alterations in the expression of cell surface adhesion receptors. The activated endothelial cells interact with the remodeled BM components and start to invade the local interstitium. After that, endothelial cells remodel their microenvironment again and interact with the modified interstitial ECM components leading to morphogenesis and cellular reorganization into tubelike structures. Finally, in the maturation phase, endothelial cells start to express new matrix components, reorganizing cell-cell interactions with pericytes and differentiating into functional blood vessels.

Given the changing interactions of endothelial cells with the ECM components in all the angiogenesis process, cell adhesion molecules may have a crucial role in this process. The main families of cell adhesion molecules include cadherins, selectins, immunoglobulin supergene family members, and integrins (Brooks 1996). Interestingly, recent studies have demonstrated the possible contribution of cell surface receptor tyrosine kinase and various proteoglycans in the regulation of cell adhesion and the possible implications in the angiogenesis process (Beauvais et al. 2009).

Integrins

Given the importance of bidirectional communication between endothelial cells and their microenvironment in the regulation of angiogenesis, the integrin family could play crucial roles in this process. Integrins are heterodimeric receptors whose function is to mediate cell-ECM interactions. The first studies about the possible importance of cell-ECM interactions in regulating cellular processes were done in the 1980s by several investigators, notably Drs. *Hynes*, *Ruoslathi*, *and Springer* (Xiong et al. 2003; Hynes 2004; Pytela et al. 1985). In these studies, some of the first described integrins were isolated and cloned, such as fibronectin receptor α5β1 and the platelet fibrinogen receptor αIIbβ3. The observation of the integrins connecting ECM molecules to the cytoskeletal provided important evidence for the concept that cells communicate bidirectionally with the local microenvironment. To date, 24 integrin heterodimers have been identified resulting between the interaction of 18α and 8β subunits, each with specific distribution and functions. In general, integrins are organized into three domains, including extracellular cation-dependent ligand-binding domain, transmembrane regions, and an intracellular tail known to interact with cytoskeletal components. Moreover, integrins contain metal ion-dependent adhesion sites (MIDAS), which are needed for their binding to integrin ligands (Mould and Humphries 2004). Importantly, many function-blocking integrin antagonists are directed to the MIDAS region blocking the interaction between the integrins with their ligands.

Integrins are not only attaching the cells to the microenvironment, but they can sense and respond to their microenvironment inducing signaling cascades. Integrin response relies on the recruitment of adaptor proteins that behave as molecular hubs for intracellular signaling and organize complex signaling networks

(DeMali et al. 2003). Importantly, the capacity of integrins to recognize and bind to their ligands depends on their activation state. Conformational changes within the cytoplasmic tails of integrins induced by intracellular signaling events cause integrin activation and enhanced ligand binding through a processed termed inside-out signaling. In contrast, molecular information of outside the cell can be transmitted to the cell interior in a process called outside-in signaling.

Different studies have demonstrated and confirmed the importance of integrins in angiogenesis and blood vessel formation. For endothelial cells, integrins are the most important partners for growth factor receptors. For example, it is well established that some growth factors, such as VEGFs, are able to activate various integrins (Byzova et al. 2000). However, the relationship between the integrin and growth factor receptor seems to be more complex than unidirectional activation. Integrins can induce growth factor signaling and vice versa; growth factors can modulate integrin activity. The best known example is the cross talk between VEGFR2 and αvβ3 integrin, which controls the adhesion and migration of endothelial cells during vascular development and VEGF-induced angiogenesis. Moreover, treatment with VEGF or integrin ligation induces the formation of a complex between these two receptors. Also the cross talk or complex could be a good target for angiogenesis regulation. A study has shown that the bone morphogenic protein antagonist, gremlin, binds to VEGFR2 stimulating the interaction with αvβ3 integrin, and finally, it induces angiogenesis (Ravelli et al. 2013).

During angiogenesis, endothelial cells interact with various ECM proteins, and therefore they need to express more than one integrin during their migration, induced by VEGF/VEGFR2. For this reason, VEGFR2 may also interact with other integrins such as β1 integrin. This synergism is mediated by tetraspanin CD63, which interacts with β1 integrins and VEGFR2, and functions as a regulator of the complex between these two receptors (Tugues et al. 2013).

Therefore, the interaction between many integrins and growth factor tyrosine kinase receptors in a coordinated manner is necessary for the correct adhesion and migration of endothelial cells during angiogenesis. Consequently, blocking integrin interactions and complex formation may inhibit angiogenesis.

Other Receptors

Apart from integrins, there are other adhesion molecules which mediate cell-cell interactions, such as cadherins, selectins, and immunoglobulin family members (Brooks 1996). During angiogenesis, endothelial cells express a particular adhesion molecule in each angiogenesis step.

The first evidence of vascular endothelial cadherin (VE-cadherin) implication in angiogenesis came from experiments using functional blocking antibodies in in vitro angiogenesis (Matsumura et al. 1997). From that moment, many studies have demonstrated the implications of VE-cadherin in the angiogenesis process and in the tumor angiogenesis. During angiogenesis, VE-cadherin expression on the adherens junction disappears and epitopes are unmasked. Moreover, VE-cadherin is also implicated in vascular proliferation and in lumen formation (Nelson and Chen 2003).

E-selectin is an endothelial membrane glycoprotein implicated in the adhesion of leucocytes to cytokine-activated endothelial cells. The possible implications of E-selectin in angiogenesis come from the observation that antibodies against this protein inhibit capillary-like tubes in vitro (Nguyen et al. 1993). Additionally, E-selectin is expressed in proliferating endothelial cells in hemangioma tumors suggesting the possible participation in angiogenesis (Kraling et al. 1996). Therefore, there is evidence of the association of E-selectin with the angiogenesis process, but the mechanism by which E-selectin contributes is still unknown.

More studies are needed to elucidate the potential roles of E-selectin, VE-cadherin, and other unknown adhesion molecules in angiogenesis. Deeper insight into the mechanism by which they regulate endothelial cell interactions will contribute to the development of new anti-angiogenic drugs.

Targeting Angiogenic Microenvironment

Inhibition of the Extracellular Matrix

As interactions between cells and ECM play an important role in angiogenesis regulation, structural and biochemical modifications of this ECM may affect the cell behavior and angiogenesis (Seiki et al. 2003). Several studies have demonstrated that the composition, structural integrity, and biochemical characteristics of the ECM are modified during many pathological processes such as tumor growth, invasion, metastasis, and angiogenesis. New ECM proteins are secreted by inflammatory cells, stromal fibroblasts, tumor cells, and endothelial cells altering the microenvironment composition. The changes in the ECM composition can also alter the integrin ligands within the local microenvironment (McCarthy et al. 2004). Therefore, alterations in the composition or biochemical characteristics of ECM proteins consequently alter the integrin mediated cross talk between cells and the ECM (Fig. 3a).

The remodeling of the ECM is a well-studied process that affects cellular behavior. Matrix-degrading enzymes are important in regulating invasive cellular processes such as angiogenesis, tumor invasion, and metastasis. Some studies demonstrated that deficient mice in MMPs, such as MMP-2 and MMP-9, present defects in tumor growth and angiogenesis (Masson et al. 2005). Additionally, other studies showed that tissue inhibitor of MMPs (TIMPs) as well as synthetic inhibitors of serine proteases, such as

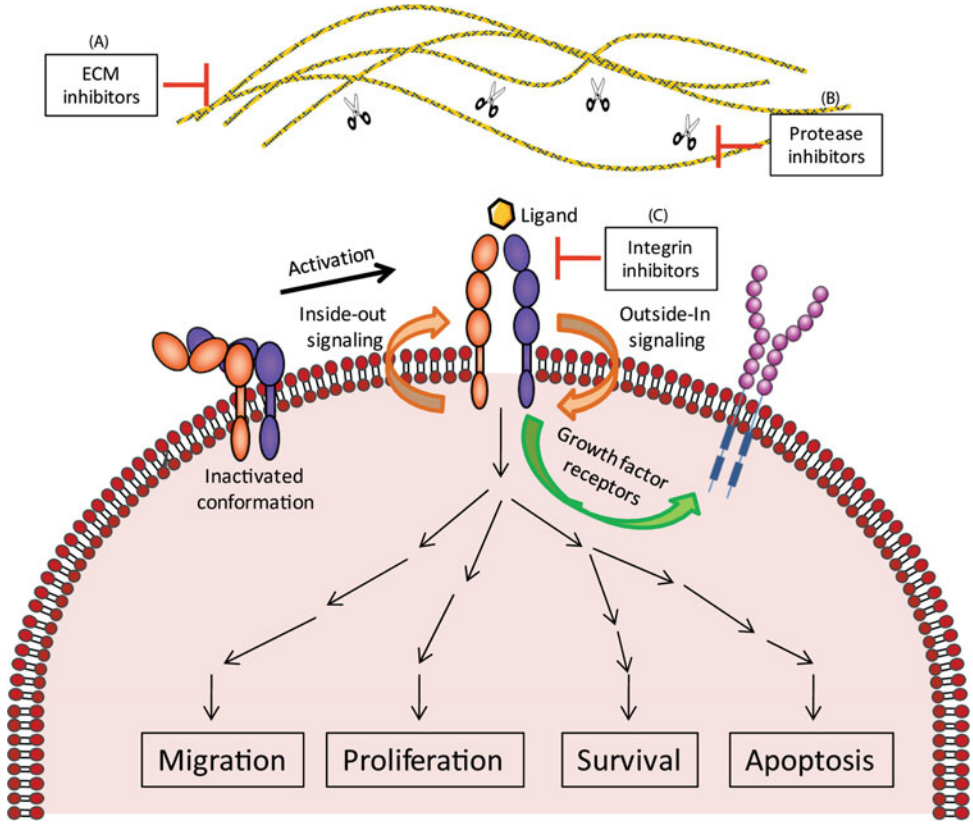

Fig. 3 *Targeting of extracellular matrix contribution in angiogenesis process*. Bidirectional communication between endothelial cells and extracellular matrix (inside-out and outside-in signaling) is important in the angiogenesis process. (**a**) Inhibition of extracellular matrix components or (**b**) protease activity produces anti-angiogenic effects. Integrins mediate the interaction between extracellular matrix and endothelial cells. (**c**) Alterations in expression, ligand binding, or activation state of integrins affect the angiogenesis process

urokinase-type plasminogen activator (uPA), can inhibit angiogenesis and tumor growth. Moreover, proteolytic enzymes can regulate invasive cellular behavior by altering ECM, creating a less restrictive microenvironment through which tumor and endothelial cells can migrate (Noel et al. 2004) (Fig. 3b). Proteolytic enzymes can also stimulate an invasive cell phenotype exposing integrin ligands of the ECM that regulate motility, proliferation, and gene expression. In addition, many other mechanisms by which proteolytic enzymes can induce invasive cellular behavior have been proposed such as a release of growth factors like VEGF by cleaving matrix-bound sites.

Many laboratories have tried to find natural angiogenesis inhibitors derived from ECM proteins. For example, arrestin, canstatin, and tumstatin inhibit angiogenesis through binding to αvβ3 integrin and β1 integrins (Petitclerc et al. 2000). Therefore, ECM fragments can inhibit the interaction between integrins and their microenvironment affecting the adhesion of endothelial cells and in consequence blocking angiogenesis.

Inhibition of Integrins

As mentioned before, integrins represent important possible targets to inhibit angiogenesis. Many integrin receptors play key roles in angiogenesis, including α1β1, α2β1, α3β1, α4β1, α5β1, α6β4, αvβ3, and αvβ5 (Hwang and Varner 2004). Granted that integrins are well studied, many strategies could be designed to alter the expression, ligand binding, or activation state by siRNA, small-molecule inhibitors, peptide memetics, and antibodies (Fig. 3c). Preclinical and clinical trials have tested many of these approaches for the control of angiogenesis and tumor progression. For example, clinical trials have studied various monoclonal antibodies directed to integrin receptors for the treatment of human cancers, such as Vitaxin (humanized monoclonal antibody directed to αvβ3 integrin) (Gutheil et al. 2000), CNTO 95 (monoclonal antibody against αv) (Trikha et al. 2004), and monoclonal antibody M200 (monoclonal antibody directed to the fibronectin receptor α5β1). Therefore, some of these anti-integrin drugs may be useful in the treatment of angiogenic neoplasm.

To optimize the effectiveness and specificity of integrin antagonists, it is very important to understand the particular functions of integrin inhibitors and identify the particular cell type in which the integrin targets are expressed. These aspects are very important given that binding specificity and functions can vary for a particular integrin, as well as a variety of cell types contribute to the regulation of angiogenesis, including endothelial progenitors, inflammatory cells, stromal fibroblasts, pericytes, and tumor cells (Jung et al. 2002). Interestingly, integrin conformation could change depending on the concentration and affinities of the integrin-binding molecule leading to activation or inhibition of integrin function.

β1 and α1 integrins play important roles in angiogenesis. In detail, mice-harboring mutation in several collagen molecules, which represent ligands for β1 integrins, presented defects in vascular development (Marneros and Olsen 2005). Collagen-binding integrins α1β1 and α2β1 contribute to the regulation of VEGF-induced angiogenesis. Therefore, the inhibition of collagen-binding integrins α1β1 and α2β1 may inhibit angiogenesis. Studies have demonstrated that mice treated with function-blocking antibodies against α1 or α2 integrin partially inhibited angiogenesis in vivo (Senger et al. 1997). This inhibition disrupted endothelial cell adhesion to collagen, suggesting the possibility that collagen-binding β1 integrin-signaling cascades play roles in VEGF-dependent angiogenesis.

Another important β1 integrin implicated in angiogenesis is the laminin-binding α3β1 integrin. α3β1 integrin is expressed in endothelial cells and angiogenic blood vessels and bound to laminin and collagen. α3β1 modulates angiogenesis by the association with thrombospondin-1 (TSP-1), an endogenous angiogenesis inhibitor, and uPA receptor (uPAR), receptor associated with proliferation and motility in angiogenesis (Short et al. 2005).

Finally, fibronectin receptors α4β1 and α5β1 are the last β1 integrin receptors implicated in new blood vessel formation. Studies in null mice for α4 and α5 suggested a role of these integrins in vascular development and blood vessel formation. Integrin α4β1 can serve as a counter-receptor for

the cell adhesion molecule vascular cell adhesion molecule-1 (VCAM-1), adhesion molecules expressed by endothelial cells during angiogenesis (Yang et al. 1995). Moreover, studies have suggested that α5β1 integrin is highly expressed in angiogenic blood vessels and can regulate cell survival and apoptosis. Antagonists of α5β1 produce an endothelial cell function inhibition in vitro and an inhibition of angiogenesis in vivo (Kim et al. 2000).

Perhaps the most studied integrin in angiogenesis is the αv integrin subfamily. Studies have shown that angiogenesis induced by fibroblast growth factor-basic (bFGF) and tumor necrosis factor-α (TNF-α) requires the integrin αvβ3 function, whereas angiogenesis induced by VEGF or transforming growth factor-α (TGF-α) requires αvβ5 integrin function (Friedlander et al. 1995). Moreover, other non-ECM proteins may also bind to αv integrins regulating angiogenesis, such as MMP-2 and uPAR. Given the evidence that αvβ3 plays significant roles in angiogenesis, many studies have been focused on the mechanisms by which αvβ3 regulates new blood vessel formation. Studies have shown that αvβ3 does not only facilitate endothelial cell adhesion and migration, it can also regulate endothelial cell survival and apoptosis. Studies in melanoma demonstrated that blocking αvβ3 induced apoptosis on human melanoma tumors suggesting that αvβ3 integrin may regulate apoptosis in both tumor cells and endothelial cells. Moreover, several studies have shown the possible association between αvβ3 integrin and VEGF or VEGFR (De et al. 2005). Importantly, αvβ3 integrin is expressed in angiogenic endothelial cells but not in quiescent endothelial cells. Therefore, monoclonal antibodies against αvβ3 integrin such as LM609 can inhibit the invasive and proliferative phenotype of endothelial cells suppressing angiogenesis (Drake et al. 1995).

Regression of Tumor Blood Vessels

The most typical approximation about inhibition of blood vessels is the inhibition of the formation of new vessels. However, preexisting tumor vasculature can be inhibited resulting in a regression of the tumor vessels. In this context, the cessation of blood flow would trigger death of endothelial cells by apoptosis or necrosis, leading to the regression of vessels, and finally tumor cell death. Several approximations aim to regress tumor blood vessels, which include vascular disruption and reduction of endothelial cells. VDAs, divided into flavonoids and tubulin-binding agents (TBAs), can cease blood flow in tumors in minutes and lead to the formation of extensive central necrosis. Moreover, directing tissue factor to antigenic epitopes expressed in tumor blood vessels would induce intravascular coagulation and cessation of blood flow (Huang et al. 1997). Growth factors, such as VEGF, not only induce the formation of new blood vessels but also regulate endothelial cell survival in existing vessels. Targeting these growth factors by anti-VEGF drugs or others, such as endogenous inhibitors TSP-1, endostatin, angiostatin, and tumstatin, decreases the permeability of tumor vessels and also increases apoptosis of endothelial cells (Inai et al. 2004). Finally, cytotoxic chemotherapeutic drugs can induce endothelial cell apoptosis besides tumor cell death. The anti-vascular effect of chemotherapeutic drugs is due to the cytotoxicity on proliferating endothelial cells contributing to their anticancer action.

Vascular-Disrupting Agents

Regression therapy has focused on the development of agents that inhibit the abnormal vasculature present in the tumor at the time of detection and treatment. This strategy uses VDAs to cause a rapid and catastrophic shutdown in the vascular function resulting in death of cells supplied by those vessels as a result of oxygen and nutrient deprivation. This approximation presents some differences in comparison with current anti-angiogenic strategies. While anti-angiogenic therapies interfere with new vessel formation preventing tumor growth and limiting metastatic potential, VDA attacks established tumor vasculature destroying tumor masses as well as preventing progression. Given these differences, the therapeutic applications of anti-angiogenics and

VDAs are complimentary and not redundant (Chaplin et al. 2006).

VDA Concept

VDAs are ligand-specific or small molecules that selectively target preexisting tumor vasculature and rapidly shut down the blood flow of tumor tissue. They produce ischemia and consequently tumor cell death. The efficacy of VDAs is variable depending on tumor type and vessel fragility, instability, and cell-to-cell endothelial junctions' defects of tumor blood vessels (Siemann et al. 2005).

Ligand-specific VDAs are antibodies, peptides, and growth factors, which selectively bind to the endothelium. The conjugation of a toxin or a procoagulant factor with them induces endothelial cell death (Thorpe et al. 2003).

The two main categories of small-molecules VDAs are flavonoids and TBAs (Lippert 2007). Flavonoids produce partial derangement of the actin cytoskeleton, DNA strand break, and apoptosis of endothelial cells, along with macrophage activation and cytokine release. TBAs induce tubulin depolymerization and disorganization of both tubulin and actin cytoskeleton, due to their binding to different sites of tubulin.

History of VDAs

Given tumoral blood vessels present functional and morphological differences in comparison to normal vessels, tumor vasculature may in principle be killed specifically, leading to tumor cell death. The first evidence was shown by *Denekamp* et al. in in vivo models. This study demonstrated that endothelial cells from tumors proliferated faster than endothelial cells from normal tissues (Denekamp and Hobson 1982). Later, this evidence was confirmed in human tumors (Eberhard et al. 2000). Based on their studies, *Denekamp* proposed vascular disruption approach to treat cancer and continued investigating the vascular collapse necessary to produce antitumoral effects (Denekamp et al. 1983). Around 1980, different studies demonstrated that some of the emergent cancer treatments presented antivascular effects. Many laboratories were interested in the identification of new molecules expressed only in the tumor vasculature to develop new drugs for cancer therapy. More recently, studies by *Burrows and Thorpe* in animal models have shown the efficacy of drugs against tumoral vascular endothelium (Burrows and Thorpe 1993).

Types of Vascular-Disrupting Agents

Flavonoids

Flavonoids are polyphenolic compounds found in a variety of vegetables, fruits, tea, and red wine. They present many pharmacological applications due to their inhibition of tumor cell proliferation and their anti-angiogenic effects (Andrea et al. 2013).

The first VDA described was flavone acetic acid (FAA). The studies in animal models were promising, but early phase clinical studies demonstrated negative results in humans (Hasani and Leighl 2011). After that, some laboratories tried to synthesize and develop many FAA analogs leading to the identification of 5,6-dimethylxanthenone-4-acetic acid ASA404 (DMXAA, Vadimezan, Novartis) and xhantenone-4-acetic acid (XAA) analogs. The development of these analogs demonstrated that vascular-disrupting activity was primarily dependent on the position of the substituents rather than on their nature. In vitro studies demonstrated that derivations of XAA analogs could stimulate human leukocytes to produce interleukin-6 (IL-6) and IL-8 and inhibit tube formation in human endothelial-like cells. However, studies using mouse models showed that the most active compound in human cells was inactive in murine models, suggesting the need for the use of appropriate in vivo animal models in selecting clinical candidates (Tijono et al. 2013).

More recently, in vivo studies have shown that ASA404 can shut down the tumor vasculature and inhibit rapidly the blood flow leading to necrosis of the tumor and hypoxia, after an hour of administration. Moreover, this flavonoid induces apoptosis of endothelial cells in the tumor vasculature and increases the vascular permeability

(Zhao et al. 2005). The mechanism of action of flavonoids as VDAs seems to be associated with the induction of local cytokine production such as TNF-α, interferons, and interleukins through the activation of the NF-xB pathway in monocytes, macrophages, vascular endothelial cells, and tumor cells (Roberts et al. 2007). Therefore, ASA404 produces the disruption of the tumor vasculature, the induction of innate immune cells, and the activation of platelets. Additionally, platelet activation induces the release of von Willebrand factor and consequently generates hypoxia. Finally, hypoxia promotes the release of VEGF that contributes with TNF-α to a positive feedback loop that increases vascular permeability, leading to tumor hemorrhagic necrosis.

Tubulin Binding Agents

TBAs were originally used as antimitotics against cancer, but anti-vascular activities were also identified. TBAs bind to tubulin and induce microtubules polymerization and stabilization or microtubule depolymerization and instability (Jordan and Wilson 2004). These VDAs modify the cytoskeleton organization of endothelial cells changing endothelial shape and leading to vessel blockage, reduction in blood flow, and disruption of the endothelial cell layer. Moreover, the exposure of the BM activates the coagulation cascade increasing the vessel permeability. Indeed, TBAs at low concentrations can affect microtubule dynamics inhibiting their contacts, such as focal adhesions and adherens junctions. Thus, TBAs inhibit endothelial cell adhesion, motility, and cell-cell interactions (Schwartz 2009).

TBAs can also interfere with the normal organization of actin stress fibers resulting in the loss of cell polarity and in the inhibition of cell contractility. Moreover, TBAs can act on focal adhesion kinase (FAK) and VE-cadherin signaling pathways disrupting adherens junction assembly. Importantly, these adherens are critical for angiogenic sprouting and for the maintenance of vascular integrity (Vincent et al. 2005).

Combrastatin-A4 (CA-4) is one of the most well-known TBAs. CA-4, isolated from the African tree *Combretum caffrum*, emerged as a promising VDA. The mechanism of action and the anti-vascular effect have been investigated in vitro and in various tumor models (Dark et al. 1997). Moreover, CA-4P (soluble prodrug of CA-4) was approved for the treatment of various thyroid cancers. However, CA-4P presented some side effects such as enhanced pain. After that, many synthetic analogs were synthesized to modify CA-4 structure in order to improve the activity and reduce adverse reactions, such as TR-644 and BNC105. In particular, TR-644 presents higher microtubule depolymerizing activity. In animal tumor models, TR-644 significantly reduced the number of vessels after 24 h from the administration of a single dose (Porcu et al. 2013).

Therapeutic Approaches of VDAs

VDAs as Monotherapy

Three different phase I clinical trials demonstrated the antitumoral activity of ASA404 at well-tolerated doses (Baguley and Siemann 2010). In phase II trials, ASA404 was administrated in combination with taxanes and carboplatin in different types of cancers. Despite the results seemed promising with improved tumor response and median survival increase, they were not demonstrated statistical significant. Finally, phase III trials failed to demonstrate survival advantages or improvement of overall survival (Lorusso et al. 2011).

Until now, phase I trials with TBAs, such as AVE8062, OXi4503, MPC-6827, ABT-751, and BNC105P, have been carried out in patients resistant to traditional therapies, with advanced solid tumors (Innocenti et al. 2013). AVE8062, CA-4 analog, causes a rapid and irreversible reduction of blood flow in different experimental tumor models. The administration of this analog as monotherapy in patients with advanced solid tumors produces antitumor effects and increases circulating endothelial cells, MMPs, and VEGF. Nevertheless, phase III trials as first lines in NSCLC and as second line in soft tissue sarcoma failed, and its development was stopped. MPC-6827 was evaluated in phase II trials, and although it was well tolerated, its activity was limited. Finally, BCN105P and CYT997 are two

other promising TBAs in clinical development (Burge et al. 2013).

Resistance to VDA Treatments

Despite, VDAs have demonstrated their efficacy in the treatment of cancer; preclinical studies and clinical trials have shown the existence of a residual viable tumor rim after treatment of solid tumors with VDAs (Wu et al. 2013). Therefore, surviving tumor cells after VDA treatments can induce resistance to this therapy. Several mechanisms to explain tumor resistance have been proposed related to hypoxia, tumor-associated macrophages, and bone marrow-derived circulating endothelial progenitor cells (Welford et al. 2011). Additionally, a variety of magnetic resonance imaging (MRI) studies have allowed to observe and to quantify the tumor resistance process. Many strategies have been suggested to improve the antitumor effects of VDAs and to prevent the acquisition of tumor resistance. The most promising strategy is the combination of VDAs with other approaches including anti-angiogenic agents, chemotherapy, and radiotherapy.

VDAs in Combination Therapy

Greater antitumor effects have been achieved when conventional chemotherapy is combined with VDAs. Cells comprising the viable rim of tumor tissue that survives after VDA treatment have a high proliferation rate and excellent nutrition. Therefore, VDA-resistant tumors show enhanced accessibility to systemically administrated agents making the surviving tumor cells susceptible to be killed by radiation and anticancer drugs (Chung et al. 2008). Therefore, the combination of VDAs with conventional therapies may improve the therapeutic potential of each strategy used as monotherapy.

Different studies have reported enhancements in antitumor activities when VDAs were combined with standard chemotherapy (Grosios et al. 2000), principally by post-chemotherapy administration.

In vivo studies in mice models demonstrated the synergism of VEGF inhibition with VDAs. Studies in patients treated with bevacizumab 4 h after CA4-P significantly reduced vascular permeability and tumor perfusion (Cesca et al. 2013). Moreover, phase II clinical trial has demonstrated that the combination of bevacizumab and CA4-P in recurrent ovarian cancer increases significantly the progression-free survival in comparison to bevacizumab alone (Mitrus et al. 2009).

Numerous studies have shown the synergistic effects of ASA404 with carboplatin and paclitaxel, but phase III trial failed (Farace et al. 2007). Another preclinical study investigated the effects of combining ASA404 and everolimus in renal cell carcinoma. The combination produced extensive necrosis and a reduction in the viable rim with respect to ASA404 alone (Wilczynski et al. 2011).

Importantly, the incorrect schedule of combined treatment can cause therapeutic failure. Therefore, more studies are needed to find the best approach and increase the therapeutic potential of the combination therapy.

Normalization of Tumor Vasculature and Microenvironment

Traditional anti-angiogenic strategies try to inhibit new vessel formation or to destroy existing vessels. Nevertheless, it is known that these therapies have insufficient efficacy and tumors can acquire resistance (Ferrara 2010). In the 1990s, preclinical studies showed the concept of vascular normalization. In these studies, VEGF signaling inhibition transiently repaired the vascular abnormalities improving tumor oxygenation and decreasing interstitial fluid pressure. Historically, vessel normalization was initially identified as vessel remodeling in human tumor xenografts. Several studies in mouse models have demonstrated the positive effects of promoting vascular normalization, such as improving tumor vessel perfusion and oxygenation (Fig. 4). Importantly, vascular normalization reduced metastasis and improved the efficacy of chemotherapies and immunotherapies (Mazzone et al. 2009).

Therefore, tumor vessel normalization may reduce the metastatic dissemination and improve

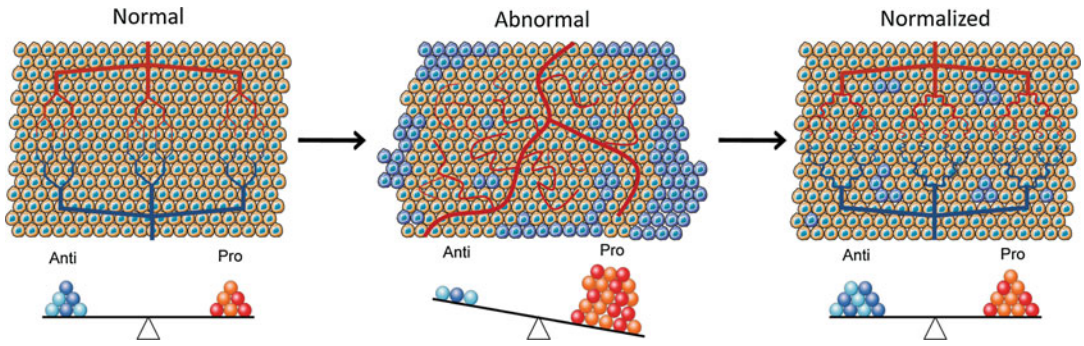

Fig. 4 *Normalization of tumor vessels in response to anti-angiogenic therapies*. (**a**) Vasculature of the tumors is structurally and functionally abnormal. Anti-angiogenic therapies improve both structure and function of the tumor vessels normalizing the tumor vasculature. (**b**) The abnormal vasculature reflects the changes in the balance of pro-angiogenic and anti-angiogenic factors in the tissue

the response to conventional therapies. The possible advantages of vessel normalization compared to traditional therapies have been debated. Nevertheless, increasing evidence indicates that vessel normalization might complement current anti-angiogenic strategies.

Abnormalities of Tumor Vessels

Overexpression of VEGF and other pro-angiogenic factors, induced in hypoxia conditions, leads to formation of a new vasculature that is structurally and functionally abnormal. Moreover, these abnormalities are exacerbated as a tumor continues to grow (Gazit et al. 1997).

Structural Abnormalities

Differentially to normal vessels, tumor vessels are tortuous; they branch irregularly in a chaotic network of tangles connecting randomly with other vessels and with stroma (Nagy et al. 2010). Moreover, tumor vessels are very heterogeneous, the vessel diameter is irregular, some vessels are oversized, and others are more immature. These anomalies have been observed in a wide range of tumor types.

The endothelial cells are also abnormal. Activated tumor endothelial cells lose their polarity, allowing endothelial cells to detach from BM and stack upon each other. Moreover, tumor endothelial cells produce extension into the lumen and form sprouts, with leading tip cells penetrating deep into the tissue. Additionally, these endothelial cells contain multiple fenestrations and other transendothelial channels, resulting in hemorrhage and increased interstitial fluid pressure (Jain 1988).

Tumor vessels are entirely affected. Therefore, all the vessel components are affected, such as pericytes or BM. Activated pericytes in cancers lose their association with endothelial cells and activate processes inside the stroma. Moreover, they change their shape and express more immature markers (Morikawa et al. 2002). In addition, vessel coverage by pericytes in tumor vessels is reduced. The pericyte-deficient condition compromises the vessel wall, favoring the intravasation of tumor cells. Finally, the tumor vessel BM often loses their interaction with endothelial cells and presents an aberrant thickness (Baluk et al. 2005).

Functional Abnormalities

The increased vascular resistance and the improper vasoregulation in tumors compromise the blood flow. Moreover, in tumors, the interstitial fluid pressure is increased and the perfusion is heterogeneous. These flow patterns create an obstacle to a uniform delivery of nutrients and drugs. In fact, properties of vascular barriers can also determine the penetration of the drugs into the tumor. Importantly, hypoxia induced by radiation and chemotherapeutics can reduce the efficacy of conventional anticancer treatments. The high metabolic demand of tumor cells produces

an excess of pro-angiogenic factors. Pro-angiogenic factors induce the formation of abnormal vessels, and these abnormal vessels are unable to fulfill the entire tumor requirements, creating a self-reinforcing vicious cycle (Rey and Semenza 2010).

The abnormal vessel structure and function increase interstitial hypertension, hypoxia, and acidosis, creating a favorable environment for tumor progression and metastasis. Tumor cells to invade must cross the tumor-blood barrier by invading the vascular BM, transmigrating across the endothelium, surviving in the blood, and finally extravasating and growing at a distant site (Kienast et al. 2010). Abnormal tumor vessels facilitate this process because the physical barrier does not exist and also because tumor endothelial cells produce proteinases, adhesion molecules, and other factors that facilitate the tumor cell migration (Sullivan and Graham 2007).

Therefore, tumor vessel abnormalities can provide a favorable environment for invasion and metastasis of tumor cells and can influence tumor responsiveness to conventional anticancer treatment.

Tumor Vessel Normalization

In tumors, the oncogenic, hypoxic, metabolic, and inflammatory pathways stimulate the production of angiogenic inductors, tipping the balance in favor of forming new vessels. Indeed, in contrast to physiological angiogenesis, the stimulation of angiogenesis persists and, consequently, tumor vessels become increasingly abnormal. Thus, targeting different components of the tumor vessel wall may restore this balance (Jain 2005).

Normalization of Endothelial Cells

VEGF is the most important and well-known pro-angiogenic factor and is implicated in many angiogenic aspects, such as endothelial cell growth, migration, and permeability. Moreover, preclinical studies have associated high levels of VEGF with vessel abnormalities. Therefore, the inhibition of the VEGF or its signaling pathway may decrease vessel abnormalities. Anti-VEGF drugs induce transient vessel normalization in preclinical models by reducing the enlarged size and tortuosity of vessels, increasing vessel maturation, enhancing pericyte coverage, and normalizing the BM (Baffert et al. 2006).

Moreover, some studies have demonstrated that blocking VEGF reduces interstitial fluid pressure, while transiently it increases perfusion, oxygenation, and drug delivery (Dickson et al. 2007). However, the prolongation of anti-VEGF treatment can finally destroy tumor vessels, and tumors can become resistant by induction of other pathways. Therefore, vessel normalization is limited to a transient window, and for this reason, some studies did not report some benefits of vessel normalization (Franco et al. 2006). In fact, new studies will help to understand whether and how the normalization window by VEGF blockade can be prolonged to enhance the benefits.

Prolyl hydroxylase domain-containing protein 2 (PHD2) is another example of angiogenic molecule that induces vessel normalization. PHD2 is an oxygen-sensing enzyme that hydroxylates HIFs when sufficient oxygen is available, and once HIFs are hydroxylated, they are targeted for proteosomal degradation. Under hypoxia conditions, PHD2 is inactive and HIFs can induce the response to increase the oxygen supply. Mouse deficiency for PHD2 in endothelial cells demonstrated that inhibition of PHD2 does not affect physiological angiogenesis but induces normalization of tumor vessels by reducing leakage, tortuosity, and remodeling and increasing endothelial cell quiescence, barrier tightening, and vessel maturation (De Bock et al. 2009). Moreover, these changes increase tumor perfusion, reduce tumor hypoxia, and shift tumor metabolism to a more aerobic glycolysis. Additionally, PHD2 deficiency decreases the invasion, intravasation, and metastasis by the induction of junctional molecules in endothelial cells, which provides a more impenetrable blood barrier.

Normalization of Vessel Microenvironment

The molecules implicated in the pericyte coverage, such as PDGFB, can also regulate the tumor vessel normalization. Sprouting endothelial cells

release PDGFB to chemo-attract pericytes that express its receptor, PDGFRB. The inhibition of PDGFB signaling causes pericyte deficiency, leading to vessel leakage, tortuosity, microaneurysm formation, and bleeding. Moreover, deficiencies in PDGFB or in pericytes form abnormal vessels (Huang et al. 2010).

Preclinical studies and studies in patients have demonstrated that deficiencies in pericyte coverage disassemble the vessel wall and promote metastasis (Yonenaga et al. 2005). In addition, overexpression of PDGFD normalizes tumor vessels and increases drug delivery. However, the inhibition of PDGFRB improves drug delivery and chemotherapy (Hellberg et al. 2010). Therefore, future studies are necessary to understand the possible role of PDGF inhibition in cancer treatment.

Regulator of G-protein signaling 5 (RGS5) is a molecule produced by activated pericytes and hypoxic endothelial cells, and its inhibition results in vessel normalization. Loss of RGS5 in pancreatic cancer model produces vessel normalization, with smaller and less leaky microvessels (Hamzah et al. 2008).

ANG-TIE (tunica interna endothelial cell kinase) receptor axis controls vessel maturation and regulates vessel normalization. The interaction between Ang-2 and its receptor, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2), on endothelial cells destabilizes vessels and promotes leakiness, whereas Ang-1, released by pericytes, induces the formation of pericyte coverage (Thurston et al. 1999). Consequently, vessel normalization is produced in glioblastomas with the inhibition of Ang-1 or the overexpression of Ang-2.

Finally, both genetic and pharmacological studies have shown that establishing perivascular gradients of nitric oxide (NO) normalizes tumor vessels. NO stimulates angiogenesis by endothelial NO synthase (eNOS), promoting the formation of stable vessels. However, tumor cells express neuronal NOS (nNOS) in human glioblastoma xenograft model, destabilizing the NO gradients. Finally, blocking nNOS in tumor cells restores the NO gradient and normalizes vessel phenotype (Kashiwagi et al. 2008).

Therapeutic Implications of Vascular Normalization

Rakesh Jain introduced the concept of vessel normalization in 2001 (Jain 2001). For this reason, the most compelling evidence about the vessel normalization stems from preclinical studies, and the translation to clinical studies has not been fully demonstrated yet. Moreover, it is difficult to obtain biopsy samples from patients during the treatment to evaluate vessel normalization. Therefore, some questions have not been answered yet, such as whether vessel normalization can be used as monotherapy or in combination with anti-angiogenic or cytotoxic therapies or whether vessel normalization can prevent metastasis.

Nevertheless, some signs of vessel normalization have been observed in cancer patients. For example, human biopsies of tumors show similar abnormalities to mouse tumors, such as high interstitial fluid pressure (Bullitt et al. 2004). Moreover, clinical studies have shown that anti-VEGF therapies induce some characteristics of vessel normalization, such as reduced numbers and size of immature tumor vessels and increased pericyte coverage, accompanied by decreased permeability, an edema, and interstitial fluid pressure. Additionally, vessel normalization was observed in patients treated with bevacizumab, but they do not demonstrate whether the beneficial effect on tumor growth inhibition was produced by normalization (Willett et al. 2009).

Vessel normalization in cancer patients has also been visualized by MRI in patients treated with anti-VEGF therapies. In patients treated with cediranib, pan-VEGFR tyrosine kinase inhibitor, MRI reveals a decrease in vessel diameter, vascular permeability, and an edema. Moreover, MRI studies have showed that survival of patients with recurrent glioblastoma, treated with cediranib, correlates with vascular normalization index. These results suggest that vessel normalization may predict efficacy of therapy (Sorensen et al. 2009).

Combination of bevacizumab with cytotoxic or cytokine therapy is approved in the treatment of some solid tumors. Some mechanisms have been proposed to explain the benefits of

combination therapy, including the improved cytotoxic drug delivery and efficacy by vessel normalization. This hypothesis is supported by preclinical findings in which VEGF blockade increases the penetration of molecules into the tumor by restoring the fluid pressure and inducing a more uniform distribution of blood flow (Wildiers et al. 2003). Moreover, the vessel normalization after VEGF blockade increases the accessibility of immune cells into the tumor (Shrimali et al. 2010). Given increased drug delivery after VEGF blockade has not been observed in all preclinical models; more investigations are required to pinpoint the effects of vessel normalization induced by bevacizumab.

Conclusion

Many mechanisms are implicated in the formation of new blood vessels. For this reason, tumor vasculature can be inhibited at different levels. Anti-angiogenics can produce the inhibition, regression, or normalization of tumor blood vessels. Inhibition of angiogenic signaling is the most utilized approach. Both the inhibition of VEGF and VEGFR by monoclonal antibodies or small molecules is effectively used in the clinic for the treatment of different solid tumors. On the other hand, EPCs contribute to the formation of new blood vessel and the metastatic niche, so the inhibition of EPC mobilization produces antitumor effects. Moreover, levels of circulating EPCs have been proposed as a diagnostic and prognostic biomarker of cancer. The bidirectional communication of endothelial cells and ECM mediated by integrins is important during the angiogenesis process. Therefore, the inhibition of integrins and ECM can block new vessel formation. Another strategy is the inhibition of the preexisting tumor vasculature by VDAs. VDAs cause a rapid and catastrophic shutdown in the vascular function resulting in death of tumor cells supplied by those vessels. Clinical trials have demonstrated the antitumoral activity of VDAs as monotherapy and in combination with other conventional therapies. Finally, normalization of the abnormal tumor vasculature is produced after anti-angiogenic therapies and improves tumor oxygenation and decreases interstitial fluid pressure, which may reduce metastatic dissemination and improve delivery of drugs to the tumor.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part IV

Anti-angiogenic Targets

The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

Napoleone Ferrara

Contents

Introduction and Historical Note 212

Identification of VEGF and Early Studies 212

VEGF Receptors 213

VEGF as a Therapeutic Target in Oncology 214
FDA Approval of Bevacizumab in Metastatic Colorectal Cancer: The First FDA Approval for Anti-angiogenic Therapy in Cancer 214
Small Molecule VEGFR TKIs 216
Protein Inhibitors 216
Targeting VEGF in Combination with Other Angiogenic Inhibitors 217
Targeting VEGF in Combination with Immunotherapy 218

Conclusions and Perspectives 218

Cross-References 219

References 219

Abstract

While multiple signaling pathways are implicated in the regulation of vasculogenesis, angiogenesis, and remodeling of the vessel wall, the vascular endothelial-derived growth factor (VEGF)-A pathway plays essential roles during development and physiological homeostasis. VEGF-A, the main focus of this chapter, belongs to a gene family that also includes VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). Two tyrosine kinases, VEGFR1 and VEGFR2, bind VEGF-A, but VEGFR2 is the main signaling receptor. The key role of VEGF-A in the pathogenesis of cancers and blinding ocular diseases has been established over the past two decades. Elucidation of the molecular and biological properties of VEGF-A led to major advances in cancer therapy and to the first effective treatment for neovascular age-related macular degeneration.

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Vascular endothelial-derived growth factor · Development and physiological homeostasis · Pathogenesis of cancers · Blinding ocular diseases · Neovascular age-related macular degeneration

Introduction and Historical Note

Angiogenesis, the growth of new blood vessels, plays essential homeostatic roles since the blood vessels deliver nutrients and regulatory substances to tissues and organs, while they remove catabolic products. Conversely, a variety of disease processes, including tumorigenesis and intraocular vascular disorders, are facilitated by uncontrolled angiogenesis (Folkman and Klagsbrun 1987). In the 1930s and 1940s, it was hypothesized that the ability to induce new vessel growth through release of tumor-derived "blood vessel growth stimulating factors" is a key event in tumorigenesis (Ide et al. 1939; Algire et al. 1945). A few years later, in 1948, it was proposed that a hypothetical diffusible factor may be responsible not only for the development of normal retinal vasculature, but also for pathological neovascularization in proliferative diabetic retinopathy and other retinal disorders (Michaelson 1948). In 1971, Judah Folkman published the hypothesis that "anti-angiogenesis" could be a strategy to treat cancer and possibly other disorders (Folkman 1971). This hypothesis generated much enthusiasm and efforts to isolate the regulators of angiogenesis soon began. Several molecules were identified and characterized as angiogenesis inducers in the subsequent decades, including EGF, TGF-α, TGF-β, aFGF, bFGF, angiogenin, etc. [reviewed in Ferrara (2002) and Folkman and Klagsbrun (1987)]. However, while these factors were able to promote angiogenesis in various bioassays, attempts to directly link them to angiogenesis were largely unsuccessful [reviewed in Ferrara (2002) and Klagsbrun and D'Amore (1991)]. It is now recognized that, while multiple pathways are implicated in the assembly of the vessel wall at different stages of development (Yancopoulos et al. 2000; Coultas et al. 2005; Hellstrom et al. 2007; Red-Horse et al. 2007), VEGF signaling mediated by its tyrosine kinase receptors plays a key role in physiological and pathological angiogenesis.

Identification of VEGF and Early Studies

In 1983, Senger et al. reported the identification of vascular permeability factor (VPF) (Senger et al. 1983), a protein that induced vascular leakage when injected in the guinea pig skin. However, these initial efforts did not yield the full purification and NH_2 terminal sequencing of VPF, thus precluding cDNA cloning and establishing the identity of VPF. In 1990, Senger et al. reported the purification and NH_2 terminal amino acid sequence of VPF (Senger et al. 1990). In 1989, we reported the isolation and cDNA cloning of vascular endothelial growth factor (VEGF), a novel heparin-binding endothelial cell mitogen secreted by bovine pituitary follicular cells (Ferrara and Henzel 1989; Leung et al. 1989). After our initial work was published, Keck et al. reported the cloning of VPF, which proved to be the same molecule as VEGF (Keck et al. 1989).

Subsequent studies established VEGF (known also as VEGF-A) as a potent, diffusible, endothelial-specific mitogen that induces both angiogenesis and vascular permeability (Dvorak 2002; Ferrara 2002; Neufeld et al. 1999). VEGF is a member of a gene family that also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF) (Ferrara et al. 2003). The human VEGF gene is located on chromosome 6p21 (Vincenti et al. 1996). Four main VEGF isoforms generated by, alternatively, mRNA splicing have been characterized as follows: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, with $VEGF_{165}$ being the predominant one. Other less common isoforms were identified such as $VEGF_{145}$ (Poltorak et al. 1997). Several "inhibitory" isoforms of VEGF have also been recently described, arising from alternative mRNA splicing or programmed read-through translation such as

including $VEGF_{165b}$ (Bates et al. 2002) and VEGF-Ax (Eswarappa et al. 2014). However, there is significant controversy regarding the mechanisms of inhibition, and in fact these variants have been reported to be weak agonists rather than inhibitors (Kawamura et al. 2008; Xin et al. 2016).

In vivo, the VEGF proteins are found as homodimers, with molecular masses ranging between 32 and 45 kDa. $VEGF_{121}$ does not bind to heparan-containing proteoglycans (HPSG) and is therefore freely diffusible, while $VEGF_{189}$ and $VEGF_{206}$ are strongly bound to HSPG and are largely sequestered in the extracellular matrix; $VEGF_{165}$ has intermediate properties. Indeed, due to their differential affinity for HPSG, the VEGF isoforms are able to generate biochemical gradients, a requirement for angiogenesis in vivo (Houck et al. 1992; Park et al. 1993). Also, proteolytic processing at the COOH terminus by plasmin or MMP3 can turn heparin-binding VEGF forms into non-heparin-binding, diffusible one [reviewed in Ferrara (2010a, b)].

VEGF Receptors

The identification of the VEGF receptors enabled important advances in our understanding of VEGF action. In 1992, the orphan receptor *fms*-like tyrosine kinase (Shibuya et al. 1990) was identified as a high-affinity receptor for VEGF (de Vries et al. 1992). In the same year, the highly related tyrosine kinase KDR (Terman et al. 1991) was also reported to be a VEGF receptor (Terman et al. 1992). These receptors are known today respectively as VEGFR1 and VEGFR2 and have each seven immunoglobulin (Ig)-like domains in the extracellular portion (Olsson et al. 2006). VEGF-A binds to both VEGFR1 and VEGFR2; VEGF-B and PlGF bind to VEGFR1; and VEGF-C and VEGF-D bind to VEGFR3 (implicated in lymphangiogenesis) but can also bind VEGFR2 after proteolytic cleavage (Pajusola et al. 1994). The mitogenic, angiogenic, and vascular permeability-enhancing activities associated with VEGF are mediated mostly by VEGFR2 (Olsson et al. 2006). Mice lacking both *vegfr2* alleles die in utero around day 8.5, most likely from aberrant hematopoietic and vascular development (Shalaby et al. 1995). Interestingly, inactivation of the *vegf* gene revealed a gene dosage-dependence such that loss of even a single *vegf* allele resulted in early embryonic lethality, largely phenocopying v*egfr2* null mice (Carmeliet et al. 1996; Ferrara et al. 1996).

The heparin-binding VEGF isoforms (or PlGF) can also bind to neuropilin 1 (NRP1), which increases their binding affinity to VEGFR2, but these molecules can also bind NRP1 independent of VEGFR2 activation (Soker et al. 1998; Whitaker et al. 2001). Two tyrosine residues in VEGFR2 have been shown to differentially regulate angiogenesis versus vascular permeability. Mice homozygous for the single substitution, tyrosine to phenylalanine, in position 1173 had defective vasculogenesis and angiogenesis and died in utero around day 8.5–9.5 (Sakurai et al. 2005). In contrast, Y949 has been implicated in the regulation of vascular permeability (Li et al. 2016). Inactivating mutations in this pathway largely abolished the direct permeability-enhancing effects of VEGF in mice (Li et al. 2016). However, these permeability-deficient mice were normal and fertile, indicating that this function of VEGF does not play essential homeostatic roles (Li et al. 2016) (see also chapter ▶ "The Role of VEGF in Controlling Vascular Permeability"). It has become clear that, while VEGFR-2 is the main signaling receptor, VEGFR-1 plays highly complex and context-dependent roles (Chung et al. 2010). Some evidence supports a role for VEGFR-1 in VEGF-mediated mitogenesis in some circumstances, while other data suggest that VEGFR-1 may act as a "decoy" receptor that competes with VEGFR-2 for VEGF binding (Park et al. 1994). Other studies indicate that VEGFR-1 mediates monocyte migration (Barleon et al. 1996). Mice embryos lacking VEGFR-1 display excessive proliferation of angioblasts, supporting a key role for VEGFR-1 as an inhibitor of VEGF activity (Fong et al. 1995, 1999). Furthermore, mice with an intact extracellular domain, but lacking the kinase domain of VEGFR-1, are apparently normal, supporting the hypothesis that a major role of VEGFR-1, at least during embryonic development, is to regulate the availability of

VEGF to VEGFR-2 (Hiratsuka et al. 1998). However, more recent studies provide evidence for a non-angiogenic role of VEGFR1, by promoting growth in response to VEGF or PlGF, at least when over-expressed in some tumor cell lines (Wu et al. 2006; Yao et al. 2011).

In spite of its less well-defined biological role compared to VEGFR2, VEGFR-1 binds VEGF with high affinity and this property has led to the development of soluble VEGFR-1 variants as potent inhibitors. Mapping the binding domain for VEGF in VEGFR-1 led to the discovery that of the seven Ig-like domains in the extracellular portion, domain 2 is largely responsible for ligand specificity and binding affinity, with a requirement of domain 3 for full binding affinity (Davis-Smyth et al. 1996; Barleon et al. 1997; Wiesmann et al. 1997; Markovic-Mueller et al. 2017). This structural information led to the construction in 1996 of Flt (1-3)-IgG (Davis-Smyth et al. 1996), the first "VEGF-trap" suitable for blocking VEGF in vivo (Ferrara et al. 1998; Gerber et al. 1999a, b). In 2002, Holash et al. (Holash et al. 2002) described a molecule known today as aflibercept, which includes the second Ig-like domain of VEGFR-1 and the third Ig-like domain of VEGFR-2, fused to Fc-IgG, which is currently widely used for the treatment of intraocular neovascularization.

VEGF as a Therapeutic Target in Oncology

VEGF secreted by tumor cells and surrounding stroma stimulates the proliferation and survival of endothelial cells, leading to the formation of new blood vessels (Ferrara 2010a, b; Jain 2003; Nagy et al. 2009). Also the VEGF mRNA is overexpressed in the majority of human tumors and correlates with invasiveness, vascular density, metastasis, and prognosis (Kerbel 2008). Over the last decades, VEGF and its receptors have become targets for anticancer therapy. Indeed, multiple strategies to inhibit the VEGF–VEGFR signaling pathway have been explored (Ellis and Hicklin 2008; Ferrara and Kerbel 2005).

Neutralizing monoclonal antibodies to human VEGF were initially produced to investigate the biological role of this growth factor (Kim et al. 1992). In 1993, the murine anti-VEGF monoclonal antibody (Mab) A.4.6.1, which recognizes all bioactive isoforms of human VEGF, was reported to inhibit the growth of human tumor xenografts in mice (Kim et al. 1993). Further studies confirmed these findings and extended them to additional tumor models (Borgstrom et al. 1996; Warren et al. 1995). However, the minimal cross-reactivity of Mab A.4.6.1 with mouse VEGF (Muller et al. 1998; Yu et al. 2008) limited the use of this reagent essentially to human tumor xenografts implanted in immune-deficient mice and precluded its use in transgenic or other models. To overcome this limitation, several novel reagents were later developed, including cross-species reactive anti-VEGF Mabs (Liang et al. 2006), soluble receptors (Davis-Smyth et al. 1996; Holash et al. 2002) or a mouse mutant expressing a humanized variant of VEGF that is recognized and blocked by antihuman VEGF Mabs (Gerber et al. 2007).

In 1997, Mab A.4.6.1 was humanized (Presta et al. 1997) to create an antibody suitable for clinical trials. The resulting antibody, bevacizumab, retained the same functional characteristics of the original monoclonal antibody (Presta et al. 1997) and was assessed for use in human clinical trials (Ferrara et al. 2004). For a more extensive discussion of clinical trials with bevacizumab and other VEGF inhibitors, see Ferrara and Adamis (2016) and Apte et al. (2019).

FDA Approval of Bevacizumab in Metastatic Colorectal Cancer: The First FDA Approval for Anti-angiogenic Therapy in Cancer

In a pivotal phase III clinical trial in 2004, bevacizumab in combination with irinitecan (I) and a 5′-FU/LV chemotherapy regimen significantly increased response rates, progression-free survival (PFS), and overall survival (OS) in previously untreated metastatic colorectal cancer (mCRC), compared to IFL alone (Hurwitz et al. 2004).

In 2004, the FDA approved the use of bevacizumab for the first-line treatment of mCRC. Bevacizumab was also approved in 2006 for second-line mCRC therapy following a positive trial (Giantonio et al. 2007).

The rationale behind combining bevacizumab to cytotoxic chemotherapy was to simultaneously target the endothelial and tumor cell components. Preclinical studies confirmed a synergistic effect of a combination of anti-VEGF and cytotoxic therapies, in part through sensitizing the endothelium to the cytotoxic agents, such that direct antivascular effects of the cytotoxic agents amplify the pro-apoptotic effects of VEGF blockade on the vascular endothelium (Gerber and Ferrara 2005; Klement et al. 2000; Sweeney et al. 2001). An alternative hypothesis postulates that VEGF inhibition can prune endothelial cells not covered by pericytes and reduce the tortuosity and high permeability of the tumor vasculature ("normalization"), thus reducing tumor interstitial pressure and enhancing delivery of cytotoxic agents (Jain 2005; Willett et al. 2004). Eliciting these effects, however, requires a "judicious" dose of anti-VEGF administered during a transient normalization window. The normalizing dose is expected to be lower than anti-angiogenic or "antivascular" doses that are instead expected to reduce drug uptake and cause hypoxia, with detrimental effects and reduced efficacy (Jain 2014). A challenge in translating such concepts has been identifying the normalization window and the normalizing doses. These appear to be dependent on the context or the tumor model (Lee et al. 2000; Tong et al. 2004; Winkler et al. 2004; Arjaans et al. 2013; Huang et al. 2012, 2013). Surprisingly, standard doses of anti-VEGF agents that are efficacious in preclinical (Arjaans et al. 2013; Pastuskovas et al. 2012) or clinical (Van der Veldt et al. 2012; Zissen et al. 2011) studies have been reported to result in a sustained reduction in tumor uptake of cytotoxic agents and antibodies, emphasizing the complex relationship between combinations of anti-VEGF therapeutic approaches with normalization strategies (see also chapter: ▶ "Benefits and Pitfalls of Tumor Vessel Normalization").

The addition of bevacizumab, either as first-line therapy to treatment naïve patients or second-line treatment to refractory patients, to conventional chemotherapies has resulted in significant clinical benefits in various advanced cancers. In non-squamous non-small cell lung carcinoma (NSCLC), increased response rates on incorporating bevacizumab with paclitaxel and carboplatin, accompanied by significantly improved PFS and OS (Sandler et al. 2006), resulted in FDA approval. In renal cell carcinomas (RCC), inactivating mutations in the VHL gene are frequent and lead to VEGF upregulation (Kaelin 2008), providing a rationale for anti-VEGF therapy. Accordingly, results from an early placebo-controlled phase II study of advanced RCC reported an increase in PFS with bevacizumab monotherapy (Yang et al. 2003). Bevacizumab treatment was also efficacious in multiple gynecological malignancies. A phase III study in patients with advanced cervical cancer found PFS and OS improvements when bevacizumab was combined with two different chemotherapy regimens (Tewari et al. 2014), leading to FDA approval in 2014. Bevacizumab has been FDA approved also for platinum-resistant ovarian cancer, in combination with chemotherapy (Pujade-Lauraine et al. 2014). Bevacizumab also demonstrated significant increases in PFS in a large randomized study in stage III or stage IV ovarian cancer patients who had undergone debulking surgery (Burger et al. 2011). Patients were randomized into three groups, one received six cycles of chemotherapy alone, the second received chemotherapy plus bevacizumab in conjunction with the chemotherapy cycles, and the third received the same treatment as group 2 plus "maintenance" bevacizumab monotherapy for up to 15 months. In agreement with preclinical studies (Mabuchi et al. 2008; Ferrara 2017), the greatest PFS benefit was observed in the group that received "maintenance" bevacizumab, emphasizing the need for long-term inhibition of angiogenesis in order to achieve maximal therapeutic benefits (Burger et al. 2011). Based on this study, the FDA approved in 2018 bevacizumab for use in combination with carboplatin and paclitaxel, followed by bevacizumab monotherapy, for the treatment of women with advanced ovarian cancer following initial surgical resection. As of today, bevacizumab has received 11 FDA approvals for six different malignancies.

Small Molecule VEGFR TKIs

Small molecule inhibitors of the VEGFR2 tyrosine kinase (TK) function were initially reported in 1996 (Strawn et al. 1996). The elucidation of the crystal structure of the VEGFR2 kinase domain (McTigue et al. 1999) facilitated the development of several families of small molecule VEGFR TKIs [reviewed in Levitzki and Mishani (2006)]. These molecules inhibit, in addition to the VEGF receptors, a number of structurally related TKs, typically PDGFRs, c-kit, Flt3, and the CSF1 receptor, with various degrees of selectivity (Levitzki 2013). Further research has evidenced a high degree of promiscuity, since many small molecules can also inhibit a spectrum of structurally unrelated TKs, including EGFR, TIE2, cMet, RET, and FGF receptors as well as serine-threonine kinases such as Raf (Levitzki 2013). Indeed, according to a study in which a panel of 242 kinases was evaluated, pazopanib significantly inhibited 94 kinases, sunitibib inhibited 147 kinases, while sorafenib inhibited 82 kinases (Kumar et al. 2009). Therefore, the antitumor activity of these molecules potentially reflects the contribution of inhibition of multiple targets in the microenvironment and, in several cases, also direct inhibitory effects on tumor cell growth (Levitzki 2013). The greater toxicity of these molecules compared to anti-VEGF (or anti-VEGFR2) Mabs likely reflects such inhibition of multiple targets.

Sorafenib was initially characterized as a RAF (serine-threonine kinase) inhibitor and was subsequently shown to inhibit also VEGFR2 autophosphorylation (Wilhelm et al. 2004). As noted above, it also inhibits a broad spectrum of kinases. It demonstrated relatively limited toxicity and encouraging efficacy in RCC (Strumberg 2005). A phase III study in patients with metastatic RCC reported that sorafenib increased the median PFS (Escudier et al. 2007) and led to patients previously treated with placebo crossing over to receive sorafenib (Escudier et al. 2009). OS was significantly improved in patients receiving sorafenib (Escudier et al. 2009).This trial led to FDA approval for the use of sorafenib in cytokine-refractory metastatic RCC. Sorafenib has been also approved for treatment of advanced hepatocellular carcinoma (Llovet et al. 2008).

Sunitinib is a broader spectrum multi-targeted oral kinase inhibitor, which was reported to prevent angiogenesis in a variety of human tumor lines in xenograft models (Mendel et al. 2003). A phase III study in previously untreated patients with metastatic RCC reported a marked increase in PFS and response rates with first-line sunitinib treatment compared to interferon α-2a (Motzer et al. 2007). Consequently, sunitinib was approved for the treatment of metastatic RCC in 2007. Other TKIs, including pazopanib (Sternberg et al. 2010) and axitinib (Motzer et al. 2013), proved efficacious in advanced RCC and also exhibited an improved safety profile compared to sunitinib, leading to FDA approval. The broad spectrum TK and RAF kinase inhibitor, regorafenib (Wilhelm et al. 2011), is the only kinase inhibitor to be FDA approved for previously treated mCRC, as monotherapy, following improved OS in a phase III study (Grothey et al. 2013). While VEGFR TKIs have been successful as monotherapy, mainly in RCC, so far they proved disappointing in combination with cytotoxic agents in breast, lung, and mCRC (Bergh et al. 2012; Robert et al. 2011; Carrato et al. 2013; Schmoll et al. 2012).

Protein Inhibitors

Given its high affinity, VEGF binding – combined with the ability to bind also VEGF-B and PlGF, the chimeric soluble VEGF rector protein Ziv-aflibercept (Holash et al. 2002) – held promise as a potentially highly effective inhibitor of the VEGF pathway. In a phase III study in second-line mCRC, it was as effective as bevacizumab, although a greater incidence of adverse events was reported (Van Cutsem et al. 2012). This trial led to FDA approval for second-line treatment of mCRC. However, in other large randomized studies, Ziv-aflibercept failed to meet its primary endpoint. For example, in patients with previously untreated mCRC, ziv-aflibercept in combination with FOLFOX6 did not improve PFS relative to FOLFOX6 alone but resulted in a greater

incidence of adverse events (Tang and Moore 2013; Folprecht et al. 2016). In addition, in a Phase III study in patients with advanced NSCLC, ziv-aflibercept in combination with doxacetal did not improve OS compared to doxacetal alone (Ramlau et al. 2012). Collectively, these findings suggest that the additional targeting of PlGF and VEGF-B compared to targeting VEGF-A alone does not confer a significant clinical advantage (Clarke and Hurwitz 2013).

Ramucirumab, a fully human monoclonal antibody targeting VEGFR2 (Krupitskaya and Wakelee 2009) has shown efficacy in multiple tumor types. Ramucirumab significantly increased OS in patients with advanced gastric or gastroesophageal junction adenocarcinoma (Fuchs et al. 2013; Wilke et al. 2014), and was FDA approved for this indication. Ramucirumab also received approval for the treatment of advanced NSCLC (Garon et al. 2014) and mCRC that progressed during or after first line chemotherapy with bevacizumab in combination with second-line FOLFIRI (Tabernero et al. 2015).

Targeting VEGF in Combination with Other Angiogenic Inhibitors

Targeting VEGF and other pathways implicated in various steps of the angiogenic process angiogenesis should, in principle, result in more effective tumor growth inhibition. This notion led to numerous efforts aiming at combining anti-VEGF agents with inhibitors of other pathways implicated in the assembly/growth of the vessel wall (Singh and Ferrara 2012) (see also chapter ▶ "Combination of Anti-angiogenics and Other Targeted Therapies").

Over the last decade, the HGF/cMet pathway generated considerable enthusiasm as a therapeutic target since it has been reported not only to mediate angiogenesis but also to be a key regulator of invasiveness and epithelial-mesenchymal transition in GBM and other tumors following VEGF blockade (Sennino and McDonald 2012; Lu et al. 2012). This hypothesis led to a number of clinical trials combining cMet inhibitors with bevacizumab or other targeted agents. Unfortunately, adding onartuzumab, a c-Met blocking antibody (or an anti-HGF antibody), did not provide any additional benefit relative to bevacizumab monotherapy in GBM and other tumors such as mCRC or breast cancer (Bendell et al. 2017a; Cloughesy et al. 2017; Spigel et al. 2016; Wakelee et al. 2017; Affronti et al. 2018; Iveson et al. 2014). The reasons for such disappointing results remain largely unclear but the failure of multiple trials targeting cMet or HGF in different clinical settings have cast considerable doubt on the significance of this signaling pathway as a therapeutic target.

A potentially promising combination of VEGF blockers is with agents targeting the angiopoietin (Ang)/Tie2 axis. This signaling system is implicated in multiple physiological and pathological processes, including blood vessel sprouting, lymphangiogenesis, recruitment of myeloid cells, and metastasis (Augustin et al. 2009; Huang et al. 2010; Saharinen et al. 2017). Indeed, preclinical studies have shown a marked additivity with VEGF inhibitors in various tumor models (Rigamonti et al. 2014), leading to clinical trials on oncology and in ophthalmology (Huang et al. 2010; Saharinen et al. 2017). Trabetanib, a peptidobody that blocks Ang1/Ang2 (Coxon et al. 2010), has been tested in breast cancer patients in combination with chemotherapy or bevacizumab (Dieras et al. 2015). Although the toxicity was manageable, there was no improvement in PFS with the addition of trebananib to paclitaxel and bevacizumab at the doses tested. In ovarian cancer patients, there was a relatively limited improvement in PFS (Monk et al. 2016). A bispecific antibody targeting VEGF and Ang 2 (Kienast et al. 2013) (vanucizumab) displayed significant activity in primary and metastatic tumor models and appeared superior to anti-VEGF alone (Baker et al. 2016). These promising data led to a phase II study in previously untreated mCRC (Bendell et al. 2017b). However, the combination of vanecizumab and chemotherapy did not result in improved PFS relative to the combination of bevacizumab and chemotherapy and was associated with a greater incidence of hypertension (Baker et al. 2016). Likewise, huMEDI3617, an anti-Ang2 mAb, was tested

alone and in combination with bevacizumab or cytotoxic chemotherapy in a phase I/Ib study in patients with advanced solid tumors. On the basis of limited clinical activity, its development has been discontinued (Hyman et al. 2018).

A combination that has been also explored in clinical trials includes bevacizumab and a humanized anti-PlGF monoclonal antibody, on the basis of a study reporting that PlGF mediates angiogenic escape and resistance to anti-VEGFR2 antibody treatment in tumor models (Fischer et al. 2007). However, subsequent preclinical studies did not fully support the hypothesis that PlGF is a major mediator of tumor angiogenesis and escape from anti-angiogenic therapy (Bais et al. 2010; Schneider et al. 2015). A series of early-stage trials combining bevacizumab with humanized anti-PlGF antibody in patients with multiple tumor types were performed several years ago. A study in GBM patients indicated lack of additional benefit from the combination, relative to bevacizumab alone (Lassen et al. 2015). Although the clinical programs combining anti-PlGF with bevacizumab have been discontinued, the same anti-PlGF antibody is being tested in medulloblastoma patients, based on a study showing that PlGF, in this context, promotes tumorigenesis through direct stimulation of tumor cell growth mediated by NRP1 signaling (Snuderl et al. 2013).

Targeting VEGF in Combination with Immunotherapy

Over the last several years, cancer immunotherapy with checkpoint inhibitors has had a major impact on cancer treatment, resulting in improvements in OS (Kelly 2018). However, despite these benefits, only subsets of the treated patients experience durable response and/or improved survival (Chen and Hurwitz 2018 (in press)), hence the need to identify suitable combinations that may enhance the benefit. One likely reason for such suboptimal responses is the fact that human cancer can utilize multiple immune inhibitory mechanisms, leading to immune escape (Chen and Mellman 2017). One such mechanism might relate to VEGF (Ott et al. 2015). Indeed, besides it well established effects on endothelial cells, VEGF has been reported to have some direct or indirect effects on multiple cells involved in immunity, including dendritic cells, T cells, regulatory T cells, and myeloid-derived suppressor cells (Khan and Kerbel 2018). Administration of anti-VEGF antibodies has been reported to increase significantly the number of tumor-infiltrating lymphocytes in animal models (Chung et al. 2013; Shrimali et al. 2010) and in humans (Wallin et al. 2016). Also, recent studies report that anti-VEGF therapy can improve anti-PD-L1 treatment in animal models, when it generates high endothelial venules (HEVs) that facilitate enhanced CTL infiltration and tumor cell destruction (Allen et al. 2017). Other studies indicate that treatment with a bispecific anti-VEGF/Ang2 antibody potentiated the activity of anti-PD-L1treatment in multiple models (Schmittnaegel et al. 2017). These considerations led to numerous clinical trials combining inhibitors of the VEGF pathway with immune checkpoint inhibitors.

Recently, in a phase III study, the addition of bevacizumab to cytotoxic chemotherapy (carboplatin, paclitaxel) and anti-PD-L1 antibody (atezolizumab) extended OS for patients with NSCLC (Socinski et al. 2018). In December 2018, the FDA approved atezolizumab, in combination with bevacizumab, paclitaxel, and carboplatin, for the first-line treatment of patients with metastatic, non-squamous NSCLC with no EGFR or ALK genomic tumor aberrations.

Additionally, a phase III study combining bevacizumab with atezolizumab in RCC has confirmed benefit for this combination in this disease (Chen and Hurwitz 2018 (in press)). Also, in phase I studies, the combination of bevacizumab with atezolizumab has been reported to result in high response rates in hepatocellular carcinoma.

Conclusions and Perspectives

The identification of VEGF as a major angiogenic mediator has revolutionized our understanding of the roles of angiogenesis in both normal physiological development and pathology. Studies into

VEGF biology have provided insights into physiologic homeostasis and molecular mechanisms of cancers.

VEGF inhibitors have shown benefits in patients with advanced and difficult-to-treat malignancies and are now a standard of care for the treatment of several metastatic cancers. The recent data showing a benefit from combining bevacizumab with immune checkpoint inhibitors in NSCLC and other malignancies emphasize the value of the anti-angiogenic approach 15 years after the initial approval of bevacizumab for mCRC. However, there remain several areas for further improvement. For example, identifying biomarkers to identify those patients who would receive the maximum benefit from anti-VEGF therapies has been so far elusive. Also, the limited predictability of preclinical tumor models has hampered progress in identifying mechanism of escape/resistance to anti-angiogenic therapy and emphasizes the challenges in designing and interpreting preclinical efficacy studies as well as the need to develop better and more predictive animal models in oncology (Singh and Ferrara 2012).

Although the focus of this chapter is on the role of VEGF in tumor angiogenesis, it is important to point out the remarkable successes of anti-VEGF therapy in ophthalmology (Ferrara 2010a, b; Ferrara and Adamis 2016). Indeed, intravitreal administration of VEGF inhibitors such as ranibizumab (Ferrara et al. 2006), bevacizumab, or aflibercept has revolutionized the therapy of intraocular neovascular disorders including age-related macular degeneration (AMD) and diabetic retinopathy (Apte et al. 2019 (in press); Ferrara and Adamis 2016). Recent studies have reported the outcomes of 5-year treatment of AMD patients with bevacizumab or ranibizumab (Comparison of Age-related Macular Degeneration Treatments Trials Research et al. 2016). Fifty percent of patients had good vision, an outcome that would have been completely out of reach before anti-VEGF agents were available (Comparison of Age-related Macular Degeneration Treatments Trials Research et al. 2016). Also, recent data show a marked reduction in the incidence rate of legal blindness due to AMD after the introduction of intravitreal VEGF inhibitors in multiple countries (Bloch et al. 2012; Borooah et al. 2015). These are dramatic advances that highlight the clinical impact of anti-angiogenesis.

Cross-References

- Angiopoietins and TIE Receptors in Lymphangiogenesis and Tumor Metastasis
- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Benefits and Pitfalls of Tumor Vessel Normalization
- Controlling Vascular Permeability: How Does It Work and What Is the Impact on Normal and Pathological Angiogenesis
- Combination of Anti-angiogenics and Other Targeted Therapies
- The Role of VEGF in Controlling Vascular Permeability

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Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors

Elina A. Kiss and Pipsa Saharinen

Contents

Introduction 228

Angiopoietin Growth Factors and TIE RTKs 229
Angiopoietins 229
TIE Receptor Signaling 229

Phenotypes of Mice with Genetic Deletions of the Angiopoietin-TIE Pathway ... 230
ANGPT1 230
ANGPT2 231
TIE1 and TIE2 231

Angiopoietins in Inflammation and Vascular Remodeling 233

Angiopoietins in Experimental Tumor Models 233
Angiopoietins in Tumor Growth and Angiogenesis 235
Combined Therapy of ANGPT2 and VEGF or Immune Checkpoint Inhibition 236
Angiopoietins in Metastasis 238

Additional Angiopoietin – TIE Associated Signaling Pathways and Their Potential as Therapeutic Targets 239
VE-PTP 239
Integrins 240

TIE2-expressing Macrophages 241

ANGPT2 – an agonist or an antagonist? 242

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Angiopoietins in Human Cancer 243
Summary 245
Cross-References 245
References 246

Abstract

Tumor blood vessel formation (angiogenesis) is essential for tumor growth and metastasis. Two main endothelial ligand–receptor pathways regulating angiogenesis are vascular endothelial growth factor (VEGF) receptor and angiopoietin-TIE receptor pathways. The angiopoietin-TIE pathway is required for the remodeling and maturation of the blood and lymphatic vessels during embryonic development after VEGF and VEGF-C mediated development of the primary vascular plexus. Angiopoietin-1 (ANGPT1) stabilizes the vasculature after angiogenic processes, via tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2) activation. In contrast, ANGPT2 is upregulated at sites of vascular remodeling. ANGPT2 is secreted by activated endothelial cells in inflammation, promoting vascular destabilization. ANGPT2 has been found to be expressed in many human cancers. Intriguingly, in preclinical models inhibition of ANGPT2 has provided promising results in preventing tumor angiogenesis, tumor growth, and metastasis, making it an attractive candidate to target in tumors. However, until now the first ANGPT2 targeting therapies have been less effective in clinical trials than in experimental models. Additionally, in preclinical models combined therapy against ANGPT2 and VEGF or immune checkpoint inhibitors has been superior to monotherapies, and these pathways are also targeted in early clinical trials. In order to improve current anti-angiogenic therapies and successfully exploit ANGPT2 as a target for cancer treatment, the biology of the angiopoietin-TIE pathway needs to be profoundly clarified.

Keywords

Angiogenesis · Angiopoietin · Angiopoietin-2 · ANGPT · ANG · TIE1 · TIE2 · TEK · Tumor metastasis · Anti-angiogenic therapy

Introduction

Tumor blood vessels promote tumor growth and, together with tumor associated lymphatic vessels, facilitate metastatic dissemination to distant organs and to lymph nodes. Master regulators of new blood vessel growth (angiogenesis) are vascular endothelial growth factor (VEGF(-A)) and its endothelial cell (EC)-specific tyrosine kinase receptor (RTK), VEGF receptor 2 (VEGFR2) (Ferrara and Adamis 2016). The importance of VEGF in physiological angiogenesis is evident as reduction in the *Vegf* gene dosage causes aberrations in vascular development and lack of a single *Vegf* allele in mice results in embryonic lethality (Carmeliet et al. 1996; Ferrara et al. 1996). Hypoxic neoplastic tumor cells as well as tumor-infiltrating inflammatory cells and tumor-associated fibroblasts express VEGF. Excess VEGF stimulates the growth of poorly matured, leaky vessels with irregular blood perfusion, causing hypoxia and stimulating further expression of VEGF in tumors. Due to the vital role of VEGF in tumor angiogenesis, VEGF has been an intensively studied target for tumor anti-angiogenic therapies, and these efforts have led to the approval of VEGF and VEGFR blocking drugs in many human cancers (Ferrara and Adamis 2016). The use of these drugs over several years has led to the understanding that blocking VEGF signaling at most delays disease progression, whereas complete responses are rare. Two types of resistance were postulated to attenuate the

efficacy of VEGF targeted drugs: evasive resistance, where initial response to anti-angiogenic therapy is lost and disease eventually progresses, and intrinsic resistance, where no antitumor response is observed (Bergers and Hanahan 2008). Several mechanisms of resistance have been suggested based on studies in preclinical models, including the action of other angiogenic factors than VEGF. Angiopoietin growth factors (ANGPT1, ANGPT2 and ANGPT4, originally termed as ANG1, ANG2 and ANG4 (the mouse ortholog was termed Ang3)) and their endothelial receptor TIE2 (also termed TEK) in association with TIE1 represent a second, almost exclusively EC-specific growth factor receptor pathway, which is known to regulate tumor angiogenesis. After angiogenic processes ANGPT1 interacts with TIE2 and promotes vascular stability and endothelial quiescence. ANGPT2 is expressed at low levels in normal tissues but is upregulated in activated ECs during inflammation and in tumor vessels, and indeed elevated ANGPT2 levels are reported in many human cancers. In line with the importance of VEGF-VEGFR and ANG-TIE systems in regulating both physiological and tumor angiogenesis, attempts of blocking both ANGPT2 and VEGF in certain tumor models have been more efficient than blocking either alone. Collectively, combinatorial inhibition of VEGF and ANGPT2 may help to overcome the challenges in current anti-angiogenic therapies, and to improve efficacy of other types of anti-tumor therapies.

Angiopoietin Growth Factors and TIE RTKs

Angiopoietins

Angiopoietin growth factors have a vital role in development, maintenance, remodeling, and repair of the blood vessels. Three angiopoietins in human have been described: ANGPT1, ANGPT2, and ANGPT4 (also termed as ANG1, ANG2, and ANG4) (Davis et al. 1996; Maisonpierre et al. 1997). Additionally in mice Ang3 has been identified, which represents a human ANGPT4 orthologue (Kim et al. 1999). The cellular effects of angiopoietins are mediated by endothelial TIE RTKs. Originally, angiopoietins have been described to bind to TIE2 RTK via the C-terminal fibrinogen-like domain (FLD) of angiopoietins (Kim et al. 2005; Davis et al. 2003). Central coiled-coil (CC) domain, which is connected to the FLD via a linker region, mediates dimerization or trimerization of angiopoietins. Especially ANGPT1 is further clustered to tetramers, pentamers, or higher oligomeric forms via an N-terminal super-clustering domain (SCD) of angiopoietins (Kim et al. 2005). Studies using recombinant angiopoietins have demonstrated the requirement for correct placing of angiopoietin FLDs for TIE2 activation (Davis et al. 2003). Initial experiments showed that a trimeric or tetrameric angiopoietin, with three to four TIE2 binding sites, was effective as a TIE2 agonist in ECs, but more recently a dimeric recombinant agonist form was created (Cho et al. 2004; Oh et al. 2015). ANGPT1 is a strong TIE2 agonist. Although the roles of Ang3 and ANGPT4 are largely unknown, they have been described to act as Tie2 agonists both in vitro and in vivo (Lee et al. 2004). In contrast, ANGPT2 functions as a weak agonist or antagonist, depending on the context (Daly et al. 2006; Yuan et al. 2009). Recently, it has been shown that angiopoietins signal also via integrins. These signaling events have been reported to involve both the FLD and N-terminal angiopoietin domains, but detailed mechanisms of angiopoietin-integrin signaling remain to be further investigated (Felcht et al. 2012; Lee et al. 2014; Hakanpaa et al. 2015).

TIE Receptor Signaling

Endothelial TIE receptors TIE1 and TIE2 mediate angiopoietin growth factor signaling. They are RTKs with an extracellular domain consisting of two immunoglobulin (Ig)-like domains, three epidermal growth factor domains (EGF), another Ig-like domain, and three fibronectin type III

(FNIII) domains followed by a transmembrane helix and a split intracellular tyrosine kinase domain (Barton et al. 2006; Macdonald et al. 2006). The ligand binding domain (LBD) includes the three Ig domains and the three EGF domains. These domains fold as a compact, arrowhead-shaped structure that binds the ANGPT1 and ANGPT2 FLDs, utilizing a lock-and-key mode of ligand recognition that is unique for RTKs (Barton et al. 2006; Macdonald et al. 2006). All angiopoietins interact with TIE2 but not with TIE1 (Maisonpierre et al. 1997; Davis et al. 1996; Kim et al. 1999; Lee et al. 2004). In fact, no ligand for TIE1 has been so far described. However, TIE1 becomes phosphorylated after ANGPT1 stimulation in primary ECs in a TIE2 dependent manner, but at a lower magnitude than TIE2 phosphorylation (Saharinen et al. 2005; Savant et al. 2015). TIE1 and TIE2 have been shown to interact on the surface of endothelial cells and angiopoietin stimulation increases TIE receptor interaction and receptor translocation to cell-cell contacts (Korhonen et al. 2016; Saharinen et al. 2008). In contacting ECs, ANGPT1 induces the formation of a receptor complex where ANGPT1 activates TIE2 in trans, by bridging TIE2 molecules from opposing endothelial cells in EC-EC junctions (Fukuhara et al. 2008; Saharinen et al. 2008). ANGPT1-induced phosphorylation of TIE2 at EC junctions leads to activation of signaling pathways such as PI3K/AKT and eNOS, whereas in motile cells ANGPT1 preferentially activates ECM-bound TIE2 RTKs in cis leading to downstream activation of ERK and DOKR (Master et al. 2001; Babaei et al. 2003; Fukuhara et al. 2008; Saharinen et al. 2008). AKT further phosphorylates the transcription factor Forkhead box protein O1 (FOXO1) involved in metabolic and growth control of endothelial cells (Wilhelm et al. 2016). Phosphorylated FOXO1 is excluded from the nucleus, resulting in suppression of FOXO1 mediated gene transcription, including the FOXO1 targets endothelial cell specific molecule 1 (*Esm1*) and *ANGPT2* (Daly et al. 2004; Korhonen et al. 2016; Kim et al. 2016). Whereas ANGPT1 induces strong TIE2 activation, ANGPT2 is a weak TIE2 agonist. The recently determined crystal structure of the TIE2 FNIII domains has provided evidence for the mechanism of TIE2 activation. The structure revealed that TIE2 dimerization *in cis* is mediated via the FNIII domains. In the dimeric TIE2 the LBDs were located far apart, facilitating TIE2 activation by multimeric but not dimeric angiopoietins (Leppanen et al. 2017; Moore et al. 2017). In line with previous reports, these results suggest that the lower oligomerization of ANGPT2 explains for its lower agonist activity. However, the TIE2 agonist function of ANGPT2 appears to be important in the lymphatic vasculature during development, whereas in inflammation ANGPT2 inhibits ANGPT1-TIE2 signaling axis via functioning as a TIE2 antagonist (Daly et al. 2006; Thomson et al. 2014; Korhonen et al. 2016; Kim et al. 2016).

Phenotypes of Mice with Genetic Deletions of the Angiopoietin-TIE Pathway

ANGPT1

Genetically modified angiopoietin mouse models have revealed a fundamental role for angiopoietins in cardio vascular development. Deficiency of *Angpt1* leads to embryonic lethality at E9.5 to E12.5 due to severe cardiovascular defects (Suri et al. 1996). In the absence of ANGPT1, cardiac development is impaired, manifesting as collapsed endocardial-myocardial interactions and a less complex endocardial structure. The mutant mice have also enlarged vessels characterized by poor pericyte coverage, and an immature, low-complexity vascular network. ANGPT1 is expressed in the myocardium of the developing heart, in perivascular cells like pericytes and vascular smooth muscle cells, and in certain other cell types, such as neurons in the retina (Suri et al. 1996; Davis et al. 1996; Kim et al. 2017). In line with the results obtained with the complete ANGPT1 knockout mice, doxycycline-driven ANGPT1 deletion in mice at E10.5 resulted in abnormal vasculature with dilated vessels in various organs and ultimately

in embryonic lethality between E17.5 and P0. Furthermore, these defects were phenocopied by a cardiomyocyte specific deletion of ANGPT1, suggesting that the vascular defects were induced via hemodynamic changes due to defective heart development in ANGPT1-deficient embryos. Surprisingly, the deletion of ANGPT1 at any point after E13.5 failed to affect the survival of the ANGPT1-deficient mice, suggesting a more specific function for ANGPT1 in the mature, quiescent vasculature (Jeansson et al. 2011). However, ANGPT1 was required for postnatal development of both superficial and deeper vascular networks in the mouse retina (Lee et al. 2013).

ANGPT2

Mice genetically deficient for *ANGPT2* are born at normal frequencies (Gale et al. 2002). However, within the first 2 weeks after birth the ANGPT2-deficient mice died due to severe chylous ascites and lymphatic dysfunction associated with patterning abnormalities in collecting lymphatic vessels and lymphatic capillaries. Additionally, the lack of ANGPT2 was observed to impair the postnatal vascular remodeling, namely the regression of the hyaloid vessels in the vitreous of the eye and formation of the retinal vasculature. Backcrossing in the C57Bl/6 background abolished the severe phenotype of ANGPT2-deficient mice (Fiedler et al. 2006). ANGPT2-deficient C57Bl/6 mice develop to adulthood, yet they exhibit impaired response to inflammatory challenges. ANGPT2 was shown to promote the endothelial cell responsiveness to tumor necrosis factor-α (TNF-α), such as upregulation of EC adhesion molecules. Interestingly, constitutive transgenic expression of ANGPT2 in mouse endothelium resulted in embryonic lethality at E9.5–E10 with major deficiency in vascular formation, a phenotype very similar to that observed in ANGPT1- or TIE2-deficient embryos (Maisonpierre et al. 1997). Besides the vital role of ANGPT2 during vascular and lymphatic development, the studies show that ANGPT2 remains as an important player in the physiology of adult vasculature. Adult mice with inducible endothelial specific expression of human ANGPT2 were reported to have impaired recovery after limb ischemia, namely reduced blood vessel growth and maturation after injury resulting in prolonged perfusion deficiency (Reiss et al. 2007). Another study showed that endothelial overexpression of human ANGPT2 in mice led to leaky vessels with compromised pericyte coverage and hemodynamic problems leading to systemic hypotension (Ziegler et al. 2013).

TIE1 and TIE2

TIE2-deficient mice die between E10.5 and E12.5 due to the defective development of the cardiovascular system (Dumont et al. 1994). The early vascular development occurs normally, but in the absence of TIE2, the primary capillary plexus fails to mature. The arteriovenous specification is defective resulting in impaired venous development in the TIE2 deficient mouse embryos (Chu et al. 2016). In addition to vascular defects, the TIE2-deficient mouse embryos have impaired cardiac development that resembles defects observed in ANGPT1-deficient embryos. Furthermore, chimeric mice derived from wild type (WT) and TIE receptor null embryonic cells showed that TIE2 was dispensable for fetal hematopoiesis, but in the presence of competing WT cells, the TIE receptor null cells failed to contribute to adult hematopoiesis in bone marrow (Puri and Bernstein 2003). Chimeric mice also demonstrated a requirement for TIE receptors in the microvasculature during late embryonic development and in the blood vessels in adult mice (Puri et al. 1999). In mice lacking TIE1 the initial phases of vascular development occur normally, but the newly formed vessels lose their integrity, which results in lethal hemorrhage between E13.5 and birth (Puri et al. 1995). After embryonic development TIE1 expression decreases but continues to be significantly expressed in some vascular beds, such as in the lungs, and is upregulated at sites of vascular remodeling and by disturbed blood flow in vessel bifurcations (Korhonen et al. 1992; Porat et al. 2004). The loss of endothelial TIE1 in adult mice does not influence the healthy vasculature

but leads to inhibition of tumor angiogenesis and growth (D'Amico et al. 2014), as well as attenuated atherosclerosis in ApoE-deficient background (Woo et al. 2011).

As well as the lack of ANGPT1 and ANGPT2, the absence of TIE1 impairs the postnatally occurring retinal vascular development in mice (D'Amico et al. 2014). However, the retinal vascular defects were more severe in mice where both TIE1 and TIE2 were deleted (Savant et al. 2015). In the retinal vasculature, ANGPT2 expression is enriched in tip cells (del Toro et al. 2010; Holopainen et al. 2012), whereas TIE2 is low in the leading vascular front. In fact, a distinct role for TIE1 was proposed in the angiogenic front, where it attenuated the expression and cell surface presentation of TIE2, in comparison to remodeling stalk cells, where TIE1 enforced TIE2 signaling (Savant et al. 2015).

Besides the fundamental role of angiopoietin-TIE pathway in blood vasculature, it is also needed in lymphatic vessel formation. Reducing the gene dosage of the orphan *Tie1* during embryonic development results in an early lymphatic phenotype at E12.5, hemorrhagies by E13.5, and lethality (Puri et al. 1995; Qu et al. 2010; D'Amico et al. 2014). In line with these studies, mice where the intracellular part of TIE1 was conditionally deleted or mice where TIE1 was deleted in developing lymphatic valves showed lymphatic defects, including subcutaneous edema and impaired formation of collecting lymph vessels and valve morphogenesis. Additionally, postnatal deletion of TIE1 led to defects in collecting lymphatic vessel maturation (Shen et al. 2014; Qu et al. 2015). More evidence for the importance of the angiopoietin-TIE pathway in lymphatic vasculature was provided by analysis of mice where ANGPT1, or both ANGPT1 and ANGPT2, were simultaneously deleted at E16.5 or where TIE2 was deleted at the time of birth or in adult mice (Thomson et al. 2014; Souma et al. 2016; Thomson et al. 2017; Kim et al. 2017). In these mice, the defective angiopoietin-TIE pathway leads to elevated intraocular pressure as well as impaired ocular drainage, due to the defects of lymphatic capillaries in the corneal limbus or of the Schlemm's canal, a hybrid vessel responsible for the drainage of aqueous humor in the anterior chamber of the eye. Consequently, the mice developed glaucoma. Importantly, loss-of-function mutations in *TIE2* or *ANGPT1* were found in patients with primary congenital glaucoma (PCG), a worldwide cause of childhood blindness due to impaired function of the Schlemm's canal (Souma et al. 2016; Thomson et al. 2017).

Recent studies have shed light on the role of TIE1 in regulating angiopoietin signaling in postnatal vascular development (D'Amico et al. 2014; Savant et al. 2015) as well as in adult mouse vasculature (Korhonen et al. 2016). Results from TIE1-deficient mice suggest that TIE1 positively contributes to angiopoietin signaling. It was observed that the conditional TIE1 deletion in mouse endothelium reduced TIE2 phosphorylation that was induced by administration of recombinant ANGPT1 in mice, indicating that TIE1 intensifies ANGPT1-induced TIE2 activation (D'Amico et al. 2014; Korhonen et al. 2016). Furthermore, TIE1 was required for ANGPT1 and ANGPT2 induced (delivered via adenoviral vectors in mice) vascular remodeling of tracheal vessels, and for ANGPT2 agonist activity in transgenic mice, where ANGPT2 expression was induced in ECs (Korhonen et al. 2016). Recently, mechanisms of TIE1 signaling have been elucidated. Angiopoietins were found to increase the direct interaction of TIE1 and TIE2 in cell junctions of cultured ECs (Korhonen et al. 2016). Loss of TIE1 impaired TIE2 trafficking in ECs, resulting in decreased TIE2 signaling. The lack of TIE1 led also to compromised TIE2 and AKT phosphorylation upon ANGPT1 binding (Savant et al. 2015; Korhonen et al. 2016). The decreased AKT phosphorylation in TIE1 silenced ECs resulted in activation and nuclear translocation of the AKT target FOXO1, which has been shown to regulate ANGPT2 expression and endothelial cell homeostasis (Korhonen et al. 2016). These results suggest that TIE1 is required for angiopoietin-induced responses in vitro and in vivo, but detailed mechanisms and vascular processes require further studies.

Angiopoietins in Inflammation and Vascular Remodeling

Whereas ANGPT1 acts as a paracrine growth factor to stabilize vessels after angiogenic processes, ANGPT2 is secreted in an autocrine fashion in ECs undergoing endothelial activation or vascular remodeling (Fig. 1a, b) (Maisonpierre et al. 1997; Jeansson et al. 2011). In ECs, ANGPT2 is stored in intracellular secretory granules, Weibel-Palade bodies, and the release of ANGPT2 is associated with a type I response of ECs, triggered by inflammatory mediators including histamine (Fiedler et al. 2004). Decreased ANGPT1-TIE2 signaling and reduced ANGPT1/ANGPT2 ratio, resulting from increased ANGPT2 expression and secretion, is observed in various diseases associated with vascular dysfunction. High ANGPT2 expression has been described in inflammatory disorders, such as sepsis, malaria, acute kidney and lung injuries, acute respiratory distress syndrome (ARDS) as well as in diabetes and in vascular malformations like cerebral cavernous malformations (for references see Saharinen et al. 2017). Furthermore, low levels of TIE1 and TIE2 receptor expression have been linked to increased vascular complications in hemorrhagic Ebola virus infection in mice (Rasmussen et al. 2014). In humans, single-nucleotide polymorphisms associated with low TIE2 expression were associated with risk for ARDS in intensive care unit patients (Ghosh et al. 2016).

Interestingly, mutations in the human *TIE2* gene have been identified in both hereditary and sporadic forms of vascular malformations. TIE2 mutations, which result in increased activity of TIE2 or mutations of the downstream target PIK3CA, cause venous malformations, characterized by enlarged venous channels, surrounded by irregularly distributed vascular smooth muscle cells (Castillo et al. 2016; Castel et al. 2016; Limaye et al. 2009). On the contrary, mutations leading to inactivation of TIE2 or ANGPT1 have been associated with PCG in humans (Souma et al. 2016; Thomson et al. 2017). These observations clearly indicate the importance of the delicate balance of angiopoietin-TIE signaling.

In both acute and chronic inflammation a switch from ANGPT1-TIE2 signaling to ANGPT2 antagonist signaling occurs (Fig. 1a, b) (Korhonen et al. 2016; Kim et al. 2016). In LPS-induced acute endotoxemia in mice, membrane bound TIE1 is rapidly cleaved, leading to impaired TIE2 phosphorylation and signaling, whereas Mycoplasma infection of mouse airways results in slower TIE1 cleavage. At the same time, TIE1, TIE2, and ANGPT1 expression is downregulated, which contributes to the loss of TIE2 signaling (Kim et al. 2016; Korhonen et al. 2016). ANGPT2 is readily released from Weibel-Palade bodies after LPS challenge and additionally, the expression of ANGPT2 is increased. ANGPT2, by competing with ANGPT1 in binding to TIE2 decreases TIE2 activation, leading to activation of FOXO1 and upregulation of *Angpt2* gene transcription via a positive feedback loop (Daly et al. 2004; Korhonen et al. 2016; Kim et al. 2016). Importantly, inflammation-induced loss of the TIE receptors appears to switch ANGPT2 from an agonist into an antagonist, impairing EC barrier function. In fact, increased ANGPT2/TIE2 ratio has been shown to promote endothelial destabilization via activating endothelial β1-integrin (Hakanpaa et al. 2015). Moreover, mice with heterozygous genetic deletion of one *Angpt2* allele, in comparison to WT mice, were observed to have advantage of survival in various sepsis models, indicating that ANGPT2 is harmful in inflammation (David et al. 2012). In contrast, TIE2 heterozygous mice lacking one *Tie2* allele were more susceptible to LPS-induced sepsis (Ghosh et al. 2016).

Angiopoietins in Experimental Tumor Models

Majority of the knowledge of angiogenesis in cancer has been obtained using methods where tumor cells are transplanted into isogenic or immunodeficient mice. In the syngeneic model, the tumor cells and the recipient mice have a common genetic background, which allows the investigation of the effect of the tumor microenvironment on tumor growth. Immunodeficient

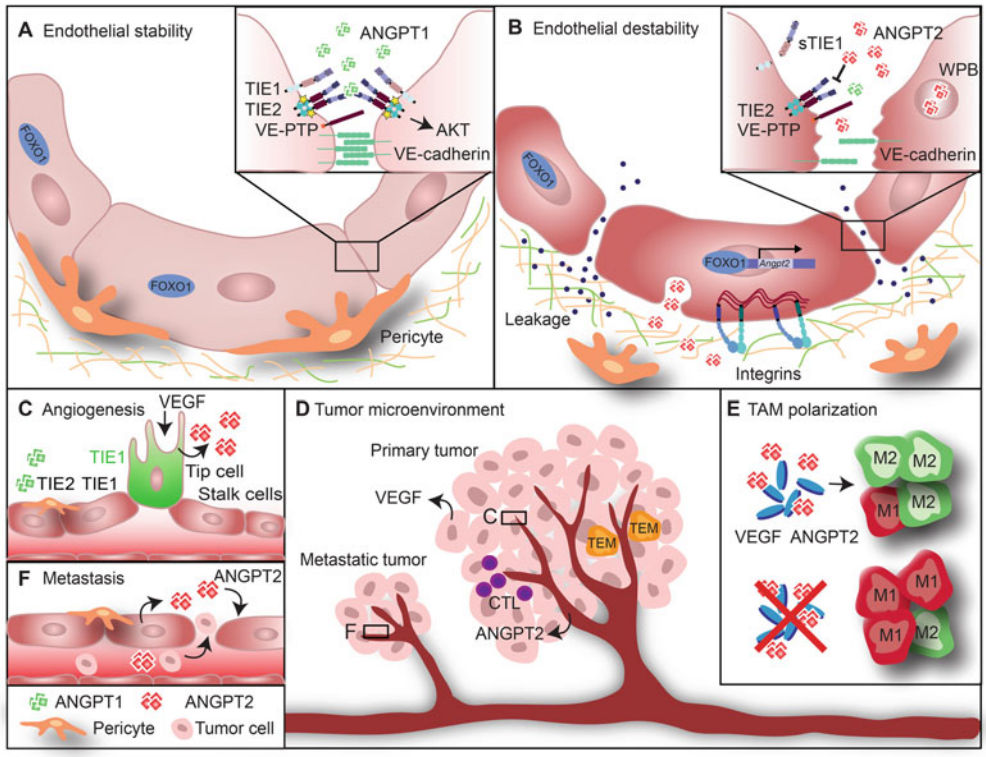

Fig. 1 The role of angiopoietins in angiogenesis and tumor microenvironment. (**a**) Perivascular cell produced ANGPT1 binds to and activates TIE2 to stabilize newly formed vessels and to limit pathological vascular responses by promoting endothelial barrier function. ANGPT1 binding to TIE2 induces an interaction between TIE2 and the orphan TIE1, enhancing TIE2 activation in endothelial cell-cell junctions. The active TIE2 induces, via the serine kinase AKT, phosphorylation of the transcription factor Forkhead box protein O1 (FOXO1), which remains in the cytoplasm. VE-PTP serves as a negative regulator of TIE signaling, but promotes the endothelial barrier function via VE-cadherin. (**b**) In activated endothelium the context-dependent agonist/antagonist ANGPT1 is released from Weibel-Palade bodies (WPB). ANGPT2 binding to TIE2 attenuates the stabilizing ANGPT1-TIE2 signaling axis. In activated endothelium TIE1 is cleaved, releasing soluble TIE1 ectodomain (sTIE1) and the expression of TIE1, TIE2, and ANGPT1 is downregulated. Moreover, in the inflammatory conditions, ANGPT2 switches into a TIE2 antagonist, further impairing TIE2 signaling. In response to diminished TIE2 signaling, FOXO1 translocates to the nucleus and promotes ANGPT2 expression via a positive feedback loop. Decreased ANGPT1-TIE2 signaling impairs endothelial cell-cell junction integrity and the stabilizing cortical actin cytoskeleton (not shown), whereas ANGPT2 signaling via endothelial integrins promotes actin stress fibers, and pericyte dropout. In tumors, blocking ANGPT2 promotes normalization of the tumor vasculature that is mediated via ANGPT1, including decreased leakiness and increased pericyte coverage of the vessels. (**c**) ANGPT2 is expressed in tip cells of angiogenic sprouts. TIE2 expression in tip cells is downregulated, but it is expressed in the stalk cells. Despite low TIE2 expression, TIE1 is present in sprouting tip cells. Inhibition of ANGPT2 in experimental tumor models decreases sprouting angiogenesis and induces vessel regression. (**d**) ANGPT2 blocking also interferes with pro-tumorigenic TIE2 expressing macrophages (TEM) that associate with tumor vasculature. In addition, combined blocking of ANGPT2 and VEGF has been found to promote antitumor immunity via increased numbers of tumor-infiltrated activated cytotoxic T cells (CTL) ANGPT2 inhibition in combination with TIE2 activation (using the antibody ABTAA) has been shown to decrease the number of regulatory T cells in some tumor models. (**e**) ANGPT2 together with VEGF have been linked to polarization of tumor-associated macrophages (TAM). It has been found that in the presence of ANGPT2 and VEGF, the proportion of protumor M2 macrophages is increased whereas the inhibition of ANGPT2 and VEGF promotes the antitumor M1 phenotype in macrophages. (**f**) Besides angiogenesis, ANGPT2 promotes lymphangiogenesis, lymph node and distant metastasis. ANGPT2 blocking antibodies or TIE2 activation reduce metastatic spread in preclinical models

mice are used in xenograft transplantation, where human-derived tumor cells or a piece of solid tumor tissue is used to introduce tumors into mice. Although this system has benefits in using tumors of human origin, the lack of immune cell involvement remains a significant limitation of the system. Despite the weaknesses of the mouse models, they are and remain as an important way to investigate tumor biology. The role of angiopoietins in tumor progression has been dissected using different approaches. Genetically engineered mouse models lacking the expression of ANGPTs or TIE receptors, or mice overexpressing ANGPT2 have provided vital information on the role of angiopoietins in tumor growth, metastasis and tumor angiogenesis. Notably, studies made with angiopoietin inhibiting antibodies or peptibodies developed for therapeutic exploitation have demonstrated the potential benefit of angiopoietin targeting in preclinical models.

Angiopoietins in Tumor Growth and Angiogenesis

Angiopoietins have a well-established role in regulating tumor growth and angiogenesis (Fig. 1c). Initial direct evidence that host-derived ANGPT2 signaling is needed for tumor growth came from studies with ANGPT2-deficient mice, where tumors grew slower than in WT mice (Nasarre et al. 2009). The growth retardation occurred specifically during early stages of tumor development. In later stages the tumor growth rates were similar in WT and in ANGPT2-deficient mice. The lack of ANGPT2 led to decreased microvessel diameter and more mature vessels with pericyte coverage, suggesting that absence of ANGPT2 leads to normalized phenotype of the tumor vasculature. Another study observed that while overexpression of ANGPT1 in mammary carcinoma cells resulted in stable tumor vasculature, the forced expression of ANGPT2 in tumor cells resulted in aberrant, leaky blood vessels lacking pericytes (Reiss et al. 2009). This report gives further evidence that ANGPT1 signaling promotes vessel stabilization and ANGPT2 the opposite. In line with the data obtained from ANGPT2-deficient mice, the opposite approach of endothelial specific overexpression of ANGPT2 in mice promoted tumor growth (Holopainen et al. 2012).

Several pharmacological approaches to inhibit ANGPT2 function have been investigated in different human tumor xenograft models and in orthotopic syngeneic and transgenic tumor models in mice. The evidence from studies employing ANGPT2-targeting agents indicates a significant role for ANGPT2 in tumor growth and angiogenesis. In general, inhibition of ANGPT2 has been shown to induce reduced microvessel density decreased vascular sprouting, vessel regression, normalization of the remaining tumor vasculature, uniform vessel diameters, reinforcement of the cell-cell junctions, tight association of pericytes with tumor blood vessels, reduction in tumor cell proliferation, and increase in tumor cell apoptosis (Holopainen et al. 2012; Falcon et al. 2009; Hashizume et al. 2010; for additional references see Saharinen et al. 2017). Several ways to block ANGPT2 function have been successfully used. First selective ANGPT2 neutralizing peptide-Fc fusion protein (peptibody) was identified by Oliner et al. and was shown in experimental tumor models to hinder tumor endothelial cell proliferation and restrict tumor growth (Oliner et al. 2004). AMG386, a peptibody, which blocks the binding of both ANGPT1 and ANGPT2 to TIE2 was exploited by pharmaceutical industry to develop trebananib for clinical studies with human patients (Herbst et al. 2009; Coxon et al. 2010). A fully human anti-ANGPT2 monoclonal antibody 3.19.3, selectively binding to C-terminal fibrinogen-like domain of ANGPT2, inhibited angiogenesis and induced vessel regression in different orthotopic and transgenic mouse tumor models (Brown et al. 2010; Mazzieri et al. 2011). Antibody administration also significantly hindered spontaneous metastasis (Mazzieri et al. 2011). In a highly metastatic lung carcinoma (LNM35) xenograft model ANGPT2 blocking with a MEDI3617 antibody resulted in reduced tumor growth and increased vessel regression (Holopainen et al. 2012). Additionally, blocking

ANGPT2 inhibited tumor lymphangiogenesis. Inhibition of ANGPT2 activity with fully human LC06 or LC08 species cross-reactive antibodies resulted in tumor growth inhibition in mouse colorectal and mammary xenograft tumor models (Thomas et al. 2013). The effect of treatment with LC06, specifically targeting ANGPT2, was superior to the treatment with LC08 recognizing both ANGPT1 and ANGPT2. This may at least in part be due to the vessel-stabilizing role of ANGPT1 signaling in the vasculature. In Colo205 colorectal cancer xenograft model, the inhibition of ANGPT2 with a peptibody (L1-7 (N)) resulted in fewer tumor vessels and promoted normalization of the remaining tumor vasculature, characterized by uniform vessel diameters, reinforcement of the cell-cell junctions, reduced sprouting, and tightly associated pericytes (Falcon et al. 2009). While inhibition of ANGPT1 alone had little effect on the tumor vasculature, the combined inhibition of ANGPT1 and ANGPT2 resulted in reduced tumor vasculature (Falcon et al. 2009; Coxon et al. 2010). However, tumor vessel normalization did not occur in the absence of ANGPT1 signaling (Falcon et al. 2009). This suggests, that ANGPT2, but not ANGPT1, regulates the vascularization of the tumors, but the vessel normalization observed in the absence of ANGPT2 is ANGPT1-dependent.

TIE1 deletion has also been shown to affect tumor angiogenesis and growth (D'Amico et al. 2014). The absence of endothelial TIE1 decreased vessel density and vascular perfusion in tumors grown in *Tie1* gene-targeted mice. Endothelial TIE1 deficiency impaired endothelial cell survival in tumors, and consequently decreased tumor cell survival. Angiogenic sprouting was also reduced in tumors grown in the absence of endothelial TIE1. Targeting of angiopoietins with adeno-associated viral vector delivery of soluble TIE2 receptor (sTIE2) together with the TIE1 deletion resulted in greater tumor growth inhibition than inhibition of either alone. Administration of sTIE2, which by capturing ANGPT1 and ANGPT2 inhibits membrane TIE2 signaling, partially impaired tumor angiogenesis and the growth of primary tumor and tumor metastases in mice (Lin et al. 1998). Interestingly, a recent report shows that an antibody binding to ANGPT2 and simultaneously activating TIE2 (ABTAA) has potent antitumor effects in combination with cytotoxic drugs, by normalizing the tumor vasculature (Park et al. 2017). In an orthotopic glioma model, the combination of temozolomide with ABTAA in comparison to antibody inhibiting ANGPT2 (ABA), reduced the tumor size and vascular leakage while increasing the pericyte coverage of tumor vessels. This tumor vessel normalization further improved perfusion, decreased hypoxia and reduced tumor lactate levels, indicating changes in tumor metabolism. The superior effect of ABTAA to vascular normalization in comparison to ABA resulted in improved drug delivery into the tumors and survival of mice after glioma cell implantation. Similar effects were also observed in Lewis lung carcinoma model and in the genetic MMTV-PyMT mouse model, where mouse mammary tumor virus (MMTV) LTR is used to drive the expression of the polyoma virus middle T-antigen (PyMT) resulting in the development of mammary tumors. Furthermore, ABTAA stimulated a favorable change in the tumor immune environment, by increasing the proportion of anti-tumorigenic M1 macrophages, and decreasing the numbers of regulatory T cells. ABTAA also decreased, whereas ABA increased, the circulating ANGPT2 levels. These results suggest that in addition to inhibiting ANGPT2, TIE2 activating drugs have beneficial effects on the tumor vasculature and the tumor microenvironment thereby increasing the efficacy of coadministered cytotoxic agents. However, at least in one model, ectopic overexpression of ANGPT1 in mice via an adenoviral vector has been reported to increase metastasis of human lung carcinoma cells (LNM35), due to ANGPT1-induced vessel dilation (Holopainen et al. 2009).

Combined Therapy of ANGPT2 and VEGF or Immune Checkpoint Inhibition

Results from preclinical tumor models have demonstrated that the dual inhibition of both VEGF and ANGPT2 has additive effects on tumor

growth inhibition, suggesting that combinatorial targeting of several angiogenic pathways may increase the efficacy of anti-angiogenic therapies. A bispecific antibody neutralizing VEGF and ANGPT2 (ANGPT2-VEGF CrossMab) retarded tumor growth in various tumor models. Especially in larger tumors the combined inhibition of VEGF and ANGPT2 was superior to the respective monotherapies (Kienast et al. 2013). Dual blockade of VEGF and ANGPT2 promoted tumor vessel regression and normalization of vessel architecture. The treatment with ANGPT2 antibody (REGN910) together with aflibercept (VEGF trap consisting of the ectodomain parts of VEGFR1 and VEGFR2) efficiently inhibited the growth of colorectal, prostate, and mammary tumors in xenograft models, decreased tumor vascularity and tumor perfusion (Daly et al. 2013). The combination of these agents was more efficacious than either of the single agent. The authors speculated that ANGPT2-TIE2 signaling increased the survival of tumor endothelial cells, thereby preventing the aflibercept-induced tumor vessel regression. Consequently, compromised EC survival after ANGPT2 blockage might have increased the efficacy of aflibercept treatment. In line with this, another study showed that VEGFR2 inhibition in pancreatic neuroendocrine tumors (PNETs) induced upregulation of ANGPT2 expression that was associated with resistance to VEGFR2 targeted anti-angiogenic therapy (Rigamonti et al. 2014). The combined blockade of ANGPT2 (using the anti-ANGPT2 monoclonal antibody 3.19.3) and VEGFR2 resulted in tumor growth inhibition. Although hypoxia was increased in tumors, the combination treatment did not provoke invasion or metastasis (Rigamonti et al. 2014). Additionally, their analysis of published microarray data suggested that high ANGPT2 expression in nontreated human sarcoma predicted poor response to VEGF neutralization therapy. In colon carcinoma xenograft model dual inhibition of ANGPT2 (using L1-7 (N)) and VEGF (using a monoclonal anti-VEGF antibody) caused tumor growth rate retardation, associated with reduction in cell proliferation and increase in tumor cell apoptosis (Hashizume et al. 2010).

High numbers of tumor associated macrophages (TAMs) have been linked to increased vascular density in human tumors (De Palma et al. 2017), and recent reports propose a role for tumor macrophages in the success of ANGPT2/VEGF therapy (Fig. 1d, e). In orthotopic syngeneic glioblastoma model combined therapy with AMG386 (ANGPT1/2 neutralizing peptibody) and aflibercept resulted in strong reduction of $F4/80^+$ TAMs (Scholz et al. 2016). Additionally, dual inhibition of ANGPT2 and VEGF decreased the number of vascular sprouts and vessels, and reduced vessel permeability via normalization of the tumor vasculature. In another study, mice with either orthotopic syngeneic glioblastoma or human xenograft glioblastoma were treated with antibodies inhibiting either VEGF alone (B20) or bispecific antibodies inhibiting both ANGPT2 and VEGF (A2V) (Kloepper et al. 2016). The dual inhibition increased the survival of mice when compared to monotherapy. As a mechanistic explanation for the better survival, it was suggested that A2V treatment favored the classical antitumor M1 macrophages. Consistent results were obtained again with mouse and human glioblastoma models from studies using ANGPT2 neutralizing antibody (MEDI3617) in combination with cediranib (a small-molecule tyrosine kinase inhibitor of the VEGFRs) (Peterson et al. 2016). Combined blocking of ANGPT2 and VEGF signaling increased the survival of tumor implanted mice when compared to either of the monotherapies. Mice receiving dual therapy exhibited improved vessel normalization. Interestingly, the superior effect of combined therapy over monotherapies was lost, when TAM recruitment to tumors was blocked by using an anti-colony-stimulating factor-1 (CSF-1) neutralizing antibody, indicating that the improved survival induced by the dual treatment was mediated by TAMs.

The abnormal tumor vasculature can also impair antitumor immunity, by limiting the extravasation of T cells, including $CD8^+$ cytotoxic T cells into the tumor bed, thereby promoting a state of immunosuppression (Lanitis et al. 2015). Thus, it has been envisioned that anti-angiogenic therapy may enhance antitumor immunity. This was shown in

a recent study where ANGPT2 and VEGF blockade (using the bispecific antibody A2V) was combined in both genetic and transplant tumor models (Schmittnaegel et al. 2017). A2V induced an anti-angiogenic response normalizing the remaining blood vessels and facilitated perivascular accumulation of activated, interferon-γ (IFNγ)–expressing $CD8^+$ cytotoxic T cells. Notably, in an immunogenic tumor model cytotoxic T cells were required for A2V antitumor effects. The therapy also upregulated the expression of the immune checkpoint ligand, programmed cell death ligand 1 (PD-L1), and blocking PD-1 in combination with A2V improved tumor control in certain tumor models, suggesting that immune cells may function as effectors of anti-angiogenic therapy.

Hypoxic tumors are often more resistant to radiation therapy. Interestingly, ANGPT2 expression is reported to increase in cancer patients of head and neck squamous cell carcinoma (HNSCC) after radiation therapy (Sridharan et al. 2016). One study observed that VEGFR2 inhibition created a short time window of vessel normalization before damaging the blood vessels, during which radiation therapy was effective (Winkler et al. 2004). This was characterized by an increase in tumor oxygenation, pericyte coverage of tumor blood vessels, and reduction in vessel diameter. VEGFR2 blockade induced upregulation of ANGPT1, which mediated the vessel normalization through TIE2. These data in part demonstrate the complexity of the tumor environment and how different treatment procedures change it. Treatment efficiency is context-dependent and optimal therapy is often a combination of different procedures.

Angiopoietins in Metastasis

Besides in tumor growth and angiogenesis, ANGPT2 has been shown to promote tumor metastasis (Fig. 1f). First indication that ANGPT2 plays a crucial role in tumor metastasis derived from studies done in MMTV-PyMT mice, which develop mammary tumors metastasizing in lungs. In these mice, treatment with ANGPT2 blocking antibody (clone 3.19.3) inhibited spontaneous tumor metastasis and additionally, restricted the growth of emerging metastases (Mazzieri et al. 2011). There are several other reports pinpointing the crucial role of ANGPT2 in tumor spreading. In mice overexpressing endothelial ANGPT2, the excess of ANGPT2 promoted B16 melanoma cell homing and early tumor growth in the lungs after their intravenous administration in mice (Holopainen et al. 2012). In these mice, ANGPT2 was shown to decrease endothelial barrier integrity, thereby likely enabling tumor metastasis. Additionally, WT mice treated with ANGPT2 expressing adenoviruses showed increased lymph node and lung metastasis of LNM35 tumor cells (Holopainen et al. 2012). Consistently, blocking ANGPT2 with an antibody (MEDI3617) in WT tumor bearing mice reduced tumor lymphangiogenesis and lymph node and lung metastasis, and improved cell junction integrity of pulmonary capillaries in mice with circulating tumor cells that homed to the lungs. ANGPT2 therefore increases metastasis at least, in part, by decreasing capillary integrity. In contrast, global ANGPT1 deficiency was shown to enhance lung metastasis without affecting primary tumor growth. The results suggested that ANGPT1 was required to inhibit the attachment and extravasation of tumor cells through pulmonary capillaries (Michael et al. 2017). Furthermore, tumor vascular normalization induced by ABTAA (the ANGPT2 inhibiting and TIE2 activating antibody), decreased tumor metastasis in the MMTV-PyMT tumors (Park at el. 2016).

ANGPT2 has been also linked to pericyte loss and subsequent vessel destabilization. Pericyte depletion induced by imatinib therapy, which inhibits platelet-derived growth factor receptor b (PDGFR-b) activity, decreased the growth of well-established tumors, but increased lung metastasis (Keskin et al. 2015). ANGPT2 was found upregulated in the imatinib treated tumors, and combination of imatinib with ANGPT2 blockage decreased both metastasis and tumor growth, suggesting that ANGPT2 enhanced metastasis in the destabilized tumor vasculature. In pancreatic ductal adenocarcinoma ANGPT2 was observed to promote lymphatic metastasis (Schulz et al. 2011). ANGPT2 mRNA expression

was localized in the ductal tumor cells, but not in normal tissue, and in cultured pancreatic cancer cells ANGPT2 was induced by TGF-β stimulation. Ectopic ANGPT2, but not ANGPT1, expression in pancreatic cancer xenografts increased peritumoral lymphatic vessel density and lymphatic metastasis, whereas of soluble TIE2 was able to counteract the harmful effects of ANGPT2.

Interestingly, ANGPT2 expression has been also associated with determining the pre-metastatic niches in tissues. Increased ANGPT2 expression induced by calcineurin-Nuclear factor of activated T-cells (NFAT) signaling was observed in early metastatic sites in lung endothelium, where it promoted angiogenesis and tumor metastasis (Minami et al. 2013). Calcineurin-NFAT signaling was suggested to be activated through increased VEGF expression induced by the primary tumor. The effect of upregulation of ANGPT2 in lung endothelium could be prevented by overexpressing Down syndrome critical region gene 1 (DSCR1), a negative regulator of calcineurin-NFAT signaling or by administration of soluble TIE2.

Combined inhibition of ANGPT2 and VEGF has been found beneficial in preventing tumor metastasis compared to corresponding monotherapies in preclinical models. In a study where H460M2 tumor cells metastasizing to the lungs were subcutaneously implanted in mice, blockade of ANGPT2 and VEGF using a bispecific antibody (CrossMab) in combination with chemotherapy completely eradicated the tumors. Besides being beneficial for reducing primary tumor size, the tumor-derived DNA in blood from circulating tumor cells was decreased following ANGPT2 and VEGF inhibition, indicating that dual blockade of ANGPT2 and VEGF impairs the early dissemination of tumor cells. Moreover, after primary tumor removal, adjuvant anti-ANGPT2-VEGF therapy decreased metastasis. Inhibition of VEGF and ANGPT2 led to regression and normalization of the tumor vessels and due to ANGPT2 inhibition also to reduced metastasis (Kienast et al. 2013). Others also report that ANGPT2 blockade combined with low-dose metronomic chemotherapy and VEGF blockade significantly reduced metastasis in a preclinical mouse model of postsurgical adjuvant therapy (Srivastava et al. 2014). The combination with ANGPT2 inhibition resulted in decreased numbers of all the macrophages and a subset of pro-tumorigenic $CCR2^{+}$ $TIE2^{-}$ metastasis-associated macrophages (MAMs) within the metastatic niche. The recruitment of $CCR2^{+}$ $TIE2^{-}$ MAMs was dependent on CCL2, and ANGPT2 antibody treatment was observed to reduce the CCL2 expression in lung metastases. Additionally, ANGPT2 stimulation induced adhesion molecule expression by endothelial cells, which may further promote the myeloid cell trafficking to the metastases.

Results from ANGPT2-deficient mice unexpectedly showed that metastatic growth of mouse colon carcinoma cells in the liver and associated tumor angiogenesis were enhanced in the absence of ANGPT2 (Im et al. 2013). Furthermore, it was observed that serum levels of circulating G-CSF and CXCL1 were increased in the ANGPT2 deficient mice, which supported the recruitment of myeloid cells into the liver. In contrast with the observation in liver colonization, but in line with previous reports, the tumor growth in lungs was impaired in the absence of ANGPT2. This study underlines the complexity of the ANGPT2-mediated effects on tumors and in vascular beds of various tissues and the need to further carefully evaluate the context-dependent role of ANGPT2.

Additional Angiopoietin – TIE Associated Signaling Pathways and Their Potential as Therapeutic Targets

VE-PTP

An important regulator of the ANGPT-TIE pathway, vascular endothelial protein tyrosine phosphatase (VE-PTP) is an EC-specific receptor tyrosine phosphatase that attenuates TIE2 activation and participates in the regulation of vascular homeostasis. VE-PTP is expressed in both arterial and venous endothelium, and its expression is

elevated in hypoxia. VE-PTP is essential for the cardiovascular development and consequently, the genetic deletion of VE-PTP results in embryonic lethality between E9.5 and E11 (Baumer et al. 2006; Dominguez et al. 2007). The VE-PTP-deficient mouse embryos suffer from remodeling defects of the vasculature, namely, the failure to form branched vascular networks. This defect was most pronounced in the yolk sac, where vessels formed large blood cavities without branches.

VE-PTP associates with TIE2 and regulates its activity via dephosphorylation. Additionally, it also associates with VE-cadherin increasing its adhesive function. Antibodies against VE-PTP, which specifically prevent its binding with TIE2 but not with VE-cadherin, provoked increased TIE2 activation, cell proliferation, and vessel enlargement in an allantois explant culture (Winderlich et al. 2009). Blocking VE-PTP function using a small molecular inhibitor (AKB-9778) or anti-VE-PTP antibodies stabilized the vascular barrier function in the mouse lungs challenged with LPS and prevented endothelial permeability induced by VEGF. Interestingly, TIE2 was a prerequisite for the effect of VE-PTP inhibition on vessel integrity, whereas VE-cadherin was dispensable, as inhibition of VE-PTP was able to stabilize the vascular endothelium even in mice with conditional deletion of VE-cadherin (*Cdh5*) (Frye et al. 2015).

Interestingly, the expression of VE-PTP in adult mouse endothelium was upregulated upon exposure to tumor cells, which may contribute to abnormal blood vessel phenotype via dampening vascular stabilizing TIE2 signaling. Indeed, inhibition of VE-PTP using AKB-9778 resulted in normalization of the vessel phenotype in mouse tumor models (Goel et al. 2013). AKB-9778 increased TIE2 activation, which promoted vascular stability. Inhibition of VE-PTP early in primary tumor development transiently delayed tumor growth by stabilizing tumor vessels, but significantly decreased tumor growth when combined with radio or chemotherapy. Additionally, VE-PTP inhibition was observed to delay metastatic progression after primary tumor excision and prolong the survival of mice.

Besides TIE2 and VE-cadherin, VE-PTP has been reported to regulate the VEGF receptor, VEGFR2. In cells stimulated with VEGF, VE-PTP silencing further increased VEGFR2 phosphorylation (Mellberg et al. 2009). Interestingly, VE-PTP, VEGFR2, and TIE2 were found together in complexes and VE-PTP was shown to regulate the activity of VEGFR2 via TIE2 (Hayashi et al. 2013). VE-PTP-deficient embryoid bodies showed disorganized sprouts and defects in lumen formation and polarization. Consistently, teratomas in mice lacking VE-PTP showed disordered and poorly functioning vasculature and noticeable VEGFR2 activation. The authors concluded that one way how TIE2 promotes vascular stability and quiescence is the inhibition of VEGFR2 receptor activation via VE-PTP. Interestingly, it was also shown that the zebrafish VE-PTP orthologue ptp-rb had an important role in regulating EC polarization and lumen formation.

Integrins

Integrins are cell surface receptors required for cell attachment and migration on the surrounding extracellular matrix, with well-established roles in tumor angiogenesis. Some integrins are highly upregulated in tumor associated blood vessels and they act together with angiogenic growth factor receptors to regulate their responsiveness to the corresponding growth factor ligands (Ivaska and Heino 2011). Recently, several reports have shown that angiopoietins can bind to certain integrins. In breast cancer cells, which do not express TIE2, ANGPT2 associated with integrin α5β1 leading to the activation of α5β1 signaling pathway and cancer metastasis in xenografts in mice (Imanishi et al. 2007). Also, in a TIE2-negative glioma cell line, ANGPT2 was observed to bind to α5β1-integrin (Lee et al. 2014). While the interaction between angiopoietins and TIE2 occurs via the C-terminal FLD, the integrin interacting domains of angiopoietins are not yet fully clarified. Initially, by using short peptides corresponding to amino acid sequences of FLD of ANGPT2, it

was observed that certain peptides were inhibiting the binding of integrin-expressing glioma cells to ANGPT2 (Lee et al. 2014). These sequences were located outside of the TIE2 binding region of the FLD, and were required for ANGPT2 interaction with α5-integrin. However, another study suggested that ANGPT2, but not ANGPT1, can activate α5β1-integrin via the ANGPT2 N-terminal domain, which is distinct from the TIE2-binding FLD (Hakanpaa et al. 2015). In this study, chimeras with either ANGPT2 N-terminal domain fused with ANGPT1 FLD or ANGPT1 N-terminal domain fused with ANGPT2 FLD were used to stimulate the fibronectin binding of α5β1-integrin. The interaction between angiopoietins and integrins is weaker in comparison to that of TIE2 receptor, indicating that TIE2 serves as the primary receptor for angiopoietins (Felcht et al. 2012). However, recently, growing amount of data describes situations where the cell surface TIE expression is diminished, while ANGPT2 is highly upregulated, potentially facilitating ANGPT2 interaction with other receptors. It remains to be seen if the interaction between angiopoietins and integrins could be targeted for future vascular therapies.

In endothelial monolayers where TIE2 was silenced, autocrine ANGPT2 was found to stimulate the formation of β1-integrin positive, elongated and centrally located matrix adhesions, which are distinct from the focal adhesions of quiescent EC monolayers. The altered substrate adhesion of the TIE2 silenced cells resulted in actin stress fiber production and decreased endothelial barrier function (Hakanpaa et al. 2015). Independent reports have shown that *Angpt2* mRNA (del Toro et al. 2010) and protein (Holopainen et al. 2012) expressions are upregulated in tip cells, whereas TIE2 was located to the stalk cells instead of the tip cells. This suggests that ANGPT2 acts in a paracrine fashion on stalk cells or via other receptors on tip cells, which express low levels of TIE2. In fact, it was observed that β1-integrin was upregulated in tip cells (del Toro et al. 2010). Based on the current data available, it is intriguing to hypothesize that the excess of ANGPT2 in tip cells, in the absence of TIE2, interacts and activates integrin receptors leading to tip cell migration, as reported by Felcht et al. They found that activated sprouting tip cells downregulated TIE2 and upregulated ANGPT2 expression (Felcht et al. 2012). TIE2 low ECs were observed to express avβ5, α5β1, and avβ3 integrins during angiogenesis. In fact, ANGPT2-induced increase in EC migration was dependent on integrin activation. In addition, αvβ3-integrin has been found in a complex with TIE2 on the cell surface (Thomas et al. 2010). ANGPT2 was shown to induce clustering of TIE2 and αvβ3-integrin at the EC junctions resulting in FAK activation, dissociation of the integrin-associated adaptor proteins, and αvβ3-integrin internalization and lysosomal degradation. Regulation of FAK-activity and integrin trafficking may represent a possible model of how ANGPT2 promotes endothelial destabilization.

Integrins have also been designated a role in fine-tuning the TIE receptor signaling. TIE2 and α5β1 integrin have been observed to form a complex, which is increased upon α5β1 activation by fibronectin (Cascone et al. 2005). Interaction of TIE2 and α5β1 facilitated the activation of TIE2 with low ANGPT1 concentrations. Additionally, blocking the α5β1 integrin function inhibited the ANGPT1-enhanced angiogenesis. Similarly, α5β1 integrin has also been shown to promote ANGPT1-induced formation of heteromeric complexes of TIE1 and TIE2, TIE receptor activation, and downstream signaling (Korhonen et al. 2016). In another study, both TIE1 and TIE2 were reported to associate with integrins α5β1 and αVβ3 through their ectodomains (Dalton et al. 2016). Furthermore, ANGPT1-mediated angiogenesis of the retinal vasculature that develops postnatally in mice requires αvβ5 integrin expression in retinal astrocytes (Lee et al. 2013).

TIE2-expressing Macrophages

In addition to endothelial cells, angiopoietin receptor TIE2 is expressed by certain hematopoietic progenitors (Ito et al. 2016) and by a unique subset of circulating monocytes (Mazzieri et al. 2011;

Coffelt et al. 2010; De Palma et al. 2005). Studies in mouse tumor models have demonstrated that circulating monocytes extravasate into tumors in response to various chemoattractants, and differentiate and mature into TAMs, a process influenced by the colony-stimulating factor 1 (CSF1) (De Palma et al. 2017). TIE2-expressing monocytes (TEMs) are recruited to tumors and have been observed in human breast cancer specimens (Kim et al. 2013; De Palma et al. 2005). Interestingly, TEMs constitutively circulate in peripheral blood but are not found in quiescent tissues suggesting that they are specifically attracted by cues from sites of tissue remodeling and angiogenesis. In fact, TEMs were observed to be proangiogenic and associate with newly formed vessels in murine tumors. TEMs were suggested to be recruited to tumors by chemokine CXCL12 via their expression of chemokine receptor CXCR4 (Welford et al. 2011). ANGPT2 interaction with TIE2 on TEMs induced TIE2 phosphorylation and upregulation of the expression of various tumor-promoting factors (Coffelt et al. 2010; Venneri et al. 2007; Murdoch et al. 2007). Blockade of ANGPT2 was shown to inhibit the transcriptional upregulation of TIE2 in TEMs and thereby impairing their association with tumor vasculature and their proangiogenic activity (Mazzieri et al. 2011). Additionally, the knockdown of TIE2 specifically in myeloid cells resulted in reduced tumor angiogenesis and perivascular association of TEMs.

In mammary carcinoma, cancer cell intravasation has been found to occur at microanatomical vascular structures, called Tumor Micro-Environment of Metastasis (TMEM), composed of a tumor cell, a perivascular TEM, and an endothelial cell. TMEMs have been also identified in human mammary carcinomas, correlating with metastasis. Recently, TIE2 blocking using rebastinib, a TIE2 kinase inhibitor, was found to reduce tumor growth and metastasis in an orthotopic metastatic mouse mammary carcinoma. Rebastinib reduced TEM infiltration, suggesting that the anti-tumor effects of rebastinib could be mediated via inhibition of protumoral TEMs (Harney et al. 2017).

ANGPT2 – an agonist or an antagonist?

Whether ANGPT2 acts as an agonist or antagonist of TIE2 in the tumor microenvironment remains as an interesting question. As discussed above, structural studies have provided insight into the molecular mechanisms behind the weak agonist activity of dimeric ANGPT2, when compared to higher activity of multimeric ANGPT1 (Leppanen et al. 2017). In general, when ANGPT2 levels are increased, such as in cancer and in various vascular leakage associated diseases, the decreased ANGPT1/ANGPT2 ratio favors ANGPT2-TIE2 signaling. This can be recapitulated using cultured endothelial cells, where excess ANGPT2 can inhibit ANGPT1-induced TIE2 activation (Maisonpierre et al. 1997). However, the outcome of ANGPT2 signaling is context-dependent. It has been suggested that the agonist activity of ANGPT2 would be important for normal homeostasis in stressed ECs that are exposed to low ANGPT1 levels (Daly et al. 2006). In addition, results using genetic mouse models have demonstrated that ANGPT2 functions as an agonist in the lymphatic vasculature (Gale et al. 2002; Thomson et al. 2014), and in transgenic mice, where ANGPT2 is expressed by endothelial cells in noninflammatory conditions (Kim et al. 2016; Korhonen et al. 2016). However, loss of TIE1 in conditional TIE1 knockout mice or during inflammation switched ANGPT2 into a TIE2 antagonist, indicating that ANGPT2 activity can be modulated by several mechanisms (Kim et al. 2016; Korhonen et al. 2016).

The levels of VEGF are known to regulate ANGPT2-dependent cellular responses. During early growth of glioma tumor cells in rodent models, ANGPT2 was reported to destabilize glioma co-opted blood vessels, before upregulation of VEGF, resulting in a secondary avascular tumor. In this hypoxic environment VEGF expression was increased, and VEGF stimulated tumor angiogenesis in synergy with ANGPT2 (Holash et al. 1999). In an analogous manner, during ischemia-induced pathological neovascularization in retina, ANGPT2 induced vessel regression when VEGF levels were low, but enhanced neovascularization when VEGF levels

were high (Oshima et al. 2005). Yet, ANGPT2 has been shown to act as an agonist in some tumor models. REGN910, a human antibody generated against the C-terminal FLD of ANGPT2, inhibits the interaction between ANGPT2 and TIE2 (Daly et al. 2013). REGN910 inhibited tumor growth in colorectal and epidermoid tumor xenograft models. Interestingly, ANGPT1 prevented the beneficial effect of REGN910 on tumor growth inhibition, suggesting that ANGPT2 served as a TIE2 agonist in these tumor models. Additionally, the expression of some TIE2 repressed genes was increased after REGN910 treatment but completely reversed with additional ANGPT1 treatment.

Angiopoietins in Human Cancer

ANGPT2 is expressed in conditions of vascular remodeling and indeed especially the circulating ANGPT2 has been found highly upregulated in a large variety of human tumors, specifically in advanced stages of cancer. In fact, the possibility to use ANGPT2 as marker for progressive tumors has been intensively investigated. Due to the challenges related to immunohistochemical staining of ANGPT2 in patients' samples, most of the evidence of ANGPT2 upregulation in cancer patients come from the increased soluble ANGPT2 levels in plasma or mRNA levels in tissue. It remains to be determined, whether the circulating ANGPT2 levels or the *ANGPT2* mRNA expression correlates with the expression of ANGPT2 protein in endothelial cells of the tumor vasculature.

The increased circulating ANGPT2 levels have been observed in many human cancers and linked to poor prognosis. In addition to circulating ANGPT2 levels, the expression of ANGPT2 mRNA or protein has been reported in various cancers. Mainly, the ANGPT2 expression is described in tumor ECs; however, some reports also indicate the ANGPT2 expression in tumor cells. In situ hybridization methods have been used to show increased ANGPT2 expression in ECs. In neuroendocrine tumors, where circulating levels of ANGPT2 correlated with advanced, metastatic disease and could be used to identify patients with high risk of rapid disease progression, ANGPT2 expression was detected in the vascular endothelium (Detjen et al. 2010). In colorectal cancer using laser capture microdissection, the *ANGPT2* expression was located in stromal cells but not in tumor cells (Goede et al. 2010). In gliomas, *ANGPT2* mRNA expression was restricted to a subset of blood vessels (Stratmann et al. 1998). In hepatocellular carcinoma, *ANGPT2* mRNA expression was associated specifically with hypervascular tumors (Tanaka et al. 1999). In human breast cancer samples *ANGPT2* was expressed by endothelial compartment but also by tumor cells (Sfiligoi et al. 2003). In prostate cancer ANGPT2 was highly upregulated in tumor epithelial cells particularly in high-grade tumors and correlated with increased microvessel density, metastases, and poor clinical outcome (Lind et al. 2005).

ANGPT2 expression has been shown in renal cell carcinoma (RCC) (Currie et al. 2002; Rautiola et al. 2016; Wang et al. 2014). ANGPT2 expression was found specifically in the tumor endothelium with weaker or no expression in tumor cells (Currie et al. 2002; Rautiola et al. 2016). Specific endothelial expression was found to correlate with vascular density (Rautiola et al. 2016). In advanced RCC ANGPT2 expression has been investigated in the context of first-line sunitinib therapy targeting multiple receptor tyrosine kinases, including VEGFRs. Interestingly, high endothelial ANGPT2 expression and CD31 expression were associated with a beneficial clinical response to sunitinib but not with patient survival. Another study showed that very high ANGPT2 expression was in general associated with better survival of RCC patients who received no anti-angiogenic therapy (Lampinen et al. 2016). In line with this, high ANGPT2 was associated with lower tumor grade and stage in this patient cohort. In another study, low circulating ANGPT2 levels in RCC patients were associated with better response to sunitinib treatment (Motzer et al. 2014). The examination whether the circulating levels of ANGPT2 correspond to the ANGPT2 expression levels in endothelial cells is needed to shed light on these results. In

another study, plasma ANGPT2 levels were significantly higher in patients with mRCC compared to healthy individuals or patients with stage I disease (Wang et al. 2014). Patient plasma ANGPT2 levels were observed to decrease during sunitinib therapy. Interestingly, the plasma ANGPT2 levels increased upon development of sunitinib treatment resistance. The correlation between ANGPT2 expression and sunitinib treatment response was not investigated. Additionally, in the majority of patients, circulating ANGPT2 levels increased during disease progression. Evaluation of ANGPT2 as a biomarker in phase 3 trial in gastric cancer identified baseline plasma ANGPT2 as an independent prognostic factor for overall survival. ANGPT2 was also associated with the incidence of liver metastasis. However, ANGPT2 did not predict bevacizumab (VEGF blocking antibody) response, nor did ANGPT2 levels increase upon progression (Hacker et al. 2016). Altogether, these data call for better understanding of ANGPT2 expression in human cancer and the correlation of tumor expressed ANGPT2 with circulating ANGPT2 levels. Furthermore, other tumor microenvironment parameters, such as hypoxia and inflammation may modulate the outcome of ANGPT2 signaling and the response to VEGF signaling inhibitors.

In glioblastoma, bevacizumab therapy was associated with decreased tumor vessel density when compared to the treatment-naïve glioblastoma (Scholz et al. 2016). Circulating ANGPT2 levels did not change in patients before and after bevacizumab treatment and in fact, bevacizumab-treated tumor vasculature was observed to highly express ANGPT2. Bevacizumab treatment was also associated with a relative increase of pro-angiogenic M2 polarized macrophages (Scholz et al. 2016). Whether the increased ANGPT2 expression and recruitment of tumor infiltrating macrophages upon bevacizumab treatment in human glioblastoma, and possible induction of therapy resistance, are linked together as suggested by data obtained from xenograft experiments remains to be determined.

Preclinical evidence has led to the development of several pharmacological ways to block angiopoietin signaling, which have progressed in clinical trials. Despite the strong preclinical data, the clinical trials have not yet been able to live up with the expectations. For example, trebananib, the ANGPT1 and ANGPT2 inhibiting peptibody, failed to improve the overall survival in combination with paclitaxel in the first phase 3 study (TRINOVA-1) with patients with ovarian cancer, although the addition of trebananib extended the progression-free survival in the same study (Monk et al. 2014, 2016). In the second study (TRINOVA-2) trebananib together with pegylated liposomal doxorubicin failed to increase progression-free survival, although trebananib increased objective response rate and duration of response (Marth et al. 2017). Although the clinical benefit of angiopoietin inhibition in clinical trials is yet to be discovered, the trials have demonstrated tolerable toxicity profile, distinct from that of VEGF inhibitors. In addition, angiopoietin-targeted drugs are tested in human cancer in combination with VEGF targeted therapy, and similarly to the combination with chemotherapy, the combination with anti-angiogenic therapy has proven tolerable in phase 2 trials (Atkins et al. 2015). In addition, MEDI3617, an anti-ANGPT2 antibody, and vanucizumab, a bispecific ANGPT2 and VEGF blocking antibody, are currently being tested in combination with immune checkpoint inhibitors in human cancer (www.clinicaltrials.gov). The results will reveal a potentially interesting concept of blocking the pro-inflammatory ANGPT2 with immune therapy.

In addition to cancer, excess growth of leaky neovessels occurs in other diseases, including vision-impairing diseases of the eye such as wet age-related macular degeneration (wAMD). VEGF-targeted anti-angiogenic therapy is widely used to treat wAMD that can lead to vision loss unless treated. Although effective in a large group of patients, in approximately 40% of patients the disease can progress despite of VEGF-targeted therapy (Ferrara and Adamis 2016). Therefore, the vascular stabilizing and anti-angiogenic features of ANGPT-TIE targeted drugs are investigated in human wAMD and diabetic macular edema (an edema causing, vision impairing vascular complication that affects diabetic patients).

These approaches include the VE-PTP inhibitor (AKB-9778), combinations of VEGF-targeted drugs with anti-ANGPT2 antibodies or bispecific antibodies targeting both ANGPT2 and VEGF.

Summary

Currently, angiopoietins are intensively studied for their properties of regulating vascular remodeling and vessel stability. Although ANGPT1 and ANGPT2 bind to the same TIE2 receptor, they tend to have opposing effects on mature blood vascular endothelium. While ANGPT1 acts as a classical agonist of TIE2 by activating the receptor, ANGPT2 acts as a context-dependent agonist or antagonist of TIE2. Therefore, ANGPT2 binding to TIE2 may occur without receptor activation and at the same time preventing ANGPT1 from activating TIE2. TIE2 activation results in suppression of EC inflammation and permeability, promoting EC survival and vessel stability. However, the angiopoietin signaling is complex, and it has been shown that EC secreted ANGPT2 has agonist activity, but this is lost during inflammation, leading to leaky vessels. In addition, the levels of VEGF have been reported to regulate ANGPT2 functions, low levels promoting vessel destabilization and high levels angiogenesis. Additionally, angiopoietins have been shown to signal via integrins, both in the presence and absence of TIE2, which may affect angiopoietin signaling in tumors, although this possibility remains to be further investigated. In addition, TIE1 was found critically required for ANGPT2 agonist activity. As TIE1 undergoes ectodomain cleavage in response to inflammatory stimuli, the level of TIE1 expression in tumors may also dictate ANGPT2 signaling outcomes. Understanding in which biological context, and possibly tumor types, ANGPT2 functions as an agonist or antagonist, or in which context it interacts with TIE2 or alternative integrin receptors, are key questions in order to harness angiopoietin signaling for therapeutic purposes.

ANGPT1 is necessary for correct vascular patterning and for the formation of nonleaky vessels of a functional vasculature. Whereas ANGPT1 is important for proper investment of vessels with pericytes, ANGPT2 can destabilize vessels via inducing pericyte dropout, facilitating VEGF-dependent angiogenesis. Tumor vasculature is unorganized and leaky, which induces variation in the interstitial fluid pressure and consequently changes in blood flow, oxygenation, and drug distribution in the tumor. As a result, the tumor microenvironment is highly hypoxic, stimulating upregulation of hypoxia-inducible genes VEGF, ANGPT2, and VE-PTP. In addition, VEGF further increases ANGPT2 expression. The leaky tumor vessels promote tumor cell dissemination and the abnormal tumor vasculature can fuel inflammation and limit antitumor immunity. ANGPT2 blocking has shown benefit in decreasing tumor metastasis, via several mechanisms and in combination with VEGF to modulate the inflammatory tumor microenvironment and to enhance anti-tumor immunity.

Although many studies have shown the beneficial effect of blocking ANGPT2 in mouse tumors that is further enhanced when provided in combination with VEGF blocking drugs or with immune checkpoint therapy, the function of ANGPT2 as a target, or prognostic, or predictive biomarker in human cancer remains to be investigated. The knowledge on the biological and molecular mechanisms of the ANGPT-TIE system should facilitate the clinical development of investigational ANGPT2-targeted drugs for the treatment of human cancer, in combination with VEGF-targeted anti-angiogenic or immune therapies.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Combination of Anti-angiogenics and Other Targeted Therapies
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part V

Pathology of Tumor Angiogenesis

Pathology of Tumor Angiogenesis

Peter Bronsert and Martin Werner

Contents

Introduction 254

Scientific Perception 255
(a) Historical Overview 256
(b) Embryological Process for Vessel Development: Vascular Genesis and Angiogenesis 257
(c) Vessel Formation Modes 257
(d) Effects of Malignant Tumors and Precursor Lesions onto Angiogenesis 258
(e) Histological Architecture 260
(f) Signaling Molecules in Angiogenesis 261
(g) Haemangiogenesis Carcinomatosa and Metastases 266
(h) Malignant Tumor Tissue 268

Future Directions 270

Cross-References 270

References 270

Abstract

Angiogenesis, commonly used to describe the process of vessel growth, defines the sprouting process from preexisting vessel and plays a pivotal role for vital beneficial processes, diseases, and harmful neoplastic lesions. Physiological angiogenesis is a temporary and locally limited process appearing in reproduction, development, and wound repair, depending on a balanced equilibrium of pro- and anti-angiogenetic directing angiogenesis.

During tumor genesis, epithelial cells have to pass a sequence, starting with epithelial dysplasia, followed by carcinoma in situ, and finally resulting in the invasive cancer. Invasive cancer has the capacity to infiltrate the surrounding tissue, to sprout to distant sides, and to form metastases. Hereby, the invasive process is accompanied with a significant cell proliferation and increasing tumor size. Without additional oxygen and nutrient support, tumors will arrest/decline their growth or even die. Tumor cells exploit the above-described physiological processes by hijacking

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local vessels and modifying the surrounding microenvironment to ensure oxygen and nutrition supply. The following chapter will present an overview into the pathology of angiogenesis focusing on embryological process of vessel development, namely, vascular genesis and angiogenesis, vessel formation modes, effects of malignant tumors and precursor lesions onto angiogenesis, and histological architecture in malignant tumors.

Keywords

Pathology of tumor angiogenesis · Breast cancer · Colorectal cancer · Lung cancer · Prostate cancer · Sarcomas

Introduction

Angiogenesis, commonly used to describe the process of vessel growth, defines the sprouting process from preexisting vessel and plays a pivotal role for vital beneficial (vessel formation during wound healing processes, development of collaterals after ischemia events) processes, diseases (e.g., diabetic retinopathy [more diseases are listed in Table 1]), and harmful neoplastic lesions. Current opinion understands physiological angiogenesis as a temporary and locally limited process appearing in reproduction (ovulation, placentation), development, and wound repair (Hanahan and Folkman 1996). Physiological angiogenesis depends on a balanced equilibrium of pro- and anti-angiogenetic directing angiogenesis in a predominant quiescent state. In numbers, only 1 of 10,000 endothelial cells is situated in the cell division cycle. Thus, besides neural cells, endothelial cells represent the second long-lasting cell population in mammals (Engerman et al. 1967; Hobson and Denekamp 1984). The process of angiogenesis differs in healthy tissue and tumors. In healthy tissue, de novo vessel formation by sprouting angiogenesis, by the recruitment of bone marrow-derived endothelial progenitor cells and the recruitment of vascular wall-resident endothelial progenitor cells, or by a process of vessel splitting known as intussusception can be observed (Carmeliet and Jain 2011) (Fig. 1).

Table 1 Diseases associated with aberrant angiogenesis

Diseases and angiogenesis		
Organ	Disease	
	Excessive angiogenesis	Insufficient angiogenesis
Blood vessels	DiGeorge syndrome (Stalmans et al. 2003), cavernous hemangioma	Atherosclerosis (Moreno et al. 2006), diabetes, hypertension
Bones and joints	Arthritis, osteomyelitis	Osteoporosis (Filipowska et al. 2017)
Eye	Persistent hyperplastic vitreous syndrome (Salvucci et al. 2015), diabetic retinopathy (Lobo et al. 2000), choroidal neovascularization	
Intestine	Inflammatory bowel disease (Moreno et al. 2006)	
Lung	Primary pulmonary hypertension (Voelkel et al. 2002; Simon and McWhorter 2002)	Neonatal respiratory distress, pulmonary fibrosis

Tumor cells exploit the aforementioned processes of angiogenesis. Furthermore, tumor cells can hijack local vessels, can go through the process of epithelial-mesenchymal transition (EMT), can generate endothelial cells by their own, and can use their own cell membranes to mimic vascular walls. Of note, contrary to non-tumorous angiogenesis, in malignant tumors angiogenesis rests for an indefinite time (Folkman 2007). During tumor genesis, epithelial cells have to pass a sequence, starting with epithelial dysplasia, followed by carcinoma in situ, and finally resulting in the invasive cancer. The pronounced difference between precursor lesions and invasive carcinomas is the integrity of the basal lamina. The basal lamina represents a barrier separating the (non-invasive) lesion from the surrounding tissue. The basal membrane is a 50 nm thick interlink between the epithelial cells and the interstitium.

Hereby a direct vascular infiltration is rarely observed (Bossi et al. 1995; Bluff et al. 2009). If the basal lamina is exceeded by the tumor, an

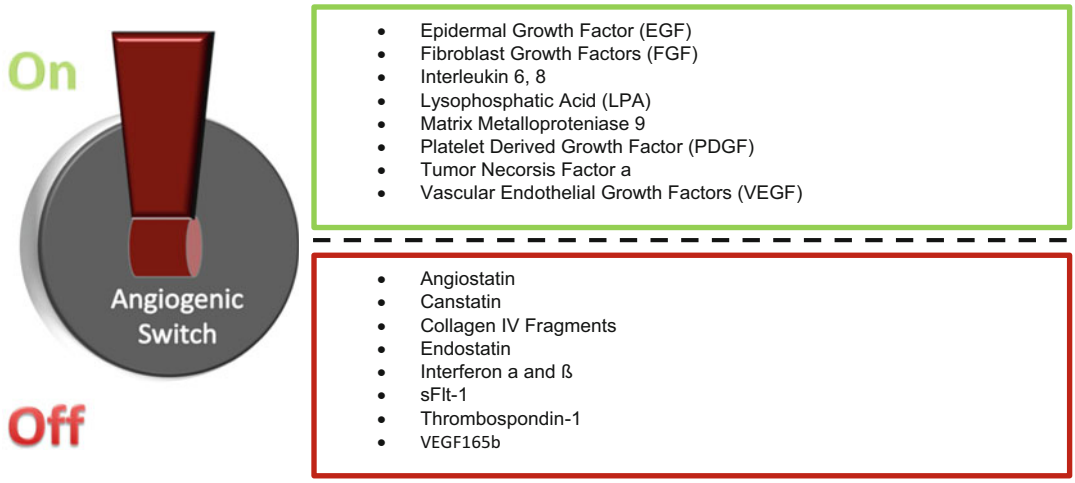

Fig. 1 Angiogenetic switch, controlling balanced equilibrium of pro- and anti-angiogenetic directing angiogenesis

invasive carcinoma is present. Invasive cancer has the capacity to infiltrate the surrounding tissue, to sprout to distant sides, and to form metastases. Hereby, the invasive process is accompanied with a significant cell proliferation and increasing tumor size. Without additional oxygen and nutrient support, tumors will arrest/decline their growth or even die. To conquer this steady state and to expand beyond the capacity of the local blood supply, tumors have to operate the so-called angiogenic switch to initiate angiogenesis.

The angiogenetic switch describes the dysbalance of pro- and anti- angiogenetic factors favoring angiogenesis. Angiogenesis represents a hallmark of cancer (Hanahan and Weinberg 2011). From a histo- and immunohisto-morphological point of view, the operated angiogenic switch is represented by proliferating endothelial cells with alternated vessel ramifications (Fig. 2) infiltrating the surrounding microenvironment. The term tumor microenvironment represents a variety of compartments and cells comprising fibroblasts, angioblasts, immune cells, and the extracellular matrix consisting of collagens. Invasive carcinomas modify the surrounding microenvironment to ensure oxygen and nutrition supply. Hereby, considerable discrepancies between tumors and their particular surrounding exist. These discrepancies are dependent of the tumor type (e.g., squamous/adenocarcinoma), grade (e.g., good/undifferentiated), stage, and the expression of angiogenic factors (Blouw et al. 2003; Morrissey et al. 2008; Jubb et al. 2011).

Scientific Perception

Oxygen is essential for tumorous and non-tumorous cells. Molecular mechanisms that monitor and adequately adapt to oxygen levels play a pivotal role during cell response. Hereby, cells can adept by two main strategies represented by an acute and a prolonged response.

Blood vessels supply every organ by distinct growth and distribution patterns. Aberrations from these physiological patterns can provoke pathological consequences culminating into numerous diseases. Diseases can be subdivided into disorders characterized or caused by exuberant angiogenesis (e.g., psoriasis, arthritis, blindness, and cancer), abnormal vascular remodeling (e.g., heart and brain ischemia and infarction, arterial and pulmonary hypertension, and preeclampsia), or vascular regression (Weis and Cheresh 2011). In its entirety, blood vessels represent a hierarchical structured organ constantly transforming in its periphery. Mechanisms causing transformation are represented by embryological, inflammatory, and tumor-specific

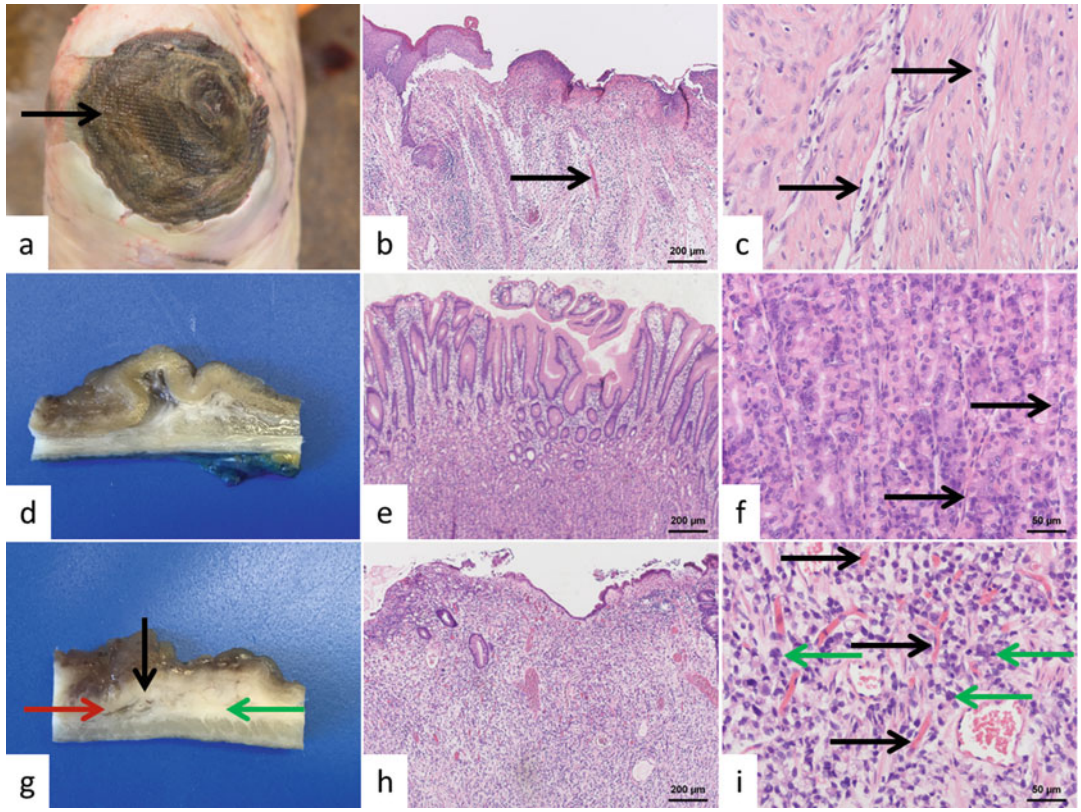

Fig. 2 (**a**–**i**): Macroscopic (**a**, **d**, **g**) and microscopic (**b**, **c**, **e**, **f**, **h**, **i**) view of an ulcus cruris (**a** [black arrow = necrotic tissue], **b** [junction between normal epithelium and ulcus, black arrow = orthogonal passing vessel, picture taken at fivefold magnification], **c** [black arrow = orthogonal passing vessel, picture taken at 20-fold magnification], normal gastric mucosa, **e** [picture taken at fivefold magnification], **f** [black arrow = orthogonal passing vessel, picture taken at 20-fold magnification]) and a gastric signet ring carcinoma, **g** [red arrow = normal mucosa, green arrow = tumor, black arrow transition between tumor and mucosa], **h** [picture taken at fivefold magnification], and **i** [black arrow = chaotic/disordered vessels, green arrow = signet ring carcinoma, picture taken at 20-fold magnification])

processes, orchestrated by a numerous number of cells and cell compartments, secreting angioblast and recruiting and rejecting factors (DNA, RNA, miRNA, proteins, and exosomes). Physiological and pathological angiogenesis differs in the balance of pro- and anti-angiogenic signaling. During physiological angiogenesis, vessels mature and become stable, whereas during pathological angiogenesis, the balance between pro- and anti-angiogenic signaling is lost.

For a structured outline, the following text will be sectioned as follows:

(a) Historical overview
(b) Embryological vascular and angiogenesis
(c) Vessel formation modes
(d) Effects of malignant tumors and precursor lesions onto angiogenesis
(e) Histological architecture
(f) Signaling molecules in angiogenesis
(g) Angiogenesis, haemangiogenesis carcinomatosa, and metastases
(h) Pathology in malignant tumors

(a) Historical Overview

The first observation of angiogenesis was noticed in 1865 by Rudolf Ludwig Karl Virchow. Virchow observed an increased number of vessels and a distinct capillary network in malignant tumors (Ferrara 2002). In 1908, Goldmann described in his

work "The Growth of Malignant Disease in Man and the Lower Animals, with special reference to the Vascular System" the vessel architecture as follows: "The normal blood vessels of the organs in which the tumor is developing are disturbed by chaotic growth, there is a dilatation and spiraling of the affected vessels, marked capillary budding and new vessel formation, particularly at the advancing border." (Fig. 2) (Goldmann 1908). Relations between tumor type, vascular architecture, and the surrounding microenvironment were described in 1927 by Lewis et al. (Lewis 1927) In 1939 Ide et al. first postulated that for the formation of new vessels in tumors, specific factors capable for the vessel stimulation are needed (Ide and Baker 1939). In 1971 Judah Folkman first demonstrated that tumors would not grow more than 2 mm without blood supply (Sherwood et al. 1971) and built the foundation of nouveau tumor angiogenesis inhibitors as an effective anticancer strategy. This limited growth was later interpreted as the vascular independent phase in tumor progression (Brem et al. 1978; Maiorana and Gullino 1978). Furthermore Folkman isolated the first tumor angiogenesis factor (TAF) by fractionating the cell line Walker 256 carcinoma (Folkman et al. 1971). The identified fractions were able to induce a vascular proliferation in vivo and in vitro (McAuslan and Hoffman 1979; Weiss et al. 1979). At the end of the 1980s, a growth factor for vascular endothelial cells in the media conditioned by bovine pituitary follicular cells was purified. This growth factor "reveals that it exerts mitogenic effects also on vascular endothelial cells isolated from several districts but not on adrenal cortex cells, lens epithelial cells, corneal endothelial cells, keratinocytes or BHK-21 fibroblasts, indicating that its target cells specificity is unlike that of any previously characterized growth factor." The vascular endothelial growth factor (VEGF) was identified (Ferrara and Henzel 1989).

(b) Embryological Process for Vessel Development: Vascular Genesis and Angiogenesis

The embryological process of vessel development and recruitment is subdivided into de novo vessel genesis – vascular genesis – and subsequently the sprout of new blood vessels from preexisting blood vessels – angiogenesis. During the early stages of embryonic development, vascular genesis – defined as de novo development of endothelial cells from angioblasts – represents the predominant process (Herbert and Stainier 2011). Angioblasts differentiate into endothelial cell-clothed cavities. The cavities become covered by pericytes and/or smooth muscle cells and represent the basis for the latter hierarchical structure (arteries, arterioles, capillaries, venules, and veins). During organogenesis these endothelial cells become nascent and induce organogenesis (e.g., in the pancreas and liver) (Butler et al. 2010). It is plausible that because of the different interactions between the endothelial cells and the infiltrated organ, vessel-specific and organ-specific heterogeneities can be observed.

(c) Vessel Formation Modes

The vessel sprouting describes a guided process attracted by pro-angiogenic signals and represents the substantial mechanism of vessel growth (Potente et al. 2011) comprising molecular activation of a migration, proliferation, and remodeling vessel program. This program is categorized into the states of quiescence, activation, and resolution of vascular cells (endothelial cells, pericytes) and extravascular matrix. As previously described, endothelial cells are situated in a quiescent state without proliferating, migrating, or dividing activities. The endothelial cells are interlinked by a tight barrier, separating the vascular lumen and surrounding tissue.

Steps of angiogenesis in preexisting blood vessels	
1.	Vasodilatation and increased permeability
2.	Pericyte separation
3.	Basement membrane degradation
4.	Endothelial cell migration
5.	Forming of the vascular stalk by tip cell-guided endothelial cell proliferation
6.	Tube formation by endothelial vacuolization and subsequent fusion
7.	Periendothelial cell recruitment (small vessels = pericytes, larger vessels = smooth muscles)

(*continued*)

Steps of angiogenesis in preexisting blood vessels	
8.	Basement membrane assembly
9.	Restitution of the quiescence state

The program starts when an endothelial cell is activated by an angiogenic stimulus (e.g., VEGF, NOTCH, ANG1, and FGF). In response to the stimulus, vascular cells change from the quiescence to the activated state. Vasodilatation and the subsequent increase in permeability with concomitant extravasation of plasma proteins represents the first step. Hereby, the vessel surrounding the matrix is remolded into a preliminary niche for vascular migration due to matrix metalloproteases. Next the vessel surrounding the pericytes separates and loses contact to the basement membrane and to the endothelial cells. Subsequently the basement membrane will be degraded via the matrix metalloproteases. The endothelial cells become motile, pass the degraded basal membrane, and extend filopodia guiding the development of the endothelial sprout. The first/guiding endothelial cells are called tip cells. The tip cells keep on moving away from the primary vessel, whereby endothelial cells from the primary vessel follow by migration and form the vascular stalk. Tip cells are navigated by guidance signals (semaphorins and ephrins) and adhere to the extracellular matrix (mediated by integrins) to migrate. The following cells of the vascular stalk recruit pericytes and synthesize the basement membranes to stabilize the vascular equilibrium. By forming vacuoles and merging together, the endothelial cells begin to hollow out the vascular stalk, form a perfused tube, and finally become a capillary. Interestingly, the fused endothelial cells share only the same lumen but not cytoplasm and remain as separated cells, only interlinked with endothelial junctions. After the formation of a new capillary, periendothelial cells (pericytes for small vessels and smooth muscles for larger vessels) will be recruited. Finally the basement membrane will be built by the endothelial cells. The specialization of endothelial cells into tip or stalk cells is transient and reversible and depends on the equilibrium between pro-angiogenic factors (e.g., VEGF and Jagged-1) and angiogenic factor suppressors of endothelial cell proliferation (e.g., Dll4). Tip cells are characterized by high expression levels of Dll-4, PDGF-b, VEGFR-2, and VEGFR-3/Flt-4 and have low levels of Notch signaling activity (Ribatti 2017).

Further Angiogenic Mechanisms

- Intussusceptive angiogenesis: Intussusceptive angiogenesis is driven by the insertion of interstitial tissue pillars into preexisting vessels that split them in two new functional vessels.
- *Vasculogenic mimicry (VM)*: The term vasculogenic mimicry describes tumor cells building pseudo-vessels surrounding blood channel structures. VM has been described for melanomas and breast, ovarian, prostate, bladder, and lung cancer (Paulis et al. 2010). These vascular-like structures contain plasma and red blood cells and participate in the blood circulation. Interestingly, endothelial cells are detectable neither by light or electron microscopy nor by immunohistochemistry (Maniotis et al. 1999; Paulis et al. 2010). Also tumor cells and endothelial cells can coexist next to each other and together surround a "blood channel" maintaining a physiological blood perfusion (Hess et al. 2007).

  The tumor cells involved in VM often express molecular profiles which are also expressed during embryonic vasculogenesis. This and the high plasticity of VM-associated tumor cells could give a hint that cancer stem cells (CSC) and/or epithelial-mesenchymal transition (EMT) are involved in VM.
- Vascular hijacking: Tumors grow by hijacking preexisting vessels in the peritumoral tissues.
- Endothelial precursors: Circulating endothelial precursor cells are recruited by tumor vessels, integrate in the vessel wall, and differentiate into ECs (Fig. 3).

(d) Effects of Malignant Tumors and Precursor Lesions onto Angiogenesis

The main characteristic to distinguish between an invasive malignant epithelial tumor and a precursor lesion (e.g., carcinoma in situ) is represented

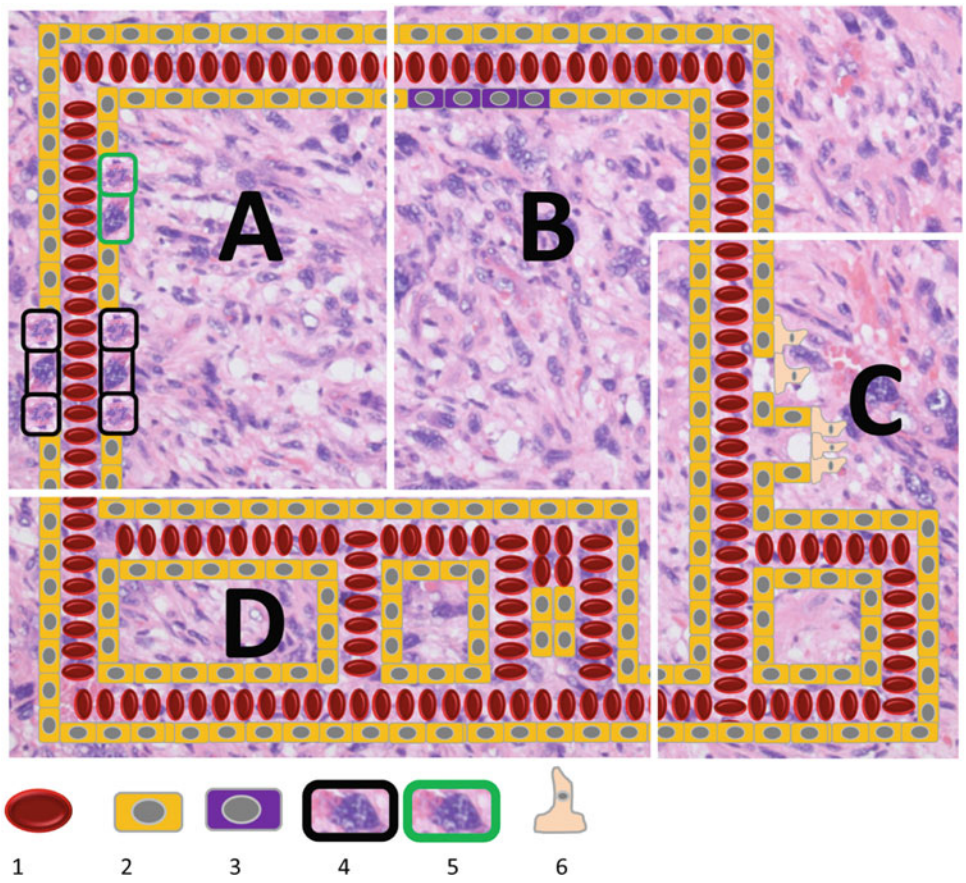

Fig. 3 Angiogenic mechanisms in tumor angiogenesis. (**a**) Vascular mimicry by cancer cells (black border) or cancer stem cells (green border) (**b**) Endothelial cells are recruited by the tumor and integrated, expanding the preexisting vessel wall (**c**) Angiogenesis of preexisting blood vessels (more details given in Fig. 5) and (**d**) intussusceptive angiogenesis by the insertion of interstitial tissue pillars into preexisting vessels and the split in two new functional vessels (Figure legend: *1* red blood cells, *2* endothelial cells, *3* endothelial cells recruited by the tumor, *4* pericytes, *5* paracrine and autocrine effects for matrix remodeling, *6* tip cell-guided endothelial cell, *7* matrix, *8* blood flow)

by the basement membrane. The basement membrane is a thin and tiny layer consisting of reticular/argyrophilic fibrils. Using the periodic acid-Schiff (PAS) reaction – a conventional examination method for light microscopy visualizing glycoproteins – the basement protrudes as a slight purple layer. Using electron microscopy, the basal membrane can be subdivided into the lamina rara, the lamina densa, and the lamina fibroreticularis, which directly merges with the connective tissue without a clear border (comparable with the tunica adventitia). The basement membrane has a thickness of 2 μm and can differ in thickness and structure as regards topography. Invasive carcinomas obtain the enzymatic ability to disassemble the basement membrane primarily by matrix metalloproteases (MMPs), elastase and trypsin. Between the basement membrane and the epithelium/endothelium, myoepithelial cells are allocated. Myoepithelial cells form a semicontinuous protective sheet separating, for instance, the human breast epithelium and the surrounding stroma. They suppress stromal invasion of tumor cells by the secretion of various anti-angiogenic and anti-invasive factors. The disruption of this cell layer results in the release of the growth factors, angiogenic factors, and reactive oxygen species causing an alteration in the microenvironment (Pandey et al. 2010).

(e) Histological Architecture

Vessels can be subdivided into arteries and veins, based on their direction, that is, reaching or departing to the heart. Arteries present a thicker structure to withstand pulsatile flow and a higher blood pressure (compared to veins) and demonstrate a divergent ramification pattern. Regarding size and function, arteries can be subdivided into large, elastic arteries (e.g., aorta, aortic branches [A. subclavian, common carotids]), medium-sized muscular arteries (e.g., A. renalis), small arteries, and arterioles (supplying tissues and organs). Contrary to arteries, veins converge to greater vessels and have thinner walls and a larger lumen. The linkage between veins and arteries is represented by capillaries. Capillaries represent the vessels with the lowest diameter, consist only of one endothelial cell layer based on an elastic membrane, and ensure the supply of oxygen and nutrients as well as the uptake of carbon dioxide and waste material.

Arteries and veins are composed of three concentric layers, the tunica intima, the tunica media, and the tunica adventitia.

The tunica intima represents the inner layer with direct contact to the blood flow, followed by the tunica media and the tunica adventitia. The outer layer, the tunica adventitia, has direct contact to the perivascular soft tissue. Interestingly, no clear boundary between the surrounding tissue and the adventitia can be defined. Blood and nutrition supply of the tunica intima and tunica media is assured by direct diffusion from the blood flow and (in case of large elastic arteries) by vasa vasorum entering the blood vessels through the tunica adventitia.

- *Tunica intima:* The inner coat, the tunica intima, comprises three layers starting with the endothelial cells, followed by a subendothelial layer, and finally the membrana elastica interna, a fenestrated layer of longitudinal orientated elastic fibers. The tunica intima varies in size depending on the diameter of the vessel. In small vessels, less than 3 mm diameter, the membrana elastica interna is only composed of stellate cells. The membrana elastica interna separates the tunica intima from the tunica media.
- *Tunica media:* The tunica media is mainly composed of smooth muscles permeated with elastic fibers, connective tissue, and foothills of the autonomic nerve system. The composition of these three elements is dependent on the vessel type. The mixture of smooth muscle and nerve cells paves the way for vasodilatation and vasoconstriction. The elastic fibers enable a continuous blood flow by facilitating a vessel expansion during the systole and passive contraction during the diastole. The lamina elastic externa, a dense elastic membrane, separates the tunica media from the tunica adventitia.
- *Tunica adventitia:* The tunica adventitia is composed of connective tissue, blood vessels (*vasa vasorum*), and autonomic nerves (*nervi vasorum*).

Tumor vessels differ from the aforementioned physiological architecture and function (Goel et al. 2011) (Fig. 2). Besides the absence of the strict hierarchical categorization in the arteries and veins, with their converging and divergent appearance, tumor vessels impair regions with a high vascular density, neighboring vessel-poor areas. Also the course of vessels varies, demonstrating irregular diameters (abnormally wide to thinly wide with dilatated or compressed lumens) and a serpentine-like course. Considering the microscopic level, tumor vessels continuously demonstrate an abnormal architecture from the inside out. In detail, endothelial cells are poorly interconnected and occasionally multilayered. The basement membrane varies in thickness and composition and is less loosely attached to the hypocontractile myoepithelial cells covering the tumor vessels (Potente et al. 2011).

The unphysiological architecture leads to an irregular perfusion and affects the flow of nutrient and oxygen. Via hypoxia and nutrition shortage, circulus vitiosus is initiated, stimulating ongoing angiogenesis and culminating in a total chaotic vessel structure with subsequent necrosis and hemorrhage. Uncontrolled proliferation and the

consequent compression of surrounding and passing malformed tumor vessels trigger the muddled situation and additionally affect lymphatic drainage.

The nutrition shortage and the hypoxic microenvironment promote evolutionary pressure onto tumor cells with a selection of a more aggressive phenotype and resistant tumor cells which can resist to these harsh conditions. The aggressive phenotype is accompanied by activated oncogenes and inter alia genes initiating epithelial-mesenchymal transition (EMT), which heightens their metastatic potential and radio-oncology resistance (Holohan et al. 2013).

(f) Signaling Molecules in Angiogenesis

Hypoxia can be defined as a condition in which the tissue is deprived of adequate oxygen supply. In malignant neoplasia, the oxygen supply differs between the neoplasia and the surrounding microenvironment. Oxygen levels below 1% can be detected in the microenvironment. The microenvironmental hypoxia triggers the angiogenic switch, represented by multiple gene expression changes affecting angiogenesis and metabolism to pave the way for oxygen and nutrition supply and subsequent tumor invasion and metastases. The histomorphological pendent of a "turned on" angiogenic switch is an increase in endothelial cell proliferation and a subsequent higher vascular density. This concept is exploited in functional in vitro assays (e.g., Matrigel capillary-like tube formation assay, 3D collagen spheroid sprout formation assay, mosaic spheroid assay, ex vivo aortic ring assay, Fig. 4.) for quantitative analyses. Hereby the expansion of cultured capillaries can be quantified via proliferation, migration, or metabolic activity.

Interestingly, in vivo hypoxia is heterogeneously represented in tumors. In one tumor anoxic regions (no blood vessel supply), hypoxic areas (generated by elevated metabolic tumor activity or immune response) and normoxic regions can be detected. Under physiological conditions, the vascular architecture represents a barrier difficult to overcome for tumor cells during intravasation and especially extravasation. In neovasculated tumors, the imbalance of pro- and anti-angiogenic factors initiates an architectural mismatch which facilitates tumor cell migration. In detail tumor vessels are characterized by a dysfunctional architecture represented by pericytes only loosely attached to the endothelium; a significant thinner/nonexistent basal lamina and a fenestrated endothelium increase the vessel permeability. Also vessel diameters show fluctuations, which provoke blood clots and local variations in blood pressure. The chaotic vessel structure and architecture of the vessel entails blood stasis and blood backflow and facilitates tumor cell migration (Paduch 2016). Histologically nonneoplastic tissues and blood vessels demonstrate a direct orthogonal alignment to

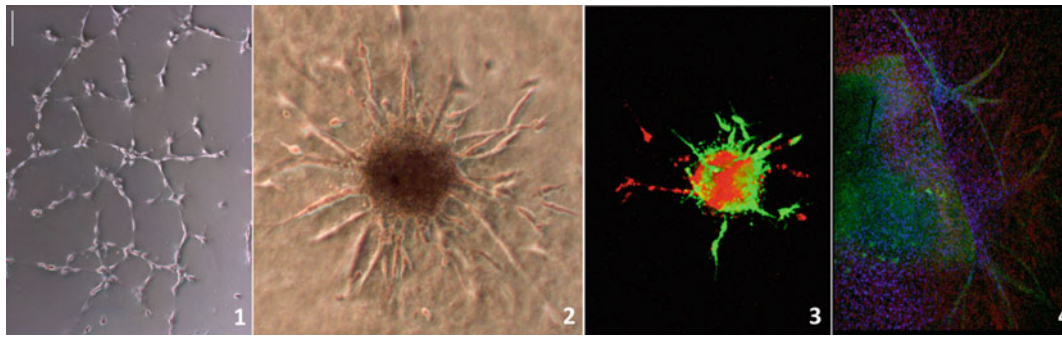

Fig. 4 From left to right: (**1**) Matrigel capillary-like tube formation assay (short: Matrigel in vitro), (**2**) 3D collagen spheroid sprout formation assay (short: spheroid assay), (**3**) mosaic spheroid assay, (**4**) ex vivo aortic ring assay (With the kind support from Jennifer Esser, University Heart Center Freiburg)

the supplied tissue, whereas in malignant tumors, blood vessels demonstrate a disordered non-orthogonal course (Fig. 2).

To compensate the insufficient oxygen and nutrient supply and to escape from tumor necrosis, tumor cells express hypoxia-inducible factors (HIF), a master transcriptional regulator of the adaptive response to hypoxia. HIF-1 is a highly conserved transcriptional heterodimer, composed of an alpha and a beta subunit. The beta subunit is a constitutively expressed aryl hydrocarbon receptor nuclear translocator. HIF-1 belongs to the PER-ARNT-SIM subfamily of the basic helix-loop-helix family of transcription factors. The alpha and beta subunits are similar in structure, both containing a bHLH domain at the N-terminus for DNA binding and a central region Per-ARNT-Sim for heterodimerization. The C-terminus recruits transcriptional coregulatory proteins. Under hypoxic conditions, HIF activates the transcription of over 40 genes, including erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor, HILPDA, and other genes, increases oxygen delivery, or facilitates metabolic adaptation to hypoxia. Furthermore, HIF plays an essential role in embryonic vascularization, tumor angiogenesis, and pathophysiology of ischemic disease. Under normoxia the enzyme prolyl hydroxylase hydroxylates the oxygen-dependent domain of HIF-1α. The hydroxylated HIF-1α is recognized by the von Hippel-Lindau (vHL) tumor suppressor protein, a component of an E3-ubiquitin ligase complex. Hereby, HIF-1α will become poly-ubiquitinated and rapidly proteasomal degraded. Hypoxia acts as an inhibitor of the prolyl hydroxylase and leads to the stabilization of HIF-1α. The hypoxic transcriptional program starts. The stabilized HIF-α subunit shuttles into the nucleus, dimerizes with the HIF-1β subunit, binds to the hypoxia response element (HRE), and activates downstream target genes for tumor angiogenesis, invasion, metabolism, and proliferation (Figs. 5 and 6).

As angiogenesis regulating factors and receptors (/R), the vascular endothelial growth factor (VEGF/R), the fibroblast growth factor (FGF/R), the platelet-derived growth factor (PDGF/R), the transforming growth factor-β (TGF-ß/R), the angiopoietins and TIE receptors, the delta/Jagged-Notch signaling, the matrix metalloproteinases, and the chemokines have been identified.

The key player supervising the angiogenetic remodeling of the extracellular matrix is represented by the vascular endothelial growth factor (VEGF) family. VEGF, originally described as an endothelial cell-specific mitogen (Ferrara et al. 1992), is not exclusively limited to the vascular system but also produced by other cell types, inter alia tumor cells, macrophages, platelets, keratinocytes, and renal mesangial cells (Iijima et al. 1993; Sunderkötter et al. 1994; Boocock et al. 1995; Frank et al. 1995; Verheul et al. 1997; Itakura et al. 2000). The five VEGFs

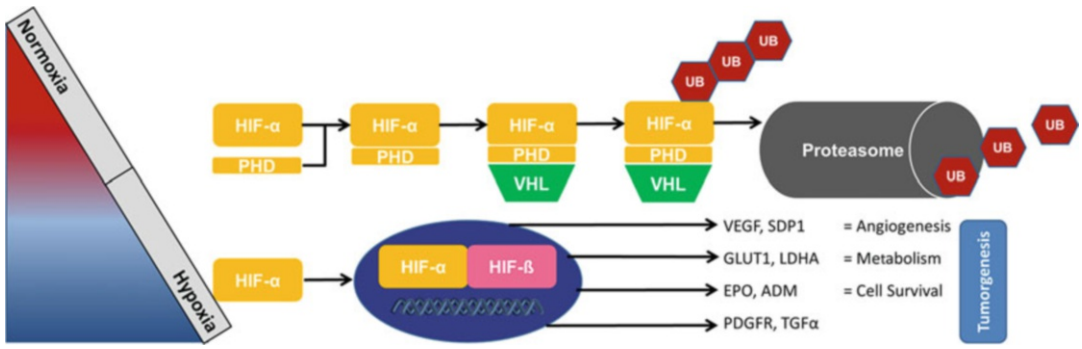

Fig. 5 Oxygen-regulated HIF-1α signaling. (Abbreviations: ADM adrenomedullin, EPO erythropoietin, GLUT1 glucose transporter, HIF-1α hypoxia-inducible factor 1α, LDHA lactate dehydrogenase A, PDGFR platelet-derived growth factor receptor, PHD prolyl hydroxylase, SDP1 , TGF-α tumor growth factor 1α, UB ubiquitin, VEGF vascular endothelial growth factor, VHL von Hippel-Lindau

are VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factors (PlGF). VEGF-A, VEGF-B, and PlGF are alternatively spliced, while VEGF-C and VEGF-D are proteolytically processed. Thereby a wide range of distinct VEGF isoforms is represented.

Under physiological conditions, VEGF modulates bone formation, hematopoiesis, and wound

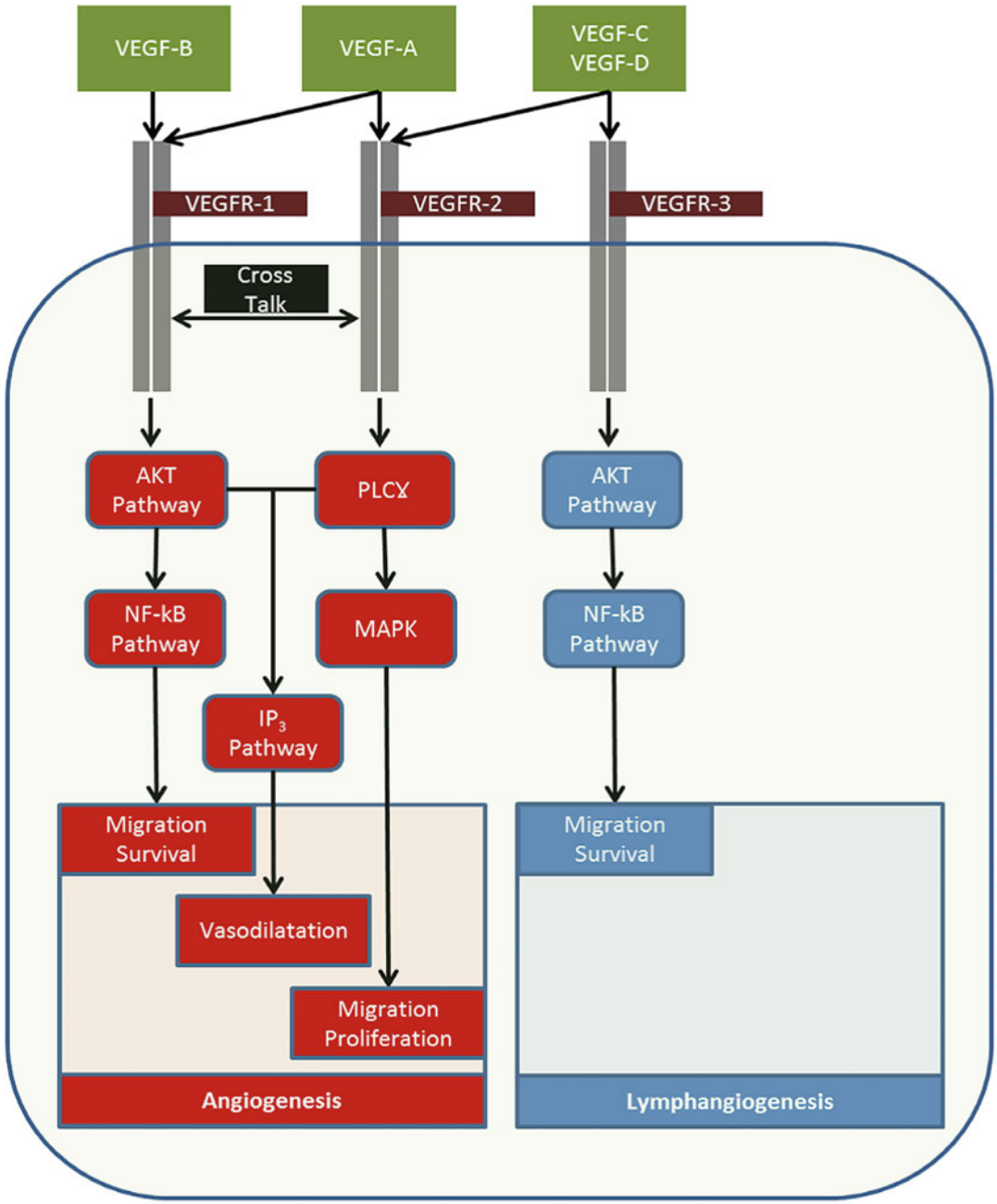

Fig. 6 Pathways of the VEGF family and VEGFR intracellular signaling cascades inter alia regulating migration, proliferation, survival, vasodilatation, as well as angiogenesis, and lymphangiogenesis is regulated. Abbreviation: *Akt* protein kinase B, *FAK* focal adhesion kinase, IP_3 inositol trisphosphate, *MAPK* mitogen-activated protein kinase, *NFκB* nuclear factor κ-light chain-enhancer of activated B cells, *PLCγ* phospholipase Cγ

healing. In vasculogenesis and angiogenesis, VEGF induces endothelial cell proliferation and migration. The VEGF family consists of five VEGF glycoproteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D) and the placental growth factors 1 and 2.

Active VEGF forms are homodimers that differ in size and have the ability of binding to heparin, heparan sulfate, or accessory transmembrane proteins called neuropilins, which limits their diffusibility and local activity (Roskoski 2017).

VEGFs modulate blood and lymph vessel formation and proliferation through the vascular endothelial growth receptors VEGFR. The VEGFR family comprises three membranous receptor tyrosine kinases (RTK) VEGFR-1, VEGFR-2, and VEGFR-3. Each tyrosine kinase consists of an extracellular segment containing seven immunoglobulin-like domains (which initiate receptor dimerization after ligand binding), one transmembrane segment, one juxtamembrane segment, and one intracellular tyrosine kinase domain (Shibuya 1995) (Fig. 7). Equivalent to other membrane-bound RTKs, the extracellularly initiated homo- or heterodimerization receptor activation subsequently initiates transmembrane signaling, and terminating the intracellular tyrosine kinase domain activation leads to an activation of the tyrosine kinase domain and subsequent autophosphorylation of tyrosine. The following signaling pathways are regulating vascular permeability as well as endothelial cell survival, migration, and proliferation.

VEGFR-1 binds to VEGF-A, VEGF-B, and PlGF. Two isoforms, a full-length and an alternatively spliced soluble form (sFlt1), are known (Kendall and Thomas 1993). sFlt-1 consists of 6 Ig-like domains with a short, 31 amino acid long tail and exhibits a strong binding ability to VEGF-A, PlGF, and VEGF-B. Both isoforms bind to VEGF-A with a higher affinity (Kd = 1~10 pM) compared to VEGFR-2. This competitive situation inhibits/retards VEGFR-2 activation via VEGF-A and serves as a model to spatially control VEGFR-2 signaling and formation of angiogenic sprouts (Kappas et al. 2008). Ambati et al. interpret the function of the sFlt1 as a counterpart of angiogenesis (Ambati et al. 2006), which could ensure the avascularity of the cornea or explain the pathogenesis of preeclampsia (Maynard et al. 2003). sFlt-1 is assumed to be involved in placentation as a biochemical barrier between fetal and maternal circulation by suppressing excess angiogenesis and abnormal vascular permeability (Maynard et al. 2003).

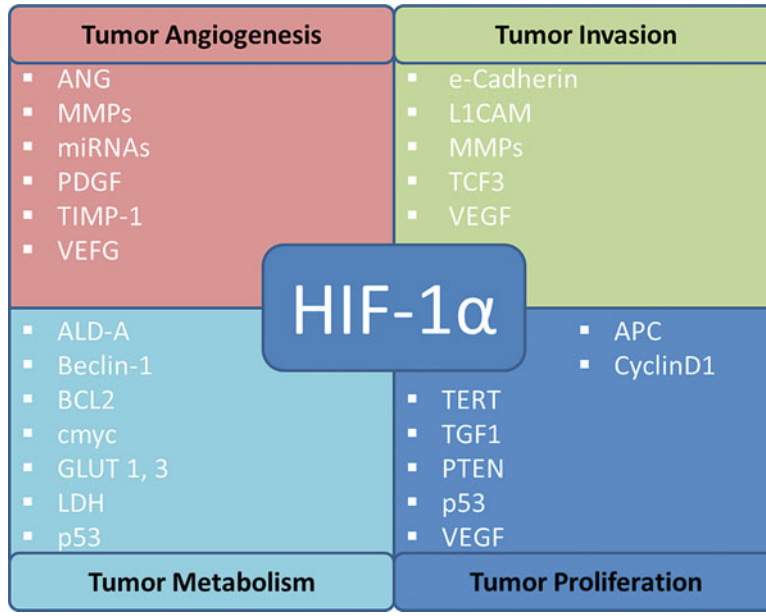

Fig. 7 Scheme of HIF-1-regulated genes involved in angiogenesis, invasion, metabolism, and proliferation

VEGFR-1-induced signaling pathways include the extracellular signal-regulated kinase (ERK), the nuclear factor κ-light chain-enhancer of activated B cells (NF-kB), the phospholipase Cγ (PLCγ), the mitogen-activated protein kinase (MAPK), the phosphoinositide 3-kinase (PI3K), the protein kinase B (AKT), and the stress kinase p38MAPK (Kerber et al. 2008). Until now, data for the biologically relevant VEGFR-1-mediated signaling pathways are ambiguous. Also the complexity of VEGFR-1 biology is further underscored by the fact that it is expressed by a broad range of cell types, including human tumor cells (Schwartz et al. 2010; Koch and Claesson-Welsh 2012).

Considering malignant tumors, VEGFR-1 participates in carcinogenesis and tumor-provoked angiogenesis. In carcinogenesis VEGFR-1 activation results in fibroblast transformation (Maru et al. 1998), EMT (Yang et al. 2006), tumor cell motility and invasiveness, reduced apoptotic activity, and subsequently decreased patients' overall survival. In a recent study, Bhattacharya et al. demonstrated that the inhibition of intracrine VEGF signaling reduces cell migration and invasion in colorectal cell lines. Interestingly, their study indicates that the reduced cell migration and invasion are not due to EMT-like changes but due to a decreased activity of cell motility-associated proteins (Bhattacharya et al. 2017). Interestingly, in tumor-provoked angiogenesis, the role of VEGFR-1 is not clear. As mentioned before, VEGFR-1 has a substantially weaker tyrosine kinase activity than VEGFR-2 and exhibits a tenfold higher binding affinity for VEGF-A (de Vries et al. 1992; Sawano et al. 1996). The present assumption is that VEGFR-1 acts as a decoy receptor sequestering VEGF-A, diminishing VEGFR-2 signaling (Schwartz et al. 2010). In vivo and in vitro VEGF-A causes VEGFR-1 activation and enhances endothelial cell migration and survival, whereas VEGFR-1 antagonist behaves in a contrary way with a reduced endothelial cell migration (Lacal et al. 2008; Ponticelli et al. 2008).

VEGFR-2. While VEGFR-1 has a higher binding affinity compared to VEGFR-2, VEGFR-2 shows up to tenfold stronger activity within the intercellular tyrosine kinase (Sawano et al. 1996). In short, the main effector of tumor angiogenesis is represented by VEGFR-2. The activation of VEGFR-2 receptors promotes migratory, proliferatory, and anti-apoptotic effects onto the endothelial cells. The classical VEGFR-2 ligand is VEGF-A, VEGF-C, and VEGF-D. VEGFR-2 activation is influenced by VEGF-A variants differing in the ability to interact with NRPs. As aforementioned, VEGFR-2 can form homodimers or heterodimers with VEGFR-1 or VEGFR-3. VEGFR-2 homodimer signaling pathways are modulated by VEGF-binding coreceptors (e.g., heparan sulfate, syndecan, glypican, and neuropilins). VEGFR-2 heterodimers were identified via signaling studies and in silico models (Mac Gabhann and Popel 2007; Deng et al. 2015). Deng et al. demonstrated that compared to the VEGF-A activation of VEGFR-2, VEGF-C-induced VEGFR-3 activation resulted in an elevated AKT activation, whereas activation of ERK1/ERK2 displayed a distinctly different kinetics. Interestingly, VEGF-C induced the formation of VEGFR-2/VEGFR-3 heterodimers (Deng et al. 2015). More unknown are the signaling effects of VEGFR-1 /VEGFR-2 heterodimers. Three effects influencing the heterodimerization can be identified: (1) the ratio between VEGFR-1 and VEGFR-2 receptors (VEGFR-1:VEGFR-2 = 1: 10), (2) the significant higher binding affinity of VEGF-A to VEGFR-1 (compared to VEGFR-2) (Imoukhuede and Popel 2011), and (3) the different extracellular localizations of VEGFR-1 and VEGFR-2 (Stefanini et al. 2009; Simons et al. 2016). Hereby, it is hard to project if VEGFR-1 and VEGFR-2 form homodimers or heterodimers.

Hereby, VEGFR-2 induced signaling pathways comprising the AKT, ERK1/ERK2, p38, PLCγ, PKCβ, MAP, and the Src kinase pathways (Takahashi et al. 1999, 2001; Shibuya 2011) and (as recently demonstrated) the YAP/TAZ pathway. Wang et al. demonstrated that VEGFR-2 modulates the Src family kinases (SFK) and Rho GTPase activity, actin dynamics, and Lats1 activity (Wang et al. 2017). Furthermore, under physiological levels, Sarabipour et al. demonstrated ligand-independent VEGFR-2 dimerization of

two monomeric VEGFR-2 (Sarabipour et al. 2016). Beside monomeric dimerization, VEGFR-1/VEGFR-2 heterodimer-mediated VEGFR phosphorylation, endothelial cell migration, sustained in vitro tube formation, and vasodilatation were also observed (Cudmore et al. 2012). Interestingly, activated VEGFR-1/VEGFR-2 heterodimers did not mediate cell proliferation, indicating that these functions are primarily controlled by VEGFR-2 homodimers. Also a soluble VEGFR-2 form (sVEGFR-2) was detected in humans and mice. While the role of sVEGFR-2 remains unclear, first data demonstrated correlations between VEGR-2 level and tumor growth (Harris et al. 2016). Beside the angiogenic functions, VEGFR-2 also plays a role in inflammation, for example, by controlling the endothelial secretion of the von Willebrand factor.

VEGFR-3. VEGFR-3 was the first identified growth factor affecting lymphangiogenesis (Kaipainen et al. 1995) and is also expressed in neural progenitor cells, macrophages, and osteoblasts. VEGFR-3 specifically binds to VEGF-C and VEGF-D and promotes lymphatic endothelial cell proliferation and migration and binds as well to anti-apoptotic signal-regulated kinase 1/2 (ERK), the phosphatidylinositol 3-kinase/AKT, and the c-Jun pathways.

Vascular Endothelial Growth Factor A–D

VEGF-A represents the best characterized family member. VEGF-A was first isolated in 1989 (Ferrara and Henzel 1989). Subsequent in vitro (Leung et al. 1989) and in vivo (Kim et al. 1993) laid the foundation for first anti-angiogenic therapy strategies. VEGF-A is an acidic and freely secreted protein with heparin-binding properties and after secretion bound avidly to heparin and the extracellular matrix. VEGF-A is expressed in all vascularized tissues. Polymorphisms in VEGF-A are associated with diabetic retinopathy in type 2 diabetes (Awata et al. 2002). Alternative splicing generates the splicing variants VEGF-A 121, 145, 165, and 189 (numbers indicating the number of amino acid residues). VEGF-A 121 is freely diffusible and binds neither to coreceptors like neuropilins (NRPs) nor heparan sulfate (HS). VEGF-A 165 and 189 bind to NRPs and HS, resulting in a withholding of the VEGF-A on the cell surface/extracellular matrix with profound effects on the VEGF-A bioactivity, affecting its diffusion, half-life, and interaction with its tyrosine kinase receptors (Stringer 2006).

(*continued*)

Vascular Endothelial Growth Factor A–D

VEGF-B, first described in 1996 (Kendall and Thomas 1993), is expressed in all tissues (especially in the heart muscle and pancreas), except the liver, and remains associated to cells and the extracellular matrix. VEGF-B modulates the growth of endothelial cells and can be released by heparin.

VEGF-C, also described in 1996 (Joukov et al. 1996), modulates angiogenesis of the venous and lymphatic vascular systems during embryogenesis by stimulating endothelial proliferation and migration of endothelial cell growth. Also VEGF-C regulates vascular permeability and vessel dilatation. Mutations affecting VEGF-C result to Milroy disease, an autosomal dominant, congenital form of primary lymphedema (Roskoski 2017).

VEGF-D, described in 1997 (Yamada et al. 1997), modulates angiogenesis of the venous and lymphatic vascular systems during embryogenesis by stimulating endothelial proliferation and migration of endothelial cell growth, affecting blood vessel permeability. VEGF-D regulates the formation of the venous and lymphatic vascular systems during embryogenesis.

Together VEGFs and VEGFRs represent the major mediators of angiogenesis. VEGF-A binds to VEGFR-1 and VEGFR-2. Hereby, VEGFR-1 has a higher affinity to VEGF-A than VEGFR-2, whereas the tyrosine kinase activity of VEGFR-1 is tenfold weaker than that of VEGFR-2.

(g) Haemangiogenesis Carcinomatosa and Metastases

The process of angiogenesis and metastatic spread via haemangiogenesis carcinomatosa accompanies each other. Hereby, metastasis describes the process of tumor migration from the primary tumor side to another nondirectly interlinked side of the body. For the process of metastases, three mechanisms are known. The process of metastatic spread can follow the (1) blood (haemangiogenesis carcinomatosa), (2) lymph vessels (lymphangiogenesis carcinomatosa), and (3) perineural sheets (perineural invasion) (Fig. 8).

Considering haemangiogenesis carcinomatosa, the intravasation, the transit into the blood vessels, the extravasation, and the setup at the secondary site are curtails not only for the tumor progression but moreover for the overall survival of the patient. Hereby, the first step, the

intravasation, represents the most critical process. Of note, all steps can be influenced by different non-tumorous cells of the microenvironment. The straightening ability along the extracellular matrix and along oxygen tension gradient (Bertuzzi and Gandolfi 2000) enables tumor cells to migrate toward the blood vessels. Tumors representing the capacity for vessel recruitment also occupy the capacity for building and generating distant metastases via haemangiogenesis carcinomatosa. Of note, tumors with a higher vascular density would be expected also to have an increased vascular invasion rate. This hypothesis is in line with a study in colorectal cancer describing the intratumoral vessel density as a predictive factor for the presence of circulating tumor cells (Tien et al. 2001). Also the pericytal coverage of tumor vessels is important. Immature microvessels, not covered by pericytes, are irregular and leaky and represent the entrance gate for metastasizing tumor cells. Using the multistage pancreatic β cell tumor model, Hannahn et al. demonstrated that neural cell adhesion molecule (NCAM) regulates metastatic tumor cell dissemination (Hanahan 1985). Functional studies observed an enhanced metastatic frequency in knockout animals with compromised blood vessel architecture (Taniguchi et al. 2001) which were documented by clinical studies (Yonenaga et al. 2005; Welén et al. 2009). Hereby, low pericyte coverage of the blood vessels correlated with distant metastasis and overall survival. An explanation could be that insufficient blood supply via immature blood vessels or pericyte depletion leads to increased hypoxia and subsequent EMT and Met receptor activation, which correlates with distant metastases and poor patients' outcome (Cooke et al. 2012).

For the invasion of larger scale vessels, tumor cells have to possess the capability (a) to degrade the vessel surrounding connective tissue (adventitia, media, and intima, depending on the vessel type), (b) to migrate into the vessel, (c) to overcome the shear stress, (d) to survive in the bloodstream, (e) to extravasate, (f) to form metastases, and (g) to recruit new vessels. Hereby, tumors with an elevated EMT activity have a significant higher captivity for blood vessel infiltration and haemangiogenesis carcinomatosa (Tsuji et al. 2009; Lapshyn et al. 2017).

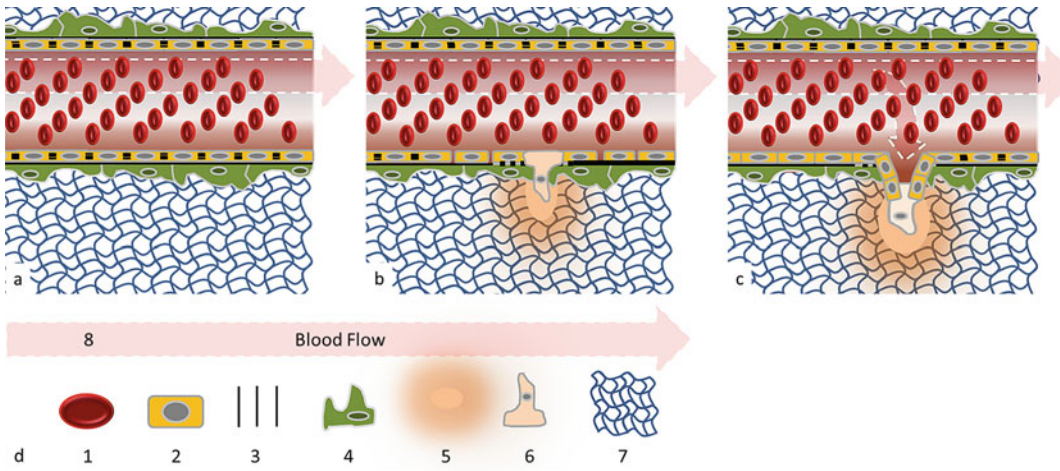

Fig. 8 Steps of sprouting angiogenesis in preexisting blood vessels. **(a)** Normal blood vessel. **(b)** Process of pericyte separation, membrane degradation, and endothelial cell migration. Hereby the vascular stalk by tip cell-guided endothelial cell and the remodeling process of the surrounding matrix are initiated **(c)** Tube formation by endothelial migration and re-vacuolization. **(d)** Figure legend: *1* red blood cells, *2* endothelial cells, *3* basement membrane, *4* pericytes, *5* paracrine and autocrine effects for matrix remodeling, *6* tip cell-guided endothelial cell, *7* matrix, *8* blood flow

(h) Malignant Tumor Tissue

After turning the angiogenetic switch, pro-angiogenetic factors are recruiting tumor-associated vessels (Fig. 2). Compared to physiological vessels, tumor-associated vessels typically acquire an aberrant morphology with multiple meandering branches, excessive fenestration caused by intermittent pericytal coverage, restricted function, and subsequent limited supply (Huang et al. 2010). Also a high degree of vascular heterogeneity represented by hypo- and hypervascular site regions is typical (Eberhard et al. 2000; Smith et al. 2011). The source of the pro-angiogenic factors (especially VEGF-A) are cancer cells. Interestingly leukocytes, recruited into the surrounding microenvironment, can sustain the availability of the pro-angiogenic factors and mediators and increase the effects of the angiogenic switch. Cells representing the microenvironment can be assigned by their origin into cells descending from the bone marrow (comprising leukocytes and endothelial and mesenchymal progenitor cells) and local cells (comprising endothelial cells, fibroblasts, adipocytes, and leucocytes). Hereby cancer-associated fibroblasts (CAF) play a pivotal role in the modulation and transformation of the tumor microenvironment. Via paracrine effects (e.g., HIF-1α, TGF-ß), local fibroblasts are transformed into CAFs. With their large spindle cell morphology, CAFs histologically resemble myofibroblasts found in wound healing processes, but differ significantly in function. Contrary to myofibroblasts, CAFs are continuously activated and do not return into the "physiological" phenotype. The origin of CAFs remains vague. Actually local tissue fibroblasts, bone marrow-derived mesenchymal stem cells, epithelial transition cells (EMT), and endothelial transition cells (endothelial-mesenchymal transition) are discussed as potential sources of CAFs. Considering the process of angiogenesis, CAFs modulate the intrusion of tumor-associated vessels by enhancing VEGF signaling and modulating the stiffness of the extracellular matrix, which are both important for tumor cell invasion and migration and the development of a metastatic disease. The CAF-based vascular recruitment of endothelial progenitor cells is modulated by VEGF, FGF, platelet-derived growth factor, insulin-like growth factor, and chemokines.

Biochemical cross-talk between cancer cells and CAFs as well as mechanical remodeling of the stromal extracellular matrix (ECM) by CAFs are important contributors to tumor cell migration and invasion, which are critical for cancer progression from a primary tumor to metastatic disease.

Breast Cancer

Breast cancer is the most frequent female malignancy and the leading cause of female cancer mortality (Steliarova-Foucher et al. 2014). From a molecular point of view, breast cancer is a heterogeneous disease and is genomically and transcriptomically classifiable (Sorlie et al. 2001; Huang et al. 2003; Voduc et al. 2010) into the following five relevant, prognostic, predictive, and intrinsic subtypes (Goldhirsch et al. 2013): luminal A, luminal B, luminal Her2, Her2-enriched, and the triple-negative subtype. In clinical routine diagnostics, the molecular subtype is immunohistologically classified by the determination of the estrogen receptor (ER) protein, the progesterone receptor (PR) protein, the membranous tyrosine kinase human epidermal growth factor receptor 1 (EGFR) and the human epidermal growth factor receptor 2 (HER2/neu), and the proliferation index Ki67. Furthermore, the triple-negative subtype can be immunohistologically subdivided in more detail. At present, the triple-negative subtype is subdivided into the triple-negative and basal-like subtype. Therefore, immunohistological stainings for cytokeratin 5/cytokeratin 6, cytokeratin 20, and EGFR are performed. Nevertheless, triple-negative breast cancers are still a cluster of breast cancer subtypes which need more precise molecular characterization. Clinically, the luminal A and luminal B subtypes are accompanied with the lowest recurrence and the best overall survival rates. Contrary, triple-negative breast cancers represent the most aggressive and deadly subtypes accompanied by high recurrence and poorest overall survival rates.

Angiogenesis in breast cancer is a well-known prognostic factor for overall survival in breast cancer patients (Horak et al. 1992). Elevated levels of the angiogenic factor VEGF are directly correlated with the tumor aggressiveness (George et al. 2001) and a poorer response to systemic treatments and radiotherapy. Also the microvessel density represents an independent prognosticator and subsequent correlates with VEGF (Foekens et al. 2001). As a direct downstream target of HER2/neu, HER2/neu overexpresses VEGF. The former unfavorable prognosis (before HER2/neu antagonist, e.g., herceptin) of luminal Her2 and Her2-enriched patients was directly linked to the increased angiogenesis (Konecny et al. 2004). Comparing luminal Her2, Her2-enriched, and triple-negative breast cancer patients, triple-negative breast cancer patients demonstrate 1.5 higher VEGF levels compared to the HER2-positive group (Linderholm et al. 2009). Beside the HER2/neu downstream-based effects, elevated VEGF levels in breast cancer are associated with the inactivation of tumor suppressor p53, especially in triple-negative breast cancer (Linderholm et al. 2009). Also the presence of VEGFR on breast cancer cells identifies the presence of autocrine pro-tumorigenic signaling including proliferation, anti-apoptotic, and migratory pathways (Perrot-Applanat and Di Benedetto 2012; Goel and Mercurio 2013).

Colorectal Cancer

Colorectal cancer (CRC) is the third most frequent cancer malignancy accounting 10% of all cancer diagnoses (Global Burden of Disease Cancer Collaboration et al. 2015) with a 5-year overall survival of 92% in stage I (early stage, regionally limited to the colon) and 11% in stage IV (advanced stage with distant metastases). Haemangiogenesis carcinomatosa is the leading process for distance metastases reflected by distant metastases in 20% of all patients during primary diagnosis of CRC and 30% during disease progression (Böckelman et al. 2015).

Tumor stages combined with histopathological grading reveal predictive and prognostic limitations for overall survival in tumor stages II and III. Therefore, molecular analyses get into the focus. Two array- and sequencing-based molecular classifications – in 2012 The Cancer Genome Atlas Network (Biankin et al. 2012) and in 2015 the Consensus Molecular Subtype Consortium (Guinney et al. 2015) – were proposed. The Cancer Genome Atlas Network classified CRC hypermutated cancers with either microsatellite instability accounting 16% comprising defective mismatch repair (∼13%) or ultramutated cancers (∼3%) and non-hypermutated, microsatellite stable (MSS) cancers with somatic copy number alterations, *APC, TP53, KRAS, SMAD4, and PIK3CA mutations accounting 84% of all CRCs* (Muzny et al. 2012).

The Consensus Molecular Subtype Consortium Gene identified four subtypes, named consensus molecular subtypes (CMS). The results were linked to patients' survival data. In short the CMS can be subdivided into the CMS-1 a hypermutated, microsatellite unstable subtype with a strong activation of the immune system, accounting proximally 14% of all tumors; the CMS-2 with molecular epithelial signatures, high chromosomal instability, WNT and MYC signaling activation, accounting proximal 37% of all cancers; the CMS-3 also combined with molecular epithelial signatures, demonstrating a higher number of *KRAS* mutations and metabolic dysregulation accounting 13%; and the CMS-4 subtype with a mesenchymal growth pattern, TGF-β activation, stromal invasion/activation, and angiogenesis, accounting 23% of all CRCs. Mixed CMS can also be observed. Hereby, the CMS-1 and CMS-4 subtype are accompanied with a worse overall survival (Guinney et al. 2015). The expression of mesenchymal genes associated with poor prognosis in CRC samples is mainly contributed to tumor-associated stromal cells rather than by epithelial tumor cells. Inflammation and angiogenesis in promoted by CMS-4 are driven by TGF-ß signaling and Th1/CD8+ T cells repressing the antitumor activity of cytotoxic T but also reinforcing angiogenesis and stroma remodeling (Calon et al. 2015; Becht et al. 2016).

Future Directions

The formation of new capillaries from preexisting vessels in epithelial and mesenchymal malignancies, known as tumor angiogenesis, plays a fundamental role in tumorigenesis, local and distant metastasis formation, and subsequent tumor prediction and prognosis. Cutting the resources of oxygen and nutrient supply by specific tumor blood supply, suppression represents the king's road in tumor therapy. Hereby the roles of HIF-1α and VEGF signaling represent essential targets for therapeutic intervention. But also tackling tumor adaptation and resistance toward anti-angiogenetic drug targeting paves the way in the cancer therapy. Hereby tumors, initially presenting a good response toward anti-VEGF treatment, establish VEGF-independent angiogenesis pathways assuring revascularization and de novo blood supply supporting tumor progression. A deeper understanding of angiogenesis signaling and the impact on tumor-derived processes of pericyte separation, membrane degradation, endothelial cell migration, vascular stalk formation, revascularization, and the remodeling process of the surrounding matrix can overcome resistances and will lead to personalized/tailored therapy regimes. Therefore, additional predictive biomarkers for categorizing patients into responders/nonresponders are crucial.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Mechanisms of Tumor Angiogenesis

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Part VI

Imaging of Tumor Angiogenesis

Imaging Tumor Angiogenesis

Gordon Jayson and James O'Connor

Contents

Introduction 278

Imaging Angiogenesis: The Technologies 279
Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI) 279

MRI Evaluation of Tumor Vasculature and Drug Development 282
Mechanisms of Action and Imaging 282

Pharmacodynamic PET Scanning 285
Studies of [^{18}F]-FDG-PET 285
Studies of [^{15}O]-H_2O and [^{18}F]-FLT 285

Emerging Imaging Technologies 286

Future Directions 287

Cross-References 287

References 288

Abstract

Angiogenesis, the formation of new blood vessels, is critical for the growth and metastasis of most solid tumors. Several cytokines, cytokine receptors, and cell adhesion molecules have been identified as potential targets for cancer treatment and in general most have been inhibited through the use of monoclonal antibodies or small molecule tyrosine kinase inhibitors.

Drug development relies on the earliest possible discrimination of active and inactive new agents, and imaging has been used to assess the anti-vascular effects of new agents for over 15 years. This has been critical for the development of new agents to the extent that any vascular endothelial growth factor (VEGF) inhibitor that does not impact dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) in early drug development can be discarded as not hitting its target.

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Much further work has looked at the value of imaging in predicting benefit from anti-angiogenic agents. However, in general, conclusions have not been uniform and it is not clear at present whether imaging can be used to select patients who are most likely to benefit from VEGF pathway inhibitors. However, new imaging biomarkers are in development and are already proving novel data on the architecture and function of tumor neovasculature.

Keywords
Angiogenesis · Imaging · DCE-MRI · CT · PET · VEGF inhibitors

Introduction

Nearly all solid tumors require formation of a neovasculature that allows them to grow and spread through vascular metastasis. Following decades of observational data, which demonstrated that the density of new blood vessels in several tumors was related to the predilection to metastasis and survival (Hasan et al. 2002), the landmark identification of vascular endothelial growth factor (VEGF) (Leung et al. 1989) as the principal angiogenic growth factor led to the development of multiple new agents targeted against this cytokine or its key signaling receptor, VEGFR2. In general these drugs have improved progression free survival (PFS) in many tumors but have not uniformly impacted on overall survival. The most sensitive tumors, such as renal and neuroendocrine cancers, can be treated with single-agent VEGF pathway inhibitors (Jayson et al. 2016), clearly impacting on survival in those situations, and some trials in moderately angio-sensitive diseases have described improvements in overall survival (Hurwitz et al. 2004) while others have not (Schmoll et al. 2012).

Drug development of anti-angiogenic agents initially focused on the optimization of monoclonal antibodies that bound VEGF, culminating in the licensing of the first effective agent, the monoclonal, anti-VEGF antibody, bevacizumab. Subsequent drug development largely focused on the receptor, yielding two main classes of drug that inhibited cell signaling through antibody-mediated inhibition of the receptor (e.g., ramucirumab) or through the elucidation of small molecule VEGFR tyrosine-kinase inhibitors, of which many examples exist (Jayson et al. 2016).

Early phase drug development relies heavily on the identification of the most effective agents as early as possible and the equally important elimination of drugs that are ineffective. Three concepts have arisen, which include proof of mechanism, where investigations are performed to determine whether a defined molecular mechanism has been inhibited, and proof of principle where, as in anti-angiogenic agents, attempts are made to test whether a new drug successfully inhibits a particular phenotype such as the tumor vasculature and proof of concept, where a drug is demonstrated to improve outcome (Workman et al. 2006).

Imaging has been used extensively in the early drug development of anti-angiogenic agents to define whether a new agent achieves proof of principle, that is, to determine whether a putative agent has impacted the tumor vasculature. By far the majority of studies have deployed dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (O'Connor et al. 2012), and this will be the principal focus of this chapter. This technology has been so uniformly useful, although susceptible to important institute-by-institute variables (O'Connor et al. 2016), that new candidate agents, which are purported to be VEGF pathway inhibitors, would be discarded if no DCE-MRI effects are described within the maximum tolerated dose of the agent. However, as we will discuss, imaging changes are necessary but not sufficient to guarantee a successful pathway to licensing.

Early phase clinical trial data inspired great confidence in imaging and its relationship to the tumor vasculature, and this led to attempts to use imaging characteristics to predict which patients would benefit from anti-angiogenic agents (O'Connor and Jayson 2012). As discussed below, this application has not been successful, and to date, no imaging biomarkers have been identified, validated, and qualified to discern which patients would benefit from VEGF

inhibitors. However, new imaging techniques are being developed and are providing novel insights into the structure and function of neovascular function.

Imaging Angiogenesis: The Technologies

The majority of imaging strategies deployed in the assessment of tumor vasculature have involved the administration and quantification of contrast during imaging. These dynamic protocols have evolved because tumor-associated vasculature is characterized by poorly functional, tortuous, dilated, and discontinuous vessels (Mancuso et al. 2006). The latter feature is a key determinant of vascular permeability; a phenotype that is critically regulated by VEGF, which itself was originally known as vascular permeability factor (Leung et al. 1989).

The relationship between VEGF and vascular permeability led to the development of imaging protocols that could quantify such changes in vasculature and hence in situ changes in VEGF biological activity. The most widely implemented technology has been dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (O'Connor et al. 2012). While DCE-CT has also been used to address the same issue, the advantage of DCE-MRI is that the technology avoids ionizing radiation and therefore can be repeated (O'Connor et al. 2009) without concern for radiation-associated consequences. In addition the relationship between contrast concentration and signal in MRI is not linear, with greater sensitivity at lower contrast levels, thereby affording DCE-MRI greater sensitivity at the segments of the contrast-signal curve that are relevant to drug development. On the other hand, CT scanning is globally available, and, as a result, a series of modifications to CT scan reporting have been developed, which is a factor in the changes in tumor density and ischemic effects of anti-angiogenic agents into reporting criteria (Choi et al. 2007). These are important because an anti-angiogenic agent may not change the diameter of a tumor deposit, yet it may have a profound impact on tumor biology.

Positron emission tomography (PET) has also been deployed in the evaluation of angiogenesis and anti-angiogenic agents. The technology relies on the incorporation of a positron-emitting isotope into a defined chemical structure. Positrons then encounter electrons, leading to the emission of photons that can be quantified, thereby offering the potential for absolute quantification of concentrations of PET tracer in tumors. However, other than [^{18}F]-FDG, which capitalizes on the Warburg effect in which glucose uptake by tumor is up to 20 times the background level, most tracers are not taken up by tumors to the same extent and are still of exploratory value. Thus, [^{15}O]-H_2O and [^{18}F]-FLT have been evaluated in several trials, but for reasons of very short half-life, in the case of the former and insufficiently convincing utility with respect to FLT, these technologies are not widely used.

Dynamic ultrasound, which relies on the detection of microbubbles in the circulation, has also been evaluated but is compromised largely by difficulties in quantitation of effect across multiple sites. Thus, the most widely used technology remains advanced MRI, and results obtained with this technology will be the main focus of the chapter.

Dynamic Contrast-Enhanced Magnetic Resonance Imaging (DCE-MRI)

Methodological Considerations

By far the majority of tumor vascular imaging studies have employed DCE-MRI, which identifies a number of critical vasculature-related parameters (Fig. 1). Before discussing the data from these studies, it is important to consider the technical and analytical issues that are only now being standardized to allow inter-site comparisons.

Attempts to standardize terminology (Tofts et al. 1999) and choice of DCE-MRI biomarkers (Leach et al. 2005) have been widely promoted over the past 15–20 years. However, many different approaches to deriving a given biomarker have been developed at different research institutions. This resulted in various different protocols

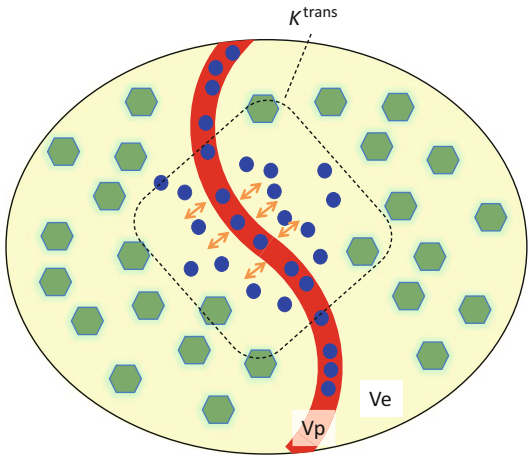

Fig. 1 DCE-MRI and derived parameters. Low molecular weight gadolinium contrast agent (*blue circles*) is injected, and images are acquired over time in the patient's tumor. Contrast in the tumor vasculature allows calculation of V_p, the tumor vascular volume. The contrast leaks out of the malformed tumor vasculature (*black dotted lines*) with kinetics dictated by the vascular permeability and endothelial surface area, parameters that are combined together to generate the analytically derived, K^{trans}. Once in the interstitial space, further analysis can also calculate a further parameter, V_e, which represents the extravascular, extracellular space. The movement of gadolinium contrast molecules is dictated by Brownian motion, and this too can be modeled through diffusion-weighted imaging (*DWI*) to identify the apparent diffusion coefficient (*ADC*). This parameter is believed to reflect cell packing and thereby potentially also serves as a biomarker of apoptosis. This is not shown here as the methodology has not been found useful in imaging angiogenesis

conducted on a range of equipment, with a number of analytical techniques being applied, rendering more complex technologies difficult to develop internationally. We and others have published a "roadmap" describing the development of imaging biomarkers (O'Connor et al. 2016). This important protocol reviews the discovery of biomarkers via a range of scientific disciplines through to validation studies, which require implementation of the technique in a few centers with a limited number of preclinical and defined clinical studies, which allow definition of precision, bias, biological relevance, and cost-effectiveness. Successful progress through these initial steps would be followed by studies in larger numbers of research groups to define further the precision of the imaging technique across many centers while determining the key application of the imaging biomarker (e.g. in screening, diagnosis, prediction or as a pharmacodynamic test). The third step would be the qualification of the imaging biomarker with technical validation and then implementation within prospective studies, through which the health benefit and cost-effectiveness of the imaging test could be defined. Global uptake of this proposal (O'Connor et al. 2016) would be transformative for imaging bioscience and drug development.

Biological Relevance of Imaging Techniques

The dynamic process of angiogenesis leads to the formation of new blood vessels, which when assessed at the tissue level can be quantified through microvessel density (MVD). When imaging techniques to examine the vasculature were developed, many investigators attempted to correlate imaging parameters with MVD, and to some extent these studies validated the hypothesis in endometrial (Haldorsen et al. 2014) and prostate cancer (Schlemmer et al. 2004) but not initially in breast cancer (Su et al. 2003). However, subsequent multi-biomarker studies in breast (Wedam et al. 2006) and colorectal cancer (Willett et al. 2004) showed that anti-angiogenic treatment reduced MVD and radiologically quantified vasculature-related parameters together.

In retrospect some of these studies were naively optimistic, at least in humans. The resolution of DCE-MRI studies in patients is at the several millimeter level, while tissue immunohistochemical studies resolve tissues at the micrometer level, reflecting several orders of magnitude in difference. Taken in conjunction with the difficulties in maintaining the orientation of tissue specimens with respect to imaging data and the impact of tissue fixation on the ability to co-localize biological and imaging data, it is perhaps surprising that any correlations were discovered between these different modalities of tumor vascular evaluation.

Given the difficulties in comparing tissue- and imaging-derived vasculature-related parameters, there was a need to understand the clinical

significance of tumor vascular imaging. We and others described simple and more complex techniques that addressed this question. Straightforward evaluation of the enhancing fraction of a tumor, the proportion of a tumor that takes up contrast, demonstrated prognostic value in ovarian and cervix cancers (O'Connor et al. 2007; Donaldson et al. 2009), while more complex mathematical models of vascular heterogeneity provided further information on outcome in colorectal cancer (O'Connor et al. 2011; Jackson et al. 2007). Thus, despite the difficulties of comparing imaging and tissue microscopic data, imaging of tumor vasculature provides clinically relevant data.

DCE-MRI Assessment of VEGF Inhibitors

The most frequently deployed imaging strategy has been T_1-weighted DCE-MRI, which has been applied to the majority of VEGF pathway inhibitors during early phase drug development. In general, the data show that all clinically effective inhibitors, whether they are antibodies or small molecule VEGF receptor tyrosine kinase inhibitors, impact on K^{trans} (Table 1), and this derived parameter has been most frequently used as the arbiter in proof of principle studies (O'Connor et al. 2012).

Simpler models of analysis have also been developed, and these require less complex modeling for their derivation. Two of the simplest are the enhancing fraction, the volume of tumor that takes up any contrast and the $IAUC_{60}$, the area under the concentration time curve in a region of tumor in the first 60 s after injection of a gadolinium-based contrast agent. These simpler analyses have also been widely deployed, and in keeping with the original vascular density studies, which were of prognostic significance, we and others have demonstrated the prognostic significance of enhancing fraction before treatment (O'Connor et al. 2007; Jackson et al. 2007; Rose et al. 2007). More complex mathematical models have been developed to allow calculation of K^{trans}, the product of endothelial surface area, and vascular permeability as well as the other parameters shown in Fig. 1. Typically these biomarkers have been derived from the pharmacokinetic models described in other review articles (Tofts et al. 1999; Tofts 1997).

Table 1 VEGF inhibitors impact on DCE-MRI parameters. The table shows a selection of VEGF pathway inhibitors that were evaluated with DCE-MRI through phase I/II clinical trials. The trials show near uniform reductions in a number of DCE-MRI parameters in keeping with the proposed mechanism of action of the drugs

	Phase of clinical trial	Tumor type	Change in DCE-MRI parameter	References
Antibody-related structures				
Bevacizumab (Anti-VEGF-A)	II	Colorectal	Ktrans, EF, WTV	O'Connor et al. (2009)
CDP791 (di-Fab anti-VEGFR2)	I	Multiple	No changes seen	Ton et al. (2007)
Ramucirumab (anti-VEGFR2 antibody	I	Multiple	Perfusion, Ktrans	Spratlin et al. (2010)
Protein therapeutics				
Aflibercept (VEGFR construct)	I	Multiple	Ktrans	Lockhart et al. (2010)
VEGFR tyrosine-kinase inhibitors				
Sunitinib	II	Hepatocellular	Ktrans	Zhu et al. (2009)
Sorafenib	II	Renal	Ktrans	Flaherty et al. (2008)
Cediranib	I	Multiple	IAUC60	Drevs et al. (2007)
Pazopanib	I	Hepatocellular	IAUC, Ktrans	Yau et al. (2011)

MRI Evaluation of Tumor Vasculature and Drug Development

Mechanisms of Action and Imaging

The application of MRI technologies to the assessment of VEGF inhibitors focused in large part on the reduction in K^{trans} as the key endpoint because VEGF was the principal mediator of vascular permeability, one of the terms incorporated into the calculation of K^{trans}. Because of the impact of these agents on vascular permeability and because sustained application of VEGF inhibitors reduced tumor interstitial pressure, we studied the time course of effects of the monoclonal anti-VEGF antibody, bevacizumab, on colorectal cancer liver metastases (O'Connor et al. 2009). These studies showed that the bevacizumab had an impact on vascular permeability within hours of drug administration and that to a large extent this was sustained for up to 12 days, thereby justifying the administration of the drug every 2 weeks. Interestingly the same study also showed acute changes in tumor volume within 12 days of single-agent bevacizumab administration, demonstrating the potential impact of such agents on tumor behavior.

The time course study of vascular changes in liver metastases revealed a reduction in K^{trans}, enhancing fraction and plasma volume fraction (Vp). Together these data were suggestive of reduced perfusion to patients' tumors. Preclinical work using thyroid models at the same time suggested that the vessels rapidly regressed when treated with VEGF inhibitors and that those that were left had a more normal structure, the concept of vascular normalization (Mancuso et al. 2006). This was then studied by Jain and colleagues in human brain studies, which suggested that one of the principal modes of action of anti-angiogenic VEGF inhibitors was vascular normalization (Batchelor et al. 2007, 2010). Further evidence suggested that vascular normalization might be of key importance in determining the optimum time to administer radiotherapy (Winkler et al. 2004).

The Search for Predictive Biomarkers

Given the strong dynamic relationship between pharmaceutical VEGF inhibition and changes in DCE-MRI, much work focused on the evaluation of pretreatment imaging characteristics as potential predictive biomarkers, tests that could be used to select the patients most likely to benefit from such drugs. However, except for some hints of predictive value in tumors that were highly sensitive to VEGF inhibitors, such as glioma, no predictive value has been detected (O'Connor and Jayson 2012).

Alternative attempts to develop predictive imaging biomarkers have focused on the quantitative evaluation of tissue VEGF concentrations through the development and evaluation of the PET tracer, [^{89}Zr]-bevacizumab. Zr was used in this imaging agent because its decay is much longer than other widely available PET isotopes. Yet this issue is still an important confounding factor in the development of the tracer. The half-life of bevacizumab in the circulation is approximately 20 days (Lu et al. 2008), whereas that of 89Zr is 3.3 days (Zhang et al. 2011).Thus, inevitably the intravascular content of [89Zr]-bevacizumab will confound interpretation of tissue levels of the tracer. Nevertheless, in the acute imaging setting specific uptake of bevacizumab has been demonstrated in vivo, where control immunoglobulin was not taken up (Nagengast et al. 2007). Further imaging studies have demonstrated uptake of the tracer in breast (Gaykema et al. 2013) and renal cancer (Oosting et al. 2015) and anti-angiogenic agents that impacted tracer uptake, and in renal cancer, patients with the largest SUV had the longest progression free survival with VEGF inhibitors (Oosting et al. 2015). Thus, despite concerns over the confounding issues of intravascular imaging agent and the need to determine bound versus free drug in the vasculature, this imaging agent appears to hold some promise but requires further validation.

Proof of Principle

A selection of illustrative early phase clinical trials that incorporated imaging into the evaluation of VEGF pathway inhibitors are listed in Table 1.

To a variable extent, these trials demonstrated that there was a dose-response effect. In other words, the higher the dose or dose level of VEGF pathway inhibitor, the greater the impact on the MRI imaging parameter. A further observation from these data was that there appeared to be a threshold effect in which clinical responses or disease stabilization were only seen in patients whose tumors manifested greater than 50% reduction in K^{trans} or $IAUC_{60}$ (O'Connor et al. 2012) following treatment with a VEGF inhibitor.

The observation of a dose-response effect increased confidence in DCE-MRI technology. Further support for the technology was derived from the observation that a di-Fab anti-VEGFR2 fragment (Ton et al. 2007) did not impact on DCE-MRI and at least in early phase evaluation did not demonstrate the same clinical efficacy or toxicity signals that were observed with other VEGF inhibitors. Critically the results seen with the di-Fab construct contrast with those seen with an intact anti-VEGFR2 antibody, ramucirumab (Spratlin et al. 2010), suggesting that the Fc domain of the antibody is of critical importance as the effector part of the drug.

Much of the 2000–2010 decade of imaging research in angiogenesis focused solely on the tumor vasculature as the critical target for VEGF inhibitors. Toward the end of this period, the relationship between VEGF and the immune system became clearer (Motz and Coukos 2011). These studies showed that VEGF inhibitors could increase immune reactivity, and thus a potential mode of action of this class of drug is through increasing the potency of the immune system. Given the rapidly developing interest in the potential for immunotherapy to augment the efficacy of radiotherapy (Sharabi et al. 2015), there remains a critical need for further mechanistic studies to understand the synergy between radiotherapy and VEGF inhibitors in vivo and in humans, albeit with increased toxicity.

To resolve the question of whether imaging analysis of the vasculature reports epiphenomenonological data whereas the principal mode of action of VEGF inhibitors is through the immune system, one approach would be to determine if other drugs with a proposed anti-vascular mechanism of action also have imaging effects. Several drugs that inhibit other vascular targets have been evaluated in the clinic, and selected studies are listed in Table 2.

The exemplary studies cited in Table 2 show that inhibition of a number of vascular targets results in changes in imaging that are related to the dose of the agent. Further, the fact that several antibody-based structures, which specifically target particular cytokines or pathways, cause imaging effects argues against the thesis presented above and assert that VEGF inhibitors induce imaging effects through an anti-vascular mode of action that is the core mechanism of action of the drugs. Taken in conjunction with other mechanistic studies (Wedam et al. 2006; Willett et al. 2004), these data demonstrate that DCE-MRI is a reproducible and sensitive method for determining whether a candidate drug has anti-vascular activity. This conclusion has been introduced into early phase drug development to the extent that some would consider that a drug with putative VEGF inhibitory characteristics that did not reduce K^{trans} within its maximum tolerated dose was not hitting its target.

Imaging Effects Are Necessary But Not Sufficient

The early success seen with bevacizumab, in particular, and the parallel interest in the emerging positive imaging data led to an exaggerated reliance on imaging for later phase clinical trial decision making. Thus, the results of early phase clinical trials of some of the agents listed in Tables 1 and 2 led to phase III clinical trials that were subsequently negative. Examples of such agents include PTK/ZK where positive imaging studies (Morgan et al. 2003) led to phase III studies that were ultimately negative (Hecht et al. 2011; Van Cutsem et al. 2011). Additional negative phase III trials of drugs that had shown positive imaging effects in phase I/II evaluation included cediranib (Schmoll et al. 2012; Batchelor et al. 2007, 2010; Hoff et al. 2012), cilengitide

Table 2 Imaging effects of drugs targeted at systems other than VEGF/VEGFR. The table shows the clinical and imaging effects of non-VEGF inhibitor, anti-angiogenic agents in early phase evaluation. In general the data show that inhibition of a number of vascular targets results in vascular changes in imaging

Target	Drug	Phase of Trial	Tumor type	Clinical effect	Imaging effect	References
Angiopoietin	AMG 386, trebananib, peptidobody	I	All	Response in ovarian cancer	Reduction in Ktrans	Herbst et al. (2009)
VEGFR2, Tie2, PDGFRβ and FGFR2	Regorafenib, low molecular weight TKi	I	All	Response in renal, colorectal and sarcoma	Dose response reduction in IAUC60	Mross et al. (2012)
PDGFRβ	CDP860, di-Fab anti-PDGFRb	I	Ovarian and colorectal	Increased ascites and peripheral edema	Increased enhancing fraction	Jayson et al. (2005)
Vascular integrins	Cilengitide, cyclic anti-vascular integrin penta-peptide	I	glioma	CR and PR seen in glioma	Association between perfusion, PK and response	Nabors et al. (2007)
	Anti-vascular integrin antibody	I	All	PR in angiosarcoma	None seen	Mullamitha et al. (2007)
Tubulins (anti-vascular agents)	Combretastatin	I	All	CR in anaplastic thyroid	Reduction in perfusion	Dowlati et al. (2002)

(Nabors et al. 2007; Stupp et al. 2014), and to a lesser extent (because of a modestly positive phase III trial) trebananib (Herbst et al. 2009; Monk et al. 2014).

The explanations for the failure of positive proof of principle imaging early phase studies to translate into positive phase III clinical trials are diverse. Suggestions to account for this failure include pharmacokinetic differences, the critical nature of the target ligand and/or lack of biomarkers to identify the patients who most benefit. On the other hand, those agents that have yielded positive results in phase III trials have all demonstrated positive imaging studies. Thus, taken together the data suggest that positive imaging data are necessary but not sufficient to identify drugs that will yield positive phase III trial outcomes for anti-vascular agents.

Why This Technology Remains Important

The above discussion has shown that imaging technology has been useful for selecting agents that are or are not biologically active but that the technology does not predict a positive outcome from phase III evaluation. Nevertheless, studies conducted over time demonstrate that VEGF inhibitors cause vascular changes that can be detected through imaging. Given the lack of predictive biomarkers for VEGF inhibitors, on-treatment changes like these are potentially the best way of detecting biological effects of these drugs. This is important because we are now entering the era of combination regimens of biologically targeted agents. Recent trials have demonstrated the efficacy of the combination of cediranib, a VEGF receptor tyrosine kinase inhibitor and olaparib, a PARP inhibitor (Liu et al. 2014). Such combinations appear active even in the absence of underlying germline BRCA gene mutations. However, this combination, which will be continued until progression, is expensive, and there is a critical need for efficacy biomarkers that can monitor and optimize use of such combinations if they are to be used in multiple health-care systems.

Pharmacodynamic PET Scanning

PET scanning relies on the incorporation of positron-emitting isotopes into chemical structures that can be administered to humans. Positrons collide with electrons to release two photons that can be quantitatively detected. Thus, the major advantage of PET scanning over most of the other imaging technologies is that it is perhaps the most quantifiable of all the techniques. That said, there are some critical logistic problems associated with the technology. These include the very short half-life of many positron-emitting isotopes, the need to often quantify the arterial input of the tracer to an imaged organ to generate entirely quantitative data, the requirement for real-time pharmacokinetic analysis to demonstrate that a novel tracer is chemically intact at the time of imaging, as well as the need for a GMP radiochemistry facility, a cyclotron and imaging equipment particularly for studies that deploy the less frequently used tracers. Thus, other than the most well-established of imaging tracers such as [18F]-FDG-PET studies, few other imaging tracers have been comprehensively studied to quantify tumor vasculature.

Studies of [^{18}F]-FDG-PET

FDG-PET has been evaluated in several studies of anti-angiogenic agents. The premise on which its use is predicated is that tumors in general take up glucose at a far greater level than surrounding tissues other than those with high background uptake, such as the brain. This differential uptake, based on the Warburg effect, allows PET imaging to be conducted.

There is a biological problem that probably accounts for the inconsistent findings associated with the use of FDG-PET in studying human tumor vasculature. The uptake of FDG is affected by the delivery of the PET tracer to a tumor, that is, by tumor perfusion, as well as the uptake of FDG into tumor cells. One of the major effects of anti-angiogenic therapy is to decrease blood supply to tumors; thus one might expect the impact on imaging would be a reduction in uptake of the tracer into tumors that have been treated with anti-angiogenic agents. On the other hand, we know that the consequence of anti-angiogenic agents at the tissue level is the induction of hypoxia, which itself can induce the expression of the glucose transporter and thereby increase uptake of the FDG radio-tracer into tumor cells. These discordant biological effects therefore compromise interpretation of FDG effects, and in accordance with this conceptual problem, the clinical data associated with anti-angiogenic agents have largely been inconsistent.

The first studies of FDG-PET in patients treated with VEGF inhibitors did not show any clear effect although these were very small investigations (Willett et al. 2004). The situation is more complicated when evaluating small molecular weight VEGF receptor tyrosine kinase inhibitors as they target kinases in addition to those of the VEGF receptor and therefore can induce greater effects on FDG imaging through direct antitumor control. However, despite this issue, imaging with conventional or Choi et al. (2007) modifications to conventional CT scan reporting were associated with greater response detection in gastrointestinal stromal tumors than FDG-PET (Benjamin et al. 2011; Yap et al. 2013; Judson et al. 2014).

Anti-angiogenic agents have been developed to target a number of cytokines or their receptors. However, despite correlative studies that suggest, for example, that FDG uptake reflects angiopoietin expression in colorectal cancer (Strauss et al. 2008), FDG has for the most part not been deployed in the pharmacodynamics evaluation of these other drugs.

Together the conflicting biological interpretation of the impact of anti-angiogenic agents on FDG uptake has led to only a few studies incorporating this technology, and largely these have not yielded consistent or useful results. Hence, FDG has not been widely used to evaluate anti-angiogenic effects.

Studies of [^{15}O]-H_2O and [^{18}F]-FLT

From a conceptual point of view, measurement of tumor perfusion through administration and quantification of [^{15}O]-H_2O should represent one

of the best strategies for the assessment of anti-angiogenic agents. Indeed early studies of water-PET with the vascular disrupting agent, combretastatin, revealed the profound effect of the drug on the tumor vasculature (Anderson et al. 2003).

Despite the significant potential to quantify perfusion through the use of [15O]-H_2O, the major limitation of the technology is that the half-life of the isotope is only 2 min, and thus the tracer has to be generated at the point of infusion into the patient, requiring major investment in infrastructure. For this reason and because of the substantial evidence base supporting MRI studies, [15O]-H_2O has not established a position in the evaluation of anti-angiogenic agents.

One of the potential consequences of successful inhibition of angiogenesis should be reduced proliferation in tumors. Thus, it was of interest to evaluate a potential tracer, the uptake of which was related to proliferation. [^{18}F]- 3′-deoxy-3-′-fluorothymidine (FLT) was developed for this purpose and has been evaluated to a limited extent in solid tumor oncology and in particular angiogenesis. The tracer is taken up by cells where it is then phosphorylated by thymidine kinase 1, thereby preventing its egress from the cell.

Correlative studies in lung cancer suggest that FLT uptake (determined by the SUV) correlates with the proliferation marker, Ki67 and CD105-determined microvessel density (Yang et al. 2012). In renal cancer, FLT-PET uptake reduced after 1–2 weeks of treatment with the VEGF receptor tyrosine kinase inhibitor, sunitinib (Horn et al. 2015), and then increased upon withdrawal of the drug (Liu et al. 2011). Thus, these limited data suggest that FLT might be a useful tracer in the evaluation of anti-angiogenic agents. However, there are two important confounding factors: Whereas the uptake of FDG is profoundly increased through the Warburg effect, this is not the case for FLT. Thus, the impact of an effective agent on tracer uptake is likely to be less apparent and harder to detect. Secondly, much drug development focuses at least at the early stages on patients with metastatic disease. With respect to FLT, background uptake of FLT in the liver is significant, and thus only the most intensive and rigorous of imaging protocols can detect changes in FLT uptake in liver metastases, which are frequently present and evaluable in patients participating in early phase clinical trials.

Emerging Imaging Technologies

We argued above that PET scanning offers the most quantitative technology for the evaluation of tumor vasculature. However, because of the number of technical issues involved in quantitative PET and the extensive literature that has arisen through MRI, the latter technology has become more widely used in the study of tumor vasculature.

Attempts to extract more information from MRI studies included the development of larger molecular weight imaging agents, which could capitalize on the leaky vasculature that characterizes human cancer. These newer tracers have included gadolinium-albumin conjugates, iron oxide tracers, and nanoparticles (summarized in Barrett et al. 2006). However, while theoretically and preclinically exciting, the development of these novel reagents into licensed imaging agents has largely not occurred, and there remains a reluctance to combine two novel factors in one clinical trial, the anti-angiogenic agent and the imaging molecule. Thus to a large extent, this field has not progressed. It is hoped that the publication of guidelines for the development and validation of newer imaging agents will be accepted and will help to introduce new imaging tracers into the clinic more efficiently (O'Connor et al. 2016).

Two further MRI technologies that do not require contrast can be used to image the vasculature. However, they have not been widely used in the study of angiogenesis or anti-angiogenic agents. They include arterial spin labeling (Barrett et al. 2007) (ASL) where a volume of blood is magnetized and its entry into an organ and the subsequent mixing with water in imaged tissue are investigated. To a large extent, this technology has been applied to vascular studies of the brain,

where motion artifact is least likely to be problematic. However, occasional studies that are not focused on the brain have highlighted the potential for this technique to quantify the tumor vasculature, e.g., in renal cancer (Zhang et al. 2016).

The second imaging approach that has been evaluated is blood oxygen level-dependent imaging (BOLD), which relies on the paramagnetic signal of deoxyhemoglobin. However, interpretation of this signal, which again has most frequently been applied in the brain, is difficult because of the confounding influences of flow, perfusion, and deoxygenation (Padhani et al. 2007). Recent refinements of the technology have demonstrated its potential to discriminate between different grades of glioma (Wiestler et al. 2016), but further studies are needed if the technology is to become more widely used in the evaluation of anti-angiogenic agents.

Tissue studies of tumor vasculature revealed a range of vascular maturity that appeared to be a relevant determinant of response to VEGF inhibitors (Sitohy et al. 2011). Vascular maturity in part reflects the degree of pericyte coverage, and thus the vessels' capacity to respond to vasodilatory influences such as carbon dioxide. This understanding led to attempts to administer CO_2 during BOLD-MRI to assess the maturity of tumor vessels as CO_2 should cause vasodilatation of mature blood vessels that are coated in pericytes. Trials were conducted in air containing 5% CO_2 or in 95% oxygen/5% CO_2 (carbogen). However, these techniques can be distressing for patients because of claustrophobia and perceived oxygen deprivation, and, despite the reported increase in tumor perfusion, these confounding factors have impacted significantly on the potential to exploit this technology further (Padhani et al. 2007; Taylor et al. 2001).

Future Directions

Several developments over the last few years have highlighted the critical need for predictive and pharmacodynamic imaging biomarkers if we are to exploit the tumor vasculature as a target for cancer treatment. One of the most exciting developments was the recent report of the combination of VEGF inhibitors with the PARP inhibitors in ovarian cancer (Liu et al. 2014). The activity seen with these two oral agents was striking and critically did not correlate with the presence or absence of germline BRCA gene mutations, which have traditionally been used to select patients for treatment with a PARP inhibitor. A second critical development focuses on the new class of immunotherapeutic agents that target a number of immune checkpoints. Given that VEGF inhibitors have potent immunomodulatory potential (Motz and Coukos 2011), there is a clear need to understand and develop combinations of VEGF inhibitors and checkpoint inhibitors (Wallin et al. 2016).

These studies are underway and it is likely that additivity will be detected. However, in both examples presented here, the cost of treating patients with combination regimens will be significant and, for many health-care organizations, prohibitive. Thus, there is a critical need for predictive and/or pharmacodynamic biomarkers that can be used to direct and then optimize therapy for our patients. As both combinations represent new paradigms in cancer treatment, e.g., there will be many further studies that evaluate novel combinations of VEGF inhibitors with other DNA repair inhibitors, one can foresee the development of multiple new, effective but expensive combination regimens. It is therefore mandatory for investigators and the pharmaceutical industry to incorporate suitable biomarker studies into the drug development strategy if we are to afford to treat our patients with these regimens.

Cross-References

- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part VII

Biomarkers for Anti-angiogenic Therapy

Biomarkers for Anti-angiogenic Therapy

Weibin Hou and Stefan Duensing

Contents

Introduction 294

Tissue-Based Biomarkers 295
Biomarkers of Pro-angiogenic Pathway Activation 295
Microenvironmental Biomarkers 296
Metabolic Biomarkers 297

Blood-Based Biomarkers 297
Cytokines and Pro-angiogenic Factors 298
Circulating Endothelial and Endothelial Progenitor Cells 298
Circulating Tumor Cells 299
Neutrophil-to-Lymphocyte Ratio 299

Genetics and Epigenetics 299
Genetic Alterations 300
Single-Nucleotide Polymorphisms (SNPs) 300
DNA Hypermethylation 301
MicroRNAs 301

Drug Toxicities as Biomarkers of Drug Efficacy 302
Hypertension and Anti-angiogenic Therapy 302
Skin Toxicity and Anti-angiogenic Therapy 302

Imaging-Based Biomarkers 303

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Conclusion 303
Cross-References 303
References 303

Abstract

The introduction of anti-angiogenic therapy has changed the clinical practice in a number of human malignancies. Despite this success, anti-angiogenic therapy frequently has only transient effects, with acquired drug resistance and tumor progression quickly to follow. The heterogeneous response to anti-angiogenic therapy has created a need for predictive biomarkers. This chapter reviews potential biomarkers for anti-angiogenic therapy focusing on tissue-based, blood-based, genetic, and epigenetic markers and lastly drug-related toxicity and imaging to determine therapeutic efficacy. Most studies summarized here were performed retrospectively in relatively small patient cohorts. Further confirmation in prospective and randomized clinical trials to establish superiority of one or a combination of biomarkers remains hence an ongoing task.

Keywords

Biomarker · Angiogenesis · Anti-angiogenic therapy · Bevacizumab · Renal cancer · Lung cancer · Breast cancer · Gastric cancer · Ovarian cancer · Glioblastoma · VEGF · VEGFR

Introduction

The idea to treat cancer by inhibiting angiogenesis originates from the late Judah Folkman more than 40 years ago (Folkman 1971). His seminal studies led to the successful development of a number of anti-angiogenic drugs including the humanized antibody bevacizumab but also tyrosine kinase inhibitors (TKIs) such as sunitinib, sorafenib, and others that are now routinely used as a treatment for several human malignancies. However, not every patient benefits from these compounds, and there is an effort to individualize treatment by developing predictive biomarkers for anti-angiogenic therapy. This approach appears not only necessary from a health economic standpoint but also with regard to the sometimes severe adverse events associated with anti-angiogenic agents.

A biomarker is defined by the National Institutes of Health as a parameter that can be objectively measured and evaluated and that indicates a pathological process or a pharmacological response to a therapeutic intervention. The use of the term biomarker has become somewhat inflationary, and it needs to be emphasized that the development of new biomarkers is cumbersome.

An overarching goal of precision oncology is to choose a drug to selectively inhibit the molecules or pathways that initiate and maintain cancer cell proliferation and survival. While most cancers harbor multiple oncogenic mutations, preclinical and clinical data support the notion that many cancers are sensitive to inhibition of a selected number of oncogenes or pathways, a concept referred to as "oncogene addiction" (Pagliarini et al. 2015). A biomarker that could indicate whether or not the patient's tumor is addicted to the angiogenic pathway is hence a potentially useful biomarker. Biomarkers of drug resistance are also suitable since drug resistance is the main reason for therapeutic failure. Factors involved in drug metabolism should not be dismissed since they could be responsible for interindividual differences in the efficacy and toxicity of the drugs. Lastly, some emerging methodologies such as next-generation sequencing, genome-wide association study (GWAS), and other "omics"-based approaches like proteomics, metabolomics, or lipidomics could deliver a more unbiased approach to biomarker development.

Based on their clinical use, biomarkers can be subdivided into four categories: biomarkers for early detection, diagnostic biomarkers, prognostic

biomarkers, and predictive biomarkers. Based on the biological material used, biomarkers can also be categorized as tissue-based, blood-based, or other body fluid-based biomarkers. A detailed analysis of the entirety of biomarkers used in anti-angiogenic therapy is nearly impossible, and the authors focus herein on predictive and prognostic biomarkers and a tissue-based, a blood-based, a genetic/epigenetic, and lastly a toxicity-based approach.

Tissue-Based Biomarkers

Since the 1980s, the search for tissue-based biomarkers has focused heavily on immunohistochemical markers. The emergence of the tissue microarray (TMA) technology has further added momentum to biomarker discovery. The TMA technology enables researchers to perform a more standardized staining procedure and at the same time reduces costs by a simultaneous analysis of hundreds of tissue specimens. Digital image analysis furthermore allows large-scale bioinformatic approaches to tissue biomarker discovery. One important caveat of TMA-based studies, however, is the bias in sample collection and the now increasingly recognized problem of intratumoral heterogeneity (Lipinski et al. 2016; Almendro et al. 2013).

Biomarkers of Pro-angiogenic Pathway Activation

One approach to identify potential predictive biomarkers for anti-angiogenic therapy is to focus on proteins that activate pro-angiogenic pathways. The activation of pro-angiogenic pathways may indicate a dependence of the tumor on neo-angiogenesis and therefore the possibility of a beneficial response to the anti-angiogenic treatment.

Based on this notion, important candidate biomarkers for anti-angiogenic therapy would be the expression of the vascular endothelial growth factor (VEGF) and the VEGF receptor (VEGFR). VEGF is highly expressed in the majority of tumors but is also expressed by benign tissue and tumor stroma. Although some studies indicated a positive correlation between VEGF expression and the therapeutic response to anti-angiogenic agents (D'Alessandris et al. 2015; Travnicek et al. 2015), follow-up studies failed to identify a predictive effect based on VEGF protein expression (Jubb et al. 2011; Kara et al. 2012).

However, several lines of evidence underscore that the expression of VEGFRs may be more informative (Gerger et al. 2011). For example, overexpression of VEGFR-2, a key regulator of pro-angiogenic signaling, has been shown to be a useful biomarker for predicting the response to anti-angiogenic therapy with the TKI sunitinib (You et al. 2015). Conversely, low VEGFR-3 expression has been found to be associated with a worse outcome in clear cell renal cell carcinoma (ccRCC) patients treated with sunitinib (Garcia-Donas et al. 2013), whereas high expression of VEGFR-3 was found to be associated with a favorable response of women with metastatic breast cancer to paclitaxel and bevacizumab (Fountzilas et al. 2011). In the same study, high expression of VEGFR-1 was associated with poor survival, which indicates that different VEGFRs play quite different roles in tumor progression (Fountzilas et al. 2011). Activated, i.e., phosphorylated, VEGFR-2 was found to correlate with a better treatment response in breast cancer patients treated with the VEGFR2 inhibitor apatinib and was furthermore found to be an independent prognostic factor for patient progression-free survival (Fan et al. 2014).

Tumor neo-angiogenesis is a complex and highly adaptive biological process. Despite the predominant role of VEGF/VEGFR signaling, multiple other pro-angiogenic factors also play an important role during angiogenesis. These include platelet-derived growth factors (PDGFs) and PDGF receptors (PDGFRs), neuropilin-1 (NRP1), fibroblast growth factors (FGFs), angiopoietins (ANGs), and various cytokines (e.g., IL-8 and others) (Carmeliet and Jain 2011). Since these pathways represent alternative pro-angiogenic triggers, their upregulation can contribute to the acquired resistance

to VEGF or VEFGR-targeted agents and are therefore candidate biomarkers for the efficacy of anti-angiogenic therapy.

Overexpression of PDGFR-β in the tumor vasculature detected by immunohistochemistry was significantly associated with a favorable response of breast cancer patients to bevacizumab (Yang et al. 2008). In gastric cancer patients treated with bevacizumab in combination with chemotherapy, a low expression of NRP1 was associated with a trend toward better survival in comparison to those patients with a high NRP1 expression of the tumor (Van Cutsem et al. 2012). Another example is glioblastoma, where ANG-2 overexpression has been suggested to be involved in the resistance to bevacizumab (Labussiere et al. 2016).

Many of the angiogenic factors expressed by tumor cells are regulated by hypoxia and transcriptional regulation by hypoxia-inducible factors (HIFs). A hypoxic microenvironment develops in most solid tumors due to the imbalance between tumor growth and oxygen supply. The HIF pathway has therefore also gained attention as a potential biomarker to anti-angiogenic therapy, especially in ccRCC, which is characterized by *VHL* mutations and hyperactivity of HIF signaling. Since one of the main downstream targets of the VHL/HIF pathway is VEGF, ccRCC was among the first tumors in which anti-angiogenic therapy was used based on a clear biological rationale (Atkins et al. 2004). While the association between *VHL* mutations and anti-angiogenic drug response did not yield informative results (Gossage and Eisen 2010), nuclear overexpression of HIF-1α or cytoplasmic overexpression of HIF-2α has been found to indicate an unfavorable prognosis in RCC patients (Fan et al. 2015). High expression of HIF-2α has been reported to be associated with a better response to sunitinib in metastatic ccRCC (Garcia-Donas et al. 2013). However, other studies did not find a correlation between HIF-1/2 protein expressions and the response to the TKI pazopanib in ccRCC patients (Choueiri et al. 2013).

Carbonic anhydrase IX (CAIX), a transmembrane protein, is a well-established HIF target that has been implicated in the regulation of cell proliferation in response to hypoxia. Lower expression of CAIX has been reported to be associated with a better clinical outcome in patients with metastatic colorectal cancer (mCRC) treated with bevacizumab in combination with chemotherapy (Hong et al. 2009). In contrast, high CAIX staining has been reported to be an independent prognostic factor for longer overall survival in patients with metastatic ccRCC treated with sunitinib (Stewart et al. 2014; Dornbusch et al. 2013).

Microenvironmental Biomarkers

The tumor microenvironment is increasingly recognized as a crucial player in tumor cell survival, drug resistance, and also neo-angiogenesis. The main components of the tumor microenvironment are blood vessels, stroma cells, and immune cells as well as the extracellular matrix (ECM) (Turley et al. 2015), all of which have been explored as potential biomarkers for anti-angiogenic therapy.

Tumor neo-angiogenesis is frequently measured by directly counting the microvessels in a given area of tumor tissue using endothelial cell-specific markers such as CD31 and others to determine the microvessel density (MVD) (Lokmic and Mitchell 2011).

MVD is a well-established parameter to reflect the neovascularization in a tumor and was hence thought to be a potentially predictive biomarker for anti-angiogenic therapy. MVD was found to be a favorable predictor in patients with non-small cell lung cancer (NSCLC) treated with chemotherapy and bevacizumab, with a higher MVD correlating with better treatment response (Zhao et al. 2012). Similarly, there are reports that a high pretreatment MVD is associated with a better pathological response to neoadjuvant bevacizumab and chemotherapy in breast cancer patients (Tolaney et al. 2015). Conversely, a low MVD was found to be associated with a less favorable response to bevacizumab patients with NSCLC (Pomme et al. 2015). Other studies, however, did not confirm these results (Jubb et al. 2006), and additional studies are very likely needed to verify the predictive relevance of MVD as a biomarker for anti-angiogenic therapy.

Tumor-associated macrophages (TAMs) are important components of the tumor microenvironment (Belgiovine et al. 2016). TAMs are usually detected by using immunohistochemical markers such as CD163 and others. Higher numbers of TAMs in the tumor tissue after anti-angiogenic therapy correlated with an impaired survival of patients with glioblastoma (Lu-Emerson et al. 2013). Depletion of TAMs, in contrast, was found to enhance the therapeutic efficacy of sorafenib in a metastatic liver cancer mouse model (Zhang et al. 2010). More evidence is needed to confirm a potential role of TAMs as predictive marker.

Neutrophils are the most abundant granulocytes and act as the first line of immune defense against invading pathogens. Their role as potential biomarker in cancer did not receive much attention until recently. Tumor-associated neutrophils (TANs) have been found to be associated with the course of disease in a variety of malignancies. For instance, high levels of TANs were found to be associated with an unfavorable recurrence-free and overall survival in various solid tumors (Shen et al. 2014). Emerging evidence suggests that TANs may not only be able to induce tumor neovascularization by the secretion of pro-angiogenic factors (Tazzyman et al. 2013) but also to promote resistance to anti-angiogenic therapy (Zhou et al. 2016).

More recently, gene expression profiles of the tumor stroma were used to develop a stroma-derived prognostic predictor in breast cancer that could predict disease outcome independently of standard prognostic parameters. Angiogenic and hypoxia-responsive genes were strongly represented and linked to poor patient outcome thus highlighting the potential usefulness of stromal gene expression as biomarker for anti-angiogenic therapy (Finak et al. 2008).

Metabolic Biomarkers

Molecular markers involved in drug metabolism may also represent potential biomarker candidates. Gene expression profiling of tumor specimens from patients with glioblastoma receiving bevacizumab in combination with chemotherapy revealed that an overexpression of FMO4 or OSBPL3 is associated with a favorable treatment response. FMO4 is part of a protein family that, after cytochrome P450, is the second-largest protein family involved in drug metabolism. OSBPL3 is one of 12 members of the oxysterol-binding protein-related protein family that play a role in lipid metabolism, vesicle trafficking, and cell signaling (Erdem-Eraslan et al. 2016).

Anti-angiogenic treatment in patient-derived intracranial glioblastoma xenografts showed decreased levels of TCA cycle enzymes including isocitrate dehydrogenase. In contrast, malectin, calnexin, and lactate dehydrogenase were increased after treatment thus providing a molecular signature of the tumor response to anti-angiogenic therapy in glioblastoma (Demeure et al. 2016), which could further be exploited for biomarker development.

Taken together, tissue-based biomarkers are promising approaches to predict the response to anti-angiogenic therapy. However, lack of standardized, large, multicentric, and prospective trials limits their application in the daily practice. The marker profile in the primary tumor may not necessarily be present in the metastatic disease treated with angiogenesis inhibitors, which further complicates the situation.

Blood-Based Biomarkers

Biomarker candidates circulating in body fluids, especially in blood, have seen a rise in the past several decades. The detection of biomarkers in body fluids is less invasive than the detection of tissue biomarkers and, more importantly, longitudinal studies to monitor course of the disease and treatment effects is feasible.

Blood-based biomarkers mostly focus on proteins; however, circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) have become more important recently. This part of our review focuses on plasma or serum protein biomarkers and CTCs; ctDNA will be discussed later.

Cytokines and Pro-angiogenic Factors

Several studies have confirmed a role of pre-treatment plasma VEGF as a biomarker for anti-angiogenic therapy. Treatment with bevacizumab was found to be associated with better progression-free survival in patients with higher VEGF-A plasma levels as compared to patients with lower VEGF-A levels (Santos et al. 2015). The pretreatment VEGF-A level has been reported to be a good prognostic marker, though not predictive biomarker for a bevacizumab-associated treatment benefit in mCRC, NSCLC, and metastatic RCC (Hegde et al. 2013). A low level of VEGF-A at baseline was associated with a better outcome in patients with epithelioid hemangioendothelioma (EHE) and angiosarcoma receiving sorafenib (Penel et al. 2014). A decrease of the serum VEGF-A levels has been suggested as a potential predictive biomarker for progression-free survival in mCRC patients receiving regorafenib (Suenaga et al. 2016). Besides VEGF-A, plasma levels of VEGF-D have been showed to predict the benefit from bevacizumab treatment in patients with pancreatic cancer (Nixon et al. 2013). There are several known isoforms of VEGF-A, including the short isoform VEGF-A121 and the longer isoforms VEGF-A145, VEGF-A165, VEGF-A189, and VEGF-A206, which differ in their abundance and receptor binding. It has been suggested that the detection of short VEGF-A isoforms could be more helpful for biomarker development since a high level of short VEGF-A isoforms may be more specific for tumor-secreted VEGF-A (Lambrechts et al. 2013; Bates et al. 2012).

Soluble VEGFRs are likewise promising candidate biomarkers for anti-angiogenic therapy. The relative change of soluble VEGFR-2 during treatment has been suggested to be a biomarker for the response in patients with metastatic breast cancer receiving bevacizumab (Lam et al. 2016). A low-soluble VEGFR-2 12 weeks after treatment initiation has been reported to be associated with a reduced efficacy in patients with advanced soft-tissue sarcoma treated with pazopanib (Sleijfer et al. 2012). Pretreatment low levels of plasma soluble VEGFR-1(sVEGFR-1) has been found to be associated with tumor regression and the development of adverse events after neoadjuvant bevacizumab and chemo-/radiotherapy (Duda et al. 2010). Low soluble VEGFR-3 has been reported to be associated with shorter progression-free and overall survival in patients with advanced neuroendocrine tumors treated with sunitinib (Zurita et al. 2015).

Growth factors like ANGs and related proteins have also been repeatedly reported to have predictive value for anti-angiogenic therapy. For example, low plasma levels of ANG-2 were predictive for primary resistance to bevacizumab in pancreatic cancer patients (Nixon et al. 2013). In women with ovarian cancer and treated with bevacizumab, high ANG-1 and low TIE-2 were found to predict a better outcome (Backen et al. 2014). In mCRC patients treated with regorafenib, high concentrations of plasma TIE-1 were found to be associated with longer overall survival compared with low TIE-1 concentrations (Tabernero et al. 2015).

Cytokines like IL-8 or IL-6 also have predictive relevance as shown in a number of studies (Zurita et al. 2015). In hepatocellular carcinoma (HCC) patients treated with bevacizumab, a high expression of IL-8 and IL-6 in plasma was predictive for shorter progression-free and overall survival (Boige et al. 2012). Changes in IL-8 levels have been reported to be predictive for the response to bevacizumab in patients with metastatic breast cancer (Lam et al. 2016).

Changes of biomarkers during anti-angiogenic therapy and their detection through longitudinal sampling may help to acquire a more dynamic assessment of therapeutic efficacy for which a number of examples exist (Nikolinakos et al. 2010; Necchi et al. 2014; Tran et al. 2012; Kopetz et al. 2010; Zhu et al. 2009).

It needs to be emphasized that large, multicenter, and prospective clinical trials are also missing for most blood-based biomarkers.

Circulating Endothelial and Endothelial Progenitor Cells

Circulating endothelial cells (CECs) consist of a population of endothelial cells that have a phenotype compatible with terminally differentiated

endothelial cells. They are believed to be shredded from the intima of blood vessels into the circulation. CECs are rarely detected in healthy individuals, but their number increases in the presence of endothelial damage. Since a disruption of tumor neovascularization may release endothelial cells into the blood stream, CECs have gained attention as biomarkers for the response to anti-angiogenic therapy. It has been reported that sunitinib treatment is associated with an early increase of CECs in RCC patients responding to the treatment (Gruenwald et al. 2010). An increase in the CEC count was associated with improved time to progression in metastatic breast cancer patients treated with bevacizumab combined with chemotherapy (Bidard et al. 2010).

In contrast, circulating endothelial progenitor cells (CEPCs) are bone marrow derived and not derived from the vasculature. CEPCs are characterized by an expression of CD34, CD133, and VEGFR-2 (Manzoni et al. 2015; Fleitas et al. 2010). CEPCs are believed to be recruited to progressing tumors, and an increased concentration of CEPCs may therefore reflect active tumor neo-angiogenesis and could potentially serve as predictive markers for anti-angiogenic therapies (Lambrechts et al. 2013). In mCRC patients treated with bevacizumab in combination with chemotherapy, reduced CEPC levels on day 4 of treatment were associated with significantly longer progression-free and overall survival (Matsusaka et al. 2011). However, other studies could not substantiate a role of CEPCs and CECs, in patient outcome and additional studies are clearly warranted (Ramcharan et al. 2015).

Circulating Tumor Cells

Solid tumors have been suggested to release cells into the circulation, but CTCs could not be isolated efficiently until recently (Alix-Panabieres and Pantel 2016). CTCs from different sites of a tumor may also represent intratumoral heterogeneity and may hence be more representative than a single biopsy. The molecular analysis of individual CTCs may hence provide insights into cancer evolution and the metastatic cascade (Krebs et al. 2014).

The usefulness of CTCs as prognostic biomarkers has been demonstrated in a number of malignancies including breast cancer, prostate cancer, and CRC (Krebs et al. 2014; Riethdorf and Pantel 2008) where higher number of CTCs are associated with shorter survival.

Several studies have attempted to link CTCs to the response to anti-angiogenic therapy. In patients with mCRC treated with bevacizumab and chemotherapy, an increased CTC count was an independent prognostic factor for poor progression-free and overall survival (Sastre et al. 2013). In patients with metastatic breast cancer, a baseline CTC count of $\geq$5 per 7.5 ml blood identified women who would benefit from bevacizumab and chemotherapy (Giuliano et al. 2011). In the another study, a simultaneous detection of CTCs and CECs showed that a rapid increase of CECs as early as in the first cycle of therapy is associated with CTC decrease, whereas a delayed increase of CECs was related to higher CTC counts and poor therapy response (Rossi et al. 2012).

Neutrophil-to-Lymphocyte Ratio

The neutrophil-to-lymphocyte ratio (NLR) is a simple and informative marker that has shown its predictive significance in a number of studies. In patients with mRCC treated with sunitinib, it was shown that the majority of patients who responded to sunitinib had a NLR of $\leq$3 (Dirican et al. 2013). Similarly, in NSCLC patients treated with bevacizumab and chemotherapy, a high NLR was associated with shorter survival (Botta et al. 2013). Recently, it has been reported that high NLR represented a negative prognostic factor in mCRC patients receiving regorafenib monotherapy (Del Prete et al. 2015).

Genetics and Epigenetics

Genetic and epigenetic alterations of tumor cells have been investigated in numerous studies for a potential use as biomarkers. Since DNA is usually stable, many genetic and epigenetic biomarkers

are more robust and reproducible than protein-based biomarkers, which are more sensitive to even small perturbations.

Genetic Alterations

The past decade has seen a number of important examples in which the identification of mutations has led to novel therapeutic interventions and/or better predictive and prognostic patient stratification. For biomarker development for anti-angiogenic therapy, genetic alterations involved in angiogenesis pathways, drug metabolism, and driver gene mutations have been widely explored.

Although genetic alterations of VEGF/VEGFR or related pathway components are rare events in human malignancies, a number of other genetic events have been successfully explored as biomarkers.

The human *RAS* genes (including *HRAS*, *KRAS*, and *NRAS*) are among the most commonly mutated oncogenes in human cancers (Downward 2003) together with the *RAF* gene family (including *ARAF*, *BRAF*, and *CRAF*), and mutations can be found especially in melanoma, lung cancer, and CRC (Davies et al. 2002; Zebisch and Troppmair 2006; Downward 2003).The *KRAS* mutational status has been successfully explored in mCRC patients treated with bevacizumab by showing that *KRAS* wild-type status is a favorable prognostic factor (Petrelli et al. 2013). Similarly, sorafenib has been demonstrated to have clinical activity in NSCLC patients when the *EGFR* status is wild-type (Blumenschein et al. 2013). The correlation of the *BRAF* and *NRAS* mutation status with clinical outcome has been investigated in patients with metastatic malignant melanoma receiving chemotherapy and/or sorafenib, and favorable clinical responses were seen in patients with *NRAS*-mutant tumors receiving the sorafenib-containing regimen (Wilson et al. 2014).

VHL gene inactivation plays a central role in the development of ccRCC and has been shown to predict better outcomes in mRCC patients treated with bevacizumab plus IFN-α (Rini et al. 2006). It has moreover been shown that a loss of *VHL* was an independent predictor of higher response rate to VEGF-targeted therapy (Choueiri et al. 2008) although this finding is not undisputed (Song et al. 2015; Choueiri et al. 2013).

In addition to specific gene mutations, the mutational burden per se can be exploited as a biomarker, and a correlation between a low mutational burden and a favorable response has been suggested in patients with HCC treated with sorafenib (Sakai et al. 2015).

Besides analysis of tumor tissue, the concept of liquid biopsy has become more and more important in the past several years for anti-angiogenic therapy (Alix-Panabieres and Pantel 2016).

It is possible to use mutations in ctDNA as biomarkers as shown for the *KRAS* and *PIK3CA* mutational status in CRC patients treated with regorafenib (Tabernero et al. 2015). Importantly, analysis of ctDNA can be used for longitudinal studies and be incorporated into future clinical trials together with the classical oncological response criteria (Dorner et al. 2015).

Single-Nucleotide Polymorphisms (SNPs)

A SNP is a single base-pair change that occurs in at least 1% of the population. Although the direct disease relevance of SNPs is often ambiguous, certain SNPs have been found to predispose individuals to develop a particular disease, response to drug in a certain fashion, or convey prognostic information.

The most widely assessed gene involved in neo-angiogenesis is *VEGF*. Improved survival has been reported in patients with breast cancer or CRC carrying *VEGF*-2578C > A and *VEGF*-1154G > A when receiving bevacizumab-containing treatment (Schneider et al. 2012). A meta-analysis of five *VEGF* polymorphisms (+936C > T, −460T > C, +405G > C, −1154G > A, and –2578C > A) in patients receiving anti-VEGF therapy revealed that only the variant *VEGF* + 405G > C was associated with a significantly improved survival across a variety of tumor types (Eng et al. 2012).

SNPs in *VEGFR2* have been reported to correlate with survival of patients with advanced HCC receiving sorafenib (Zheng et al. 2014). SNPs in *VEGFR1* have been demonstrated to be predictive in mRCC patients receiving sunitinib as first-line therapy. Here, the CC-variant in rs9582036 of *VEGFR1* predicted a worse treatment response, and both the CC-variant in rs9582036 and the AA-variant in rs9554320 of *VEGFR1* predicted an unfavorable progression-free and overall survival (Beuselinck et al. 2014).

Since the inhibition of the VEGF/VEGFR pathway is known to decrease the activity of endothelium-derived nitric oxide synthase (eNOS) via the PI3K/AKT pathway, thus reducing the nitric oxide (NO) levels, *eNOS* gene polymorphisms have been studied in relationship to the clinical outcome of patients with HCC receiving sorafenib as well as mCRC patients receiving bevacizumab and chemotherapy. A specific haplotype in the *eNOS* gene might be able to identify a subgroup of HCC patients resistant to sorafenib (Casadei Gardini et al. 2016a) but also a subset CRC patients being more responsive to bevacizumab (Ulivi et al. 2015).

There are also genetic polymorphisms in genes for drug metabolizing enzymes, receptors, transporters, and other targets, which are believed to be responsible for interindividual differences in the efficacy and toxicity of certain drugs (McLeod and Yu 2003).

For example, sunitinib can be transported outside a cell by proteins like ABCB1 (ATP-binding cassette subfamily B member 1) or ABCG2 (ATP-binding cassette member G2) and subsequently metabolized by cytochrome P4503A enzymes (CYP3A4/CYP3A5). The association of SNPs with sunitinib efficacy and safety among ten candidate genes has been analyzed in a cohort of 333 mRCC patients. It was found that the presence of CGT in the *ABCB1* haplotype was associated with better progression-free survival, while the presence of *CYP3A5*1* was associated with enhanced toxicity requiring dose reduction (Diekstra et al. 2015). These results were consistent with some studies but disagree with results obtained in patients from Asia, thus underscoring population-specific difference (Teo et al. 2016).

DNA Hypermethylation

DNA hypermethylation of promoters containing CpG islands in the presence of a general hypomethylation of the genome is found in many human cancers. DNA hypermethylation usually leads to an inhibition of gene expression, which frequently affects tumor suppressor genes (Heyn and Esteller 2012). DNA methylation markers are relatively stable and hence good candidates for biomarker development (Van De Voorde et al. 2012).

This is underscored by the finding that the absence of DNA hypermethylation of *VEGFRs* correlates with favorable response to TKI treatment (Kim et al. 2012). In patients with mRCC and treated with TKIs or bevacizumab, hypermethylation of *CST6*, LAD1, and *NEFH* was associated with both shortened progression-free and overall survival (Peters et al. 2014; Dubrowinskaja et al. 2014).

MicroRNAs

MicroRNAs (miRNAs) are small, noncoding RNA molecules of approximately 19–25 nucleotides in length that play important role in differentiation, cell proliferation, apoptosis, and stress responses by binding to complementary sites on mRNAs in order to inhibit translation or induce their degradation. It has been estimated that more than 30% of mRNAs are regulated by microRNAs. MiRNAs are frequently altered in cancer patients. Since they are at the same time robust and occur in all bodily fluids, they have also been tested as biomarkers for anti-angiogenic therapy (Bartel 2004; Croce 2009).

Remarkably, a number of miRNA could be linked to angiogenesis (Wurdinger et al. 2008). They have been shown to promote angiogenesis by targeting negative regulators of pro-angiogenic signaling (Wang and Olson 2009). Examples are the miR-17-92 cluster, miR-21, miR-31, miR-126, and several others (Wang and Olson 2009; Borges et al. 2016; Kong et al. 2014).

The predictive value of serum miR-126 has been analyzed in patients with mCRC treated with

bevacizumab and chemotherapy, and a significant correlation between changes in miR-126 and the treatment response was found (Hansen et al. 2015).

Another example is miR-378, which has been reported as an independent predictor for progression-free survival in women with ovarian cancer treated with bevacizumab (Chan et al. 2014).

In HCC patients treated with sorafenib, elevated expression of miR-425-3p in the tumor tissue was associated with better time to progression and progression-free survival (Vaira et al. 2015).

In mRCC patients receiving sunitinib, several miRNAs were found to correlate with the oncological outcome including miR-31, miR-126, and miR-221 (Gamez-Pozo et al. 2012). Another study showed that miR-221 overexpression in tumor tissue was associated with poor progression-free survival in mRCC patients receiving sunitinib in line with the function of miR-221 in angiogenesis and cellular proliferation (Khella et al. 2015).

Drug Toxicities as Biomarkers of Drug Efficacy

Therapeutic efficacy and toxicity have been found to often be linked. Several drug-induced toxicities such as hypertension, hypothyroidism, proteinuria, skin toxicity, and myelosuppression have been reported to correlate to the oncological outcome (Shah et al. 2013). Although not a biomarker in the classical sense, these adverse events may under certain conditions convey predictive information.

Hypertension and Anti-angiogenic Therapy

Hypertension is a common side effect of VEGF inhibitors. The exact mechanism responsible for anti-angiogenic therapy-induced hypertension is not known in detail but may entail effects on NO production resulting in vasoconstriction (Robinson et al. 2010a). Capillary rarefaction is another potential mechanism (Robinson et al. 2010b).

When hypertension was utilized to assess the efficacy of sunitinib in patients with mRCC, patients with drug-induced hypertension had a significantly better outcome with respect to response rate and survival (Rini et al. 2011). Similar results were seen in CRC and pancreatic cancer patients treated with bevacizumab (Tahover et al. 2013; Pant et al. 2014), patients with soft-tissue sarcoma treated with pazopanib (Duffaud et al. 2015) and patients with HCC receiving sorafenib (Casadei Gardini et al. 2016b).

Skin Toxicity and Anti-angiogenic Therapy

Skin toxicity is also a common adverse effect of anti-angiogenic therapy. The incidence of skin toxicity has been increasing, partly due to physicians' increased awareness and partly due to the use of higher drug dosages. Skin toxicities include rash, mucositis, alopecia, depigmentation, pruritus, xerosis, acneiform rashes, and, importantly, hand-foot syndrome, which often is serious and may lead to dose reduction or discontinuation of treatment. Skin toxicities are more common in patients treated with sorafenib and sunitinib and less common in patients receiving pazopanib or bevacizumab (Zhang et al. 2011; Balagula et al. 2012).

The etiology of skin toxicities in association with anti-angiogenic therapy is unclear. It has been proposed that a synergistic effect of inhibition of VEGFR and PDGFR leads to a compromised capillary endothelium and poor reparative response to ordinary trauma at high friction areas such as the hands and feet. This idea is supported by the facts that sorafenib and sunitinib, the two anti-VEGFR TKIs most strongly associated with hand-foot skin reaction, inhibit both VEGFR and PDGFR (Massey et al. 2015).

Remarkably, sorafenib-induced hand-foot syndrome in mRCC patients was associated with a favorable response and significantly better progression-free survival (Nakano et al. 2013).

Imaging-Based Biomarkers

Imaging technology plays an increasingly important role in the assessment of therapy responses. Computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and more recently ultrasound (US) have all showed their ability in evaluating, quantifying, and monitoring changes in the vascular architecture and function of tumors in preclinical models and in patients, which makes them potentially very important biomarkers for predicting and tracking response to anti-angiogenic therapy. Dynamic contrast-enhanced (DCE) imaging techniques (DCE-CT, DCE-MRI, and DCE-US) have been validated as effective surrogates to monitor early anti-angiogenic response in several malignancies. In a phase II trial to evaluate the role of DCE-CT in monitoring the response to neoadjuvant bevacizumab and radiation therapy in resectable soft-tissue-sarcomas, DCE-CT parameters correlated with MVD and can be used for monitoring early and late response to bevacizumab (Kambadakone et al. 2015). The predictive and prognostic role of DCE-MRI in patients with recurrent glioma treated with a bevacizumab-based regimen has been demonstrated in a recent meta-analysis (Choi et al. 2016). DCE-US using microbubble contrast is a new quantitative imaging with several advantages like non-invasiveness and cost-effectiveness (Hudson et al. 2015) and suitable for monitoring response to anti-angiogenic therapy (Lassau et al. 2014). In addition, PET tracer, such as the VEGF-A-binding ^{89}Zr-bevacizumab, has been used to image and quantify whole-body VEGF-A (Oosting et al. 2015) and could be, among others, used as biomarkers for anti-angiogenic therapy (Leu et al. 2013).

In summary, imaging-based methods are promising and rapidly evolving as biomarkers for anti-angiogenic therapy, and prospective clinical trials are needed to validate and optimize their use for clinical practice.

Conclusion

Anti-angiogenic treatment modalities have changed the landscape of oncology in the past decades. Numerous biomarkers have been explored as highlighted by this book chapter in order to personalize their application to reduce unnecessary toxicities and costs and to improve patient outcomes. However, there is currently no validated biomarker in routine use to select patients for anti-angiogenic treatment. Inter- and intratumoral heterogeneity appears to be important reasons for this overall lack of success. Novel markers on the horizon that should particularly be given attention are based on functional imaging. In addition, whole genome sequencing of surprise/long-term responders may help to develop novel and improved molecular markers.

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Cross-References

- ▶ Imaging Tumor Angiogenesis
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis

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Part VIII

Mechanisms of Resistance in Anti-angiogenic Therapy

Anti-angiogenic Cancer Therapy: Development of Resistance

Domenico Ribatti

Contents

Introduction 314
Anti-angiogenesis 314
Development of Resistance 315
Normalization of Tumor Blood Vessels and Pericyte Coverage 315
Hypoxia 316
Recruitment of Inflammatory Cells and Immature Myeloid Cells 317
Alternative Mechanisms of Tumor Vessel Formation 317
Genomic Instability of Tumor Endothelial Cells and Increase of Metastatic Potential 318
Conclusion 319
Cross-References 320
References 320

Abstract

Therapeutic resistance is the major cause for a poor prognosis in cancer patients. Clinical results of anti-angiogenic therapies are very modest, resulting in a moderate improvement of overall survival, and the clinical outcome is associated with the development of resistance. The clinical benefit of anti-angiogenic drugs is due to several intrinsic and acquired limitations including tumor indifference to anti-angiogenic therapy; selection of resistant clones and activation of alternative mechanisms that lead to activation of angiogenesis, even when the target of the drug remains inhibited; therapy-induced reduction of oxygen levels within the tumor and accumulation of infiltrating cancer stem cells; activation of pro-invasive mechanisms and increased dissemination and metastasis; normalization of tumor blood vessels; recruitment of inflammatory cells and immature myeloid cells; alternative mechanisms of tumor vessel formation; and genomic instability of tumor

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endothelial cells. In this context, the concept and strategies of anti-angiogenic therapies should be extensively reconsidered and reevaluated. In particular, rational combinations of anti-angiogenic agents based on pharmacokinetic and pharmacodynamics data are needed to overcome resistance.

Keywords
Angiogenesis · Anti-angiogenesis · Resistance · Tumor Growth · VEGF

Introduction

In 1971, Judah Folkman first advanced the hypothesis that tumor growth also depends on the formation of new blood vessels from the preexisting vascular bed (Folkman 1971). It is now generally accepted that tumor growth is angiogenesis dependent and that any increment of tumor growth requires an increase in vascular growth (Ribatti et al. 1999). Most human tumors arise and remain in situ without angiogenesis for a long time before they switch to an angiogenic phenotype (Ribatti et al. 2007a). Dormant tumors have been discovered during autopsies of individuals who died of causes other than cancer (Black and Welch 1993). Activation of the angiogenic switch has been attributed to the synthesis and release of angiogenic factors or reduction of the concentration of endogenous angiogenic inhibitors, including endostatin, angiostatin, and thrombospondin (Ribatti 2009).

Angiogenic factors can be exported from tumor cells, mobilized from the extracellular matrix (Mignatti and Rifkin 1993), or released from the inflammatory cells recruited to the tumor (Ribatti and Crivellato 2009). Tumor angiogenesis is regulated by numerous "classic" pro-angiogenic factors, including fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and placental growth factor (PlGF). Moreover, evidence has been accumulated that in addition to the "classic" factors, many other "nonclassic factors," including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and erythropoietin (EPO), play an important role (Ribatti et al. 2007b). As a result of the imbalance of angiogenic activators and inhibitors, tumor blood vessels display many structural and functional abnormalities (Ribatti et al. 2007c).

Anti-angiogenesis

In 1971, Folkman proposed a seminal hypothesis: "prevention of new vessel sprouts from penetrating into an early tumor" will keep the "tiny tumor" in a "dormant" state. Beginning in the 1980s, the pharmaceutical industry began to exploit the field of anti-angiogenesis for creating new therapeutic molecules in angiogenesis-dependent diseases.

In 1993, Ferrara et al. demonstrated that VEGF-blocking antibodies reduced tumor growth and vascular density in animal models (Kim et al. 1993). Bevacizumab (Avastin) was the first angiogenesis inhibitor approved by the Food and Drug administration (FDA) for the treatment of colorectal cancer, in combination with irinotecan, 5-fluorouracil, and leucovorin (Hurwitz et al. 2004). Subsequently, bevacizumab in combination with chemotherapy extended overall survival in metastatic non-small cell lung cancer and advanced cervical cancer (Sandler et al. 2006; Tewari et al. 2014). In multiple randomized phase III clinical trials, bevacizumab conferred a survival benefit only when administered in combination with chemotherapy. Different mechanisms may be involved to explain how anti-angiogenic agents boost the efficacy of chemotherapy (Table 1). Angiogenesis inhibitors enhance the efficacy of certain chemotherapeutics by prolonging their contact time with tumor cells (Cesca et al. 2013).

Table 1 Mechanisms explaining how anti-angiogenic agents boost the efficacy of chemotherapy

Direct effect on tumor cell viability
Induction of cytotoxicity independently of the vascular effects
Block of pro-survival signals
"Normalization" of the tumor microenvironment causing increasing intratumoral delivery of chemotherapy
Temporary improvement of oxygen and nutrients to tumor cells rendering them more sensitive to cytotoxic agents
Stimulation of the host immune response

Table 2 Approved anti-angiogenic VEGF inhibitors and their indications

Bevacizumab (metastatic colorectal cancer; non-small cell lung cancer; renal cell carcinoma; ovarian cancer; breast cancer)
Regorafenib (refractory metastatic colorectal cancer)
Ramucirumab (gastric or gastroesophageal junction cancers; metastatic colorectal cancer; non-small cell lung cancer)
Sorafenib (hepatocellular carcinoma; renal cell carcinoma; thyroid cancer)
Sunitinib (renal cell carcinoma; pancreatic neuroendocrine tumors)
Pazopanib (renal cell carcinoma; soft tissue sarcoma)
Axitinib (renal cell carcinoma)
Vandetanib (medullary carcinoma of thyroid)
Lenvatinib (thyroid cancer)
Nintedanib (non-small cell lung cancer)
Aflibercept (colorectal cancer)

Several strategies to inhibit the VEGF/VEGF receptor (VEGFR) signaling pathway for the treatment of cancer have been explored. Anti-angiogenic therapy is essentially anti-VEGF/anti-VEGFR therapy (Table 2). In addition to monoclonal antibodies, alternative approaches of inhibiting VEGFRs by using small VEGFR tyrosine kinase inhibitors (TKIs) have been investigated. TKIs target signaling pathways of VEGFR-1, VEGFR-2, VEGFR-3, and other pro-angiogenic pathways such as the platelet-derived growth factor (PDGF) receptor (PDGFR) and FGF receptor families. TKIs could be more effective than antibody-based therapy that solely target one component of the VEGF/VEGFR pathway. Trials that have combined monoclonal antibodies and TKIs have given rise to an increase of adverse side effects profile.

Development of Resistance

The clinical benefits of anti-angiogenic treatments are relatively modest, because the drugs merely slow down tumor progression and prolong survival by only a few more months. When VEGF-targeted therapies are discontinued, the tumor vasculature is rapidly reestablished (Mancuso et al. 2006), suggesting that prolonged use of VEGF-targeted therapy is necessary to achieve maximal therapeutic effect. Continuation of bevacizumab treatment beyond progression was associated with greater benefit in terms of overall survival (Grothey et al. 2008).

Intrinsic resistance is characterized by inefficacy of tumor treatment with anti-angiogenic anti-VEGF, fusion proteins that trap VEGF (Lockhart et al. 2010), or anti-VEGFR TKIs (Batchelor et al. 2010; Gotink and Verheul 2010). Acquired resistance develops as a result of sequential genetic and epigenetic changes that confer to the tumor cells a complex drug-resistant phenotype. Decreased drug uptake, expression of new drug-efflux pumps, drug metabolism, repair of DNA-damage, alterations of cell proliferation, and/or apoptotic mechanisms (Gottesman 2002) are involved in acquired resistance. In acquired resistance, alternative mechanisms lead to activation of angiogenesis even when the target of the drug remains inhibited (Bergers and Hanahan 2008).

Normalization of Tumor Blood Vessels and Pericyte Coverage

VEGF inhibition could temporarily restore or normalize the function of tumor-associated vasculature, decreasing vascular permeability in conjunction with restoration of sustained pressure gradients and thereby enhancing systemic delivery of oxygen or perfusion of cytotoxic agents to intratumoral sites (Jain 2001). Abrogation of VEGF signaling increases collagenase IV activity, leading to restoration of normal basement membrane, which generally in tumors has an abnormally thickness.

Moreover, tumor vascular normalization is accompanied by increased pericyte coverage. It has been suggested that pericyte protects the endothelium against drugs (Cooke et al. 2012). In this context, an increase in pericyte coverage as a consequence of angiogenesis inhibition might induce a reduced sensitivity to the drug and acquired resistance.

Experimental models. Pericyte coverage promotes resistance through direct support, or paracrine

interactions with endothelial cells and tumor vessels covered by pericytes are less sensitive to VEGF blockade (Ribatti et al. 2011). Pericytes can activate compensatory PDGFR-mediated pro-angiogenic signaling under anti-VEGF therapy (Song et al. 2009). Combined treatment or pretreatment with anti-PDGF-B/PDGFR-β reducing pericyte coverage increases the success of anti-VEGF treatment in the mouse RIP1-TAG2 model (Bergers et al. 2003). However, extensive regression of endothelial cells was not observed in tumors after inhibition of PDGFR-β signaling (Abramsson et al. 2003). After treatment of RIP1-TAG-2 tumors and Lewis lung carcinomas with VEGF-Trap, surviving pericytes may become more tightly associated with endothelial cells or have no apparent association with tumor vessels (Inai et al. 2004). Treatment of RIP1-TAG2 tumors with anti-PDGFR-β antibody reduces pericytes, increases endothelial cell apoptosis, but does not seem to reduce tumor vascular density (Song et al. 2005). Treatment with a DNA oligonucleotide aptamer (AX102) that selectively binds PDGF-B leads to progressive reduction of pericytes in Lewis lung carcinomas (Sennino et al. 2007). Tumors in platelet-depleted mice show diminished pericyte recruitment, resulting in reduced blood vessel density, maturation, and perfusion (Li et al. 2014).

Clinical evidence. VEGFR-2 blockade can lead to the upregulation of angiopoietin-1 (Ang-1) that increases pericyte coverage of the vessels (Winkler et al. 2004). In glioblastoma patients, the Ang-1/Ang-2 ratio correlates with survival (Sie et al. 2009) and vascular normalization, whereas high Ang-2 levels correlate with resistance to anti-VEGF therapy (Batchelor et al. 2010). Blockade of VEGF signaling with the TKI cediranib significantly reduced levels of Ang-2 in the same patients (Batchelor et al. 2010). Ectopic expression of Ang-2 had no effect on vascular permeability, tumor growth, or survival, but it resulted in higher vascular density, with dilated vessels and reduced mural cell coverage (Chae et al. 2010). When combined with anti-VEGFR-2 treatment, Ang-2 destabilized vessels and compromised the survival benefit of VEGFR-2 inhibition by increasing vascular permeability. This suggests that VEGFR-2 inhibition normalized tumor vasculature, whereas ectopic expression of Ang-2 diminished the beneficial effects of VEGFR-2 blockade by inhibiting vessel normalization.

Inhibitors of the VEGF/VEGFR pathway used to treat malignancies of the central nervous system normalize tumor vasculature and decrease tumor interstitial pressure, leading to an improved access of cyto-reductive drugs and radiotherapy efficacy, due to an increased oxygen delivery (Mc Gee et al. 2010). However, these agents may also restore the low permeability characteristics of normal brain microvasculature, counteracting beneficial effects.

Vascular normalization may change the immune response. Inhibitors of VEGF signaling and of prostaglandin E2 suppress Fas ligand expression in tumor endothelial cells, resulting in infiltration of $CD8^+$ T cells (Motz et al. 2014).

Hypoxia

Tumors are hypoxic in spite of high vascularization due to the poor structure and functionality of tumor blood vessels. Hypoxia in tumors develops in the form of chronic hypoxia resulting from long diffusion distances between perfused tumor vessels and loci of acute hypoxia, resulting from transient collapse of tumor vessels. Abnormal tumor vasculature reduces blood flow tumor sites, hindering the delivery of chemotherapeutic drugs and favoring hypoxic microenvironment which, in turn, induced the upregulation of pro-angiogenic factors (Semenza 2014). Moreover, hypoxia mediates immune cell recruitment, and these cells concentrate at the tumor periphery, while in the tumor core, hypoxia provides an aggressive selection for cancer stem cells (CSCs) (Semenza 2014).

Hypoxic areas of tumors are refractory to chemotherapy and radiotherapy and contribute to select tumor cell populations able to escape to metastatic sites and pro-angiogenic CSCs (Blagosklonny 2001, 2004). The improvement in tumor oxygenation seems to last 2–4 days after anti-VEGF treatment (Jain 2013). At later times, increased tumor hypoxia has been reported after

bevacizumab treatment (Keunen et al. 2011). VEGF blockade aggravates hypoxia, which in turn upregulates the production of angiogenic factors or increases tumor cell invasiveness (Bergers and Hanahan 2008; Paez-Ribes et al. 2009). Hypoxia-induced expression of surface molecules in tumor endothelial cells directs mobilization of EPCs in growing tumor vessels (Moschetta et al. 2014).

Tumor cells respond to hypoxia by becoming tolerant and modifying their metabolic characteristics to resist to low oxygenation (Rapisarda and Melillo 2009), selecting more invasive metastatic clones of cancer cells resistant to anti-angiogenic agents (Semenza 2014). Invasiveness is enhanced through the production of pro-migratory proteins, such as stromal cells derived factor-1 alpha (SDF-1α) and hepatocyte growth factor-scatter factor (HGF-SF) and pro-invasive extracellular matrix proteins (Finger and Giaccia 2010; Semenza 2014). Hypoxia in highly metastatic tumors may cause excessive VEGF production and gene instability in tumor endothelial cells (Taylor et al. 2010).

Recruitment of Inflammatory Cells and Immature Myeloid Cells

The most aggressive human cancers, including malignant melanoma, breast carcinoma, and colorectal adenocarcinoma, are associated with the recruitment of various inflammatory cells which are involved in therapy resistance, including macrophages, mast cells (Ribatti 2013), $CD11b^+$ $Gr1^+$ myeloid cells (myeloid-derived suppressor cells, MDSCs) (Shojaei et al. 2007), $Tie2^+$ monocytes (TEMs) (De Palma et al. 2005; 2009), tumor-associated fibroblasts (TAFs) (Raffaghello et al. 2015), and lymphocytes (Ding et al. 2011).

Tumors, refractory to anti-VEGF therapy, display an increased number of MDSCs (Shojaei et al. 2007), and MDSCs derived from these tumors stimulate tumor growth in the presence of anti-VEGF antibodies (Shojaei et al. 2007). TEMs contribute to resistance against anti-VEGF therapy (aflibercept or bevacizumab) and promote glioma cell invasiveness in the xenograft U-87 MG mouse glioma model (Gabrusiewicz et al. 2004). TAFs secrete PDGF-C, and neutralizing antibodies against PDGF-C ameliorate TAF-induced angiogenesis (Crawford et al. 2009). TAFs isolated from tumors, refractory to anti-VEGF therapy, could promote tumor growth of anti-VEGF-sensitive tumors during VEGF-targeted therapy (Crawford and Ferrara 2009). Inhibition of angiogenesis stimulates the infiltration of the subclass of $CD4^+$ T cells (Ding et al. 2011). TAFs-derived exosomes promoted chemoresistance of colon cancer cells upon treatment with 5-fluorouracil or oxaliplatin by increasing the CSC population (Hu et al. 2015). TAFs can induce tamoxifen resistance in MCF7 breast cancer cell line, and metformin can resensitize these cancer cells to tamoxifen (Martinez-Outschoorn et al. 2011). Inflammatory cells secrete other pro-angiogenic factors, including FGF-2, interleukin-8, -17 (IL-8, IL-17) and Ang-2 (Azam et al. 2010; Casanovas et al. 2005; Huang et al. 2010; Chung et al. 2013; Rigamonti et al. 2014). In particular, IL-17 induces the G-CSF-dependent recruitment of $CD11b^+Gr1^+$ immature myeloid cells (Chung et al. 2013). IL-8 secreted by bone marrow stromal cells (BMSCs) in multiple myeloma patients contributed to BMSC-induced NF-kB activity, responsible in turn of resistance to bortezomib (Markovina et al. 2010).

GM-CSF stimulates macrophages to produce soluble VEGFR-1 (sVEGFR-1), leading to sequestration of VEGF and subsequent inhibition of angiogenesis, tumor growth, and metastasis (Eubank et al. 2009). Moreover, GM-CSF promotes MDSC survival and renders these cells resistant to sunitinib (Fink et al. 2011).

Alternative Mechanisms of Tumor Vessel Formation

Other modes of tumor vascularization may be less sensitive to anti-angiogenic therapies. Intussusceptive microvascular growth (IMG) generates vessels more rapidly with a less metabolic demand as compared to sprouting angiogenesis

and is a strategy that tumors can use for rapid adaptation to milieu changes (Ribatti and Djonov 2012). IMG occurs in several tumors, including colon carcinoma, mammary carcinomas, melanoma, B-cell non-Hodgkin's lymphoma, and glioma (Crivellato et al. 2003; Djonov et al. 2001; Nico et al. 2010; Patan et al. 1996; Ribatti et al. 2005). Treatment of mammary carcinoma allografts with a TKI results in transient reduction in tumor growth rate with decreased tumor vascularization. After cessation of therapy, the tumor vasculature re-expands prevalently by IMG (Hlushchuk et al. 2008). In this context, anti-angiogenic therapy causes a switch from angiogenesis to IMG, representing an escape mechanism and accounting for the development of resistance. In the course of the so called "vasculogenic mimicry," blood vessels are generated without the participation of endothelial cells and independent of classical angiogenic factors, including FGF-2 and VEGF (Maniotis et al. 1999). Stimulation with VEGF does not enhance vasculogenic mimicry (van der Schaft et al. 2005), and vasculogenic mimicry might be dependent on CSCs (El Hallani et al. 2010).

Vascular co-option occurs in site of metastases or in densely vascularized organs. Tumor cells co-opt and grow as cuffs around adjacent vessels (Holash et al. 1999). Vessel co-option has been reported in liver metastases (Vermuelen et al. 2001), non-small cell lung cancer, and lung metastases (Pezzella et al. 1996, 1997). Co-opted vessels initiate an apoptotic cascade mediated by Ang-2 followed by regression of the vessels. Shortly after regression, hypoxic tumor cells expressing VEGF upregulate the angiogenic response (Holash et al. 1999). Treatment of glioma with a monoclonal antibody against VEGFR-2 induces co-option of quiescent cerebral vessels (Kunkel et al. 2001). Similar findings have been reported for cerebral melanoma metastases after treatment with the anti-angiogenic agent ZD6474 (Leenders et al. 2004). More recently, Kuczynski et al. (2016) demonstrated that co-option of liver vessels and not-sprouting angiogenesis drives acquired sorafenib resistance in hepatocellular carcinoma. CSCs secrete VEGF to stimulate tumor angiogenesis; tumor vasculature, in turn, supports CSC self-renewal and maintaining (Alvero et al. 2009). Moreover, CSCs recruit endothelial precursors involved in revascularization and tumor regrowth (Ribatti 2012). Treatment with sunitinib induces elevated plasma levels of SDF-1, potentially contributing to the development of resistance under anti-angiogenic treatment (Ebos et al. 2007). Antibody-mediated blockade of SDF-1, which abrogates EPC-endothelial cell binding can counteract drug resistance (Ceradini et al. 2004).

Orthotopic or subcutaneous injection of glioblastoma stemlike cells in immunocompromised mice generated large anaplastic tumor xenografts, showing a vessel wall formed by human endothelial cells derived from glioblastoma stemlike cells (Ricci-Vitiani et al. 2010). Postnatal vasculogenesis contribute to tumor vascular supply throughout endothelial precursor cells (EPCs), which migrate from the bone marrow and differentiate in the stromal environment of tumors (Asahara et al. 1999).

Genomic Instability of Tumor Endothelial Cells and Increase of Metastatic Potential

Until recently, tumor endothelial cells were believed to be genetically stable. However, they are different from normal endothelial cells (Table 3) and may also be heterogeneous among organs or tumor types. The heterogeneity of tumor endothelial cells may be dependent on the tumor microenvironment, tumor stage, or treatment progress.

Table 3 Differences between normal and tumor blood vessels

Normal blood vessels	Tumor blood vessels
Hierarchical branching pattern	Unorganized branching pattern
Pericyte coverage	Tortuous vessels
Polarized	Abnormal basement membrane
Quiescent endothelial cells	Loose of pericytes
	Loose of endothelial cell interconnections
	Leaky vessels
	High interstitial fluid pressure (IFP)

Colorectal cancer endothelial cells overexpress specific transcripts as compared to endothelial cells of the normal colorectal mucosa (St Croix et al. 2000). A distinct gene expression pattern related to extracellular matrix and surface proteins characteristic of proliferating and migrating endothelial cells has been demonstrated in glioma and invasive breast carcinoma (Madden et al. 2004; Parker et al. 2004) . Moreover, endothelial cells isolated from various tumors acquired genotype alterations (Hida et al. 2004). Proximity of tumor cells and endothelial cells within the tumor microenvironment may be responsible for the genotype alterations (Gabrusiewicz et al. 2004; Hida and Klagsbrun 2005). Renal carcinoma endothelial cells are resistant to vincristine (Bussolati et al. 2003), while hepatocellular carcinoma endothelial cells are resistant to 5-fluorouracil and adriamycin (Xiang et al. 2009; Akiyama et al. 2012).

Inhibition of the VEGF/VEGFR signaling pathway may exert potential metastasis-promoting effects. Short-term treatment with sunitinib prior to intravenous inoculation of breast and melanoma cells could accelerate metastasis and short survival, despite cessation of treatment (Ebos et al. 2009). Moreover, sunitinib increases metastasis in orthotopic mouse models of breast and colon cancer (Shojaei et al. 2012). Increased invasiveness might result from enhanced expression of VEGF and PlGF or recruitment of EPCs that promote the formation of a pre-metastatic niche (Ebos et al. 2007). Moreover, hypoxia generated by angiogenesis inhibition triggers pathways that make tumors more aggressive and metastatic and less sensitive to anti-angiogenic treatment (Ebos et al. 2009). Finally, VEGF-targeted therapy can allow an epithelial-mesenchymal transition, which could in turn promote increased invasion and metastasis (Lu et al. 2012).

Conclusion

Anti-angiogenic treatment induces a reactive resistance which is mediated by the HIF/VEGF pathway, allowing both endothelial and cancer cells to resist to therapy (Blagosklonny 2005). Resistance to VEGF pathway inhibitors involves different mechanisms, including normalization of tumor blood vessels, alternative mechanisms of vessel formation, hypoxia, recruitment of inflammatory cells, and immature myeloid cells. All of these mechanisms deserve further investigation both in animal models and in humans to clarify their significance and importance.

VEGF blockade aggravates tumor hypoxia, which upregulates the production of other angiogenic factors in the tumor microenvironment. In this context, targeting VEGF and other pathways implicated in angiogenesis should result in more effective tumor growth inhibition. Moreover, more rational combinations of anti-angiogenic agents based on pharmacokinetic and pharmacodynamic data are needed to overcome resistance, and it is extremely important to determine the optimal duration and scheduling of anti-VEGF agents. The importance of the time interval of the normalization effects of anti-angiogenesis, the so-called window of normalization, has been underlined (Weissleder 2002). Metastatic effects of preclinical anti-angiogenic therapy with an antibody-targeting mouse VEGFR-2 are prevented by concurrent chemotherapy (Paez-Ribes et al. 2015). The identification of specific predictive biomarkers (Table 4) remains an important endpoint even if biomarkers that are predictive of anti-VEGF therapy may be specific to different tissues and tumor subtypes.

Table 4 Biomarkers to predict response to angiogenesis inhibitors

Functional imaging [dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI); positron emission tomography (PET)]
Hypertension
Circulating proteins (baseline plasma VEGF concentration; baseline plasma levels or treatment-induced changes in PlGF, soluble VEGFR-2)
Circulating cells (circulating endothelial cells; circulating tumor cells)
Single nucleotide polymorphisms (SNPs)
Tumor biomarkers (tumor vascularity; VEGF pathway components; markers of tumor cells, endothelial cells, and inflammatory cells)

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Cross-References

- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Biomarkers for Anti-angiogenic Therapy
- Mechanisms of Anti-angiogenic Therapy
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part IX

Mechanisms of Synergy in Combinations of Anti-angiogenics and Other Targeted Therapies

Cytotoxics and Anti-angiogenics: Metronomic Therapies

Andreas Pircher, Normann Steiner, and Eberhard Gunsilius

Contents

Introduction 328
The Combination of Cytotoxic Drugs and Anti-Angiogenic Compounds 328
Metronomic Chemotherapy 330
Resistance to Metronomic Chemotherapy 340
Future Directions 340
Cross-References 341
References 341

Abstract

A vascular bed brings nutrients and oxygen to malignant tumors and is a prerequisite for their growth and spread throughout the body. There are many compounds that can inhibit angiogenesis in cancer. Twenty years ago, the anti-angiogenic effects of conventional cytotoxic drugs have been described, leading to a plethora of preclinical and clinical evaluations of virtually all known chemotherapeuticals. The anti-angiogenic effects are observed at much lower doses than conventionally given and the concept of anti-angiogenic chemotherapy or metronomic chemotherapy evolved, i.e., the administration of low doses of cytotoxic agents over a prolonged period instead of maximum tolerated doses in repeated cycles in conventional chemotherapeutic regimens. Here, we will focus on the principles of the anti-angiogenic effects of chemotherapeuticals, the combination of anti-angiogenic compounds with conventional chemotherapeutic agents and metronomic chemotherapy, including a compilation of the clinical effects in patients with cancer.

Keywords

Anti-angiogenesis · Cytotoxic drugs · Metronomic chemotherapy · Advanced cancer · Vessel-normalization · Palliative cancer therapy

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Introduction

Despite recent advantages in the treatment of malignancies, e.g., the development of small-molecules that target specific intracellular pathways or monoclonal antibodies specific for tumor-associated surface molecules, systemic chemotherapy with cytotoxic agents remains a standard of care in most patients with cancer, even if metastasized or in a curative setting.

Usually, cytotoxic drugs are given as a single agent or in combination regimens at the maximum tolerated dose that was established in phase-I clinical trials to kill cancer cells as much as possible. Due to toxicities, also in healthy tissues as the bone marrow or mucous membranes, prolonged breaks lasting several weeks must be made between the chemotherapy cycles, allowing the regeneration of normal tissues. These treatment-gaps allow the tumors to regrow and to acquire additional genomic changes leading to resistant clones.

Elucidating the indispensable role of the vascular support for the maintenance and the growth of malignant tumors, Judah Folkman in 1971 proposed treatment strategies that target not only the tumor cells but aiming at the tumor-supportive microenvironment, especially the tumor vasculature (Folkman 1971).

Dysregulation of angiogenesis is found in most cancer types and plays an important role in invasion, metastasis, and cancer progression (Folkman 2007). Various proangiogenic factors and their associated receptors, such as vascular endothelial growth factor (VEGF and VEGFR), platelet-derived growth factor (PDGF and PDGFR), fibroblast growth factor (FGF and FGFR), and angiopoietins are essential for angiogenesis and tumor growth. Therefore, different anti-angiogenic treatments targeting directly VEGF signaling or VEGF-independent pathways, inhibition of the mammalian target of rapamycin (mTOR)-signaling, inhibition of multiple protein kinases, and inhibition of the angiopoietin pathways, as well as vascular targeting agents or vascular disrupting agents (VDA), are used and/or in development in modern strategies to fight cancer with the aim to cause a rapid and selective shutdown of the established tumor vasculature, thereby leading to secondary tumor-cell death and necrosis of the tumor.

On the other hand, it was discovered already in the 1980s that conventional chemotherapy, even "old-fashioned" drugs as cyclophosphamide or mitoxantrone, can exert anti-angiogenic activities when administered in appropriate schedules.

In this chapter, we will mainly focus on the anti-angiogenic effects of established cytotoxic agents in combination with "classical" anti-angiogenics as well as their use as anti-angiogenics by administering low doses over prolonged time, a strategy that is called metronomic chemotherapy.

The Combination of Cytotoxic Drugs and Anti-Angiogenic Compounds

The vessel network in malignant tumors is structurally and functionally abnormal, with malformations as arterial-venous shunts, atypical branching, meandering, narrowing, and dilatations leading to chaotic blood flow. Their coverage by pericytes is chaotic and there are gaps between the endothelial cells. The penetration of cytotoxic drugs into malignant tumors can be additionally hindered by a low vascularization by such abnormal vessels leading to hypoxic and acidic regions and the presence of a high interstitial pressure. As nutrient deficient, hypoxic and acidic areas can turn on resistance mechanisms, the efficacy of cytotoxic drugs is not only hampered by suboptimal concentrations within the tumor tissue but also by the fact that regions of resistant cells within the tumor tissue are present (Minchinton and Tannock 2006).

One might have the assumption that anti-angiogenic treatment reduces further the already altered blood flow within the tumor tissue, thereby limiting the efficacy of systemically delivered cytotoxic drugs. However, anti-angiogenic compounds can induce a process called vascular normalization, where the tumor vasculature regains, at least in part, a normal function (Jain 2013). This process leads to better penetration of cytotoxics into the tumor tissue also by reducing the high interstitial pressure.

As an example, it has been shown in an animal model that treatment with bevacizumab, a monoclonal antibody against VEGF, can decrease vessel permeability and the interstitial pressure leading to an increase of the blood flow in the tumor tissue for one week (Dickson et al. 2007). This "window of opportunity" allowed increased penetration of the cytotoxic agents topotecan and etoposide into the tumor when they were administered 1 or 3 days after bevacizumab, but not if they were given simultaneously or after seven days, indicating that treatment strategies combining cytotoxics and anti-angiogenic compounds must be appropriately designed. Keeping in mind the spatial heterogeneity of the tumor vasculature there is room to speculate that this combined strategy works not in all areas of malignant tumors.

Direct evidence of vascular normalization was reported in patients with rectal adenocarcinoma receiving bevacizumab infusions prior to their subsequent tumor treatment. Using functional computed tomography, a substantial and significant decrease in tumor blood perfusion was found (Willett et al. 2004). Correspondingly, a significant reduction in microvessel density was found in all five patient's tumors and in 4/4 analyzed patients the interstitial fluid pressure (IFP) declines from 15 +/ 2 mmHg to 4 +/− 2.2 mmHg. Moreover, an increase in vessels positive for α-smooth muscle actin indicated vessel normalization.

In clinical trials, anti-angiogenic drugs such as bevacizumab have shown little activity against malignant tumors when administered as a single treatment. However, when given in combination with standard cytotoxic drugs, a significant improvement in the clinical outcome of patients was observed in various cancer types as colorectal cancer, non-small cell lung cancer, cervical cancer, advanced breast cancer, and ovarian cancer. Side effects are moderate with proteinuria and hypertension. Rarely, bowel perforation was reported. The development and the use of bevacizumab as the prime example of successful combination of an anti-angiogenic with conventional chemotherapy is comprehensively reviewed by Napoleone Ferrara (Ferrara and Adamis 2016).

In a pivotal randomized phase-III trial in patients with metastatic colorectal cancer, the addition of bevacizumab to standard chemotherapy (irinotecan, 5-fluorouracil, leukovorin) resulted in a 10% higher response rate and significantly prolonged progression free survival and overall survival (Hurwitz et al. 2004). This beneficial effect of adding bevacizumab to chemotherapy was confirmed in other clinical trials and is now considered a standard of care.

Also, the addition of the monoclonal antibody ramucirumab, which binds to the extracellular domain of the VEGFR2, to standard chemotherapy significantly prolonged progression free (PFS) and overall survival (OS) in patients with colorectal cancer when given as second-line treatment (Tabernero et al. 2015). The combination of ramucirumab and conventional chemotherapy resulted in improved PFS and OS in randomized phase-III studies in patients with advanced gastric cancer. Significant positive effects were also observed in randomized trials in patients with non-small cell lung cancer, metastatic renal cell carcinoma, advanced ovarian cancer, and malignant brain tumors (for details see (Ferrara and Adamis 2016)).

Aflibercept, a fusion protein with high affinity to VEGF (VEGF-trap), given in combination with irinotecan, 5-fluorouracil and leukovorin as a second-line treatment after failure on a oxaliplatin-based regimen, resulted in superior response rates and prolonged PFS and OS significantly in patients with colorectal cancer (Van Cutsem et al. 2012).

The benefit of combining anti-angiogenics with conventional chemotherapy compared to chemotherapy alone usually lies in the range of several months, indicating that especially in metastatic cancer there is a huge unmet need for further effective treatment strategies. Moreover, malignant tumors frequently acquire resistance during therapy against both, classical cytotoxic agents and anti-angiogenics, respectively. Intriguingly, experiments using chemotherapy resistant mouse tumors revealed that, if the same cytotoxics are given in much lower doses over a prolonged period, the resistance disappeared. This is attributed to the fact that, regarding conventional-dose

chemotherapy, in the treatment interval that is necessary to allow the regeneration of normal tissues and that takes about three weeks, also the damaged endothelium within tumor vessels regenerates. This is not the case if lower doses of the drug are administered continuously (Browder et al. 2000). This strategy is termed metronomic-chemotherapy (Hanahan et al. 2000).

Metronomic Chemotherapy

Endothelial cells are one of the most quiescent cell compartments in the body and rarely divide under physiological conditions (Eelen et al. 2015). However, endothelial cells can adapt to their microenvironment and malignant tumors can induce endothelial cell activation via upregulation of various endothelial cell growth factors and cytokines, which lead to changes in the endothelial cell phenotype (Dudley 2012). Thereby, tumor endothelial cells (TEC) are characterized by a hyper-proliferative and hyper-motile state (Hida et al. 2016a, b). Nevertheless, most of the preclinical studies testing the influence of chemotherapeutics on endothelial cells were performed on human umbilical vein endothelial cells (HUVEC) and not on isolated TEC (Montiel et al. 2009). Therefore most of the studies deduce the effect from HUVEC to TEC and only a few studies tested cytotoxic and anti-angiogenic drugs on isolated TEC (Hida et al. 2016a). Theoretically, the proliferative phenotype of TEC makes them more sensitive to standard cytotoxic drugs than resting endothelial cells. Unfortunately, TEC from animal tumors, especially from highly metastatic cancers, showed resistance to standard chemotherapeutics (Ohga et al. 2012). However, anti-angiogenic activity of cytotoxic drugs was proven at substantial lower doses as required for the cytotoxic activity on cancer cells.

There is a high correlation of several standard chemotherapeutics with vascular complications as thrombosis, hypertension, atherosclerosis, and vascular damage. It is thought that these vascular complications are undesired "off target effects" of the used chemotherapeutics and induced by the inhibition of important pathways required for normal vascular function. Platinum compounds are used in the treatment of many forms of cancer and they are well known to induce vascular thrombosis. The pathophysiological mechanisms are not completely understood but impaired endothelial function, vascular and renal damage, and oxidative stress might play an important role. Key features of endothelial dysfunction induced by chemotherapeutics are loss of vasoreagibility (leading to hypertension) by decreased endothelial nitric oxide (NO) bioavailability and induction of inflammation and endothelial cell activation (being potentially pro-thrombogenic).

Cytotoxic anticancer drugs are conventionally administered in a pulsatile manner at maximum tolerated doses (MTD) to induce cancer cell apoptosis. Thereby, higher doses of cytotoxics should induce more cancer cell death and eradicate the bulk tumor cell mass. A major handicap of MTD-guided therapy is that also healthy proliferating cells will be affected and toxicities occur, especially hematotoxicity, alopecia, and mucosal damage. These adverse effects limit the increase in dose and the necessary intervals between the treatment cycles of usually three weeks' duration (to allow regeneration of normal tissues) lead to the development of resistance mechanisms against standard cytotoxics. Moreover, chemotherapy administered in classical schedules causes mobilization of circulating endothelial progenitor cells that can promote the revascularization of the tumors, a phenomenon that does not occur if cytotoxic drugs are given at lower doses repeatedly (Bertolini et al. 2003). To avoid these unwanted effects of classical chemotherapy, prolonged treatment using much lower doses characterizes the concept of metronomic chemotherapy. Metronomic chemotherapy schedules include a more continuous administration of conventional cytotoxic agents at lower doses (subtoxic levels), whereby the cumulative dose over time often exceeds that of conventional 3-week regimens. In contrast to conventional chemotherapy, the primary goal of metronomic chemotherapy is not the destruction of the tumor cell compartment directly but to inhibit

tumor endothelial cells within the cancer vasculature, thereby cutting of tumors from their blood supply (Fig. 1) (Polverini and Novak 1986; Bocci et al. 2002).

In women with advanced breast cancer treated with metronomic cyclophosphamide and methotrexate (with or without thalidomide), the number of circulating endothelial cells (CEC) was significantly lower in patients with no clinical benefit to metronomic chemotherapy whereas an CEC count >11 cells/microliter after two months of treatment was associated with a longer progression free and overall survival. The increase in CEC was mostly due to an increased fraction of apoptotic CEC, as a result of the effect of the metronomic chemotherapy on the tumor vasculature (Mancuso et al. 2006).

An additional anti-angiogenic mechanism of metronomic chemotherapy is the inhibition of the transcription factor HIF-1α. It has been shown that continuous low doses of anthracyclines as doxorubicin and daunorubicin inhibit the transcription of HIF-1α target genes resulting in a reduced tumor vascularization in an animal model (Kim et al. 2013).

One of the best investigated chemotherapeutic drug showing a dual role either by its anti-angiogenic activity or by its cytotoxic activity against cancer cells is paclitaxel (Cesca et al. 2013; Belotti et al. 1996). Paclitaxel is a tubulin-binding drug, which leads to microtubule polymerization and finally inhibits mitosis due to impairment of microtubule functions. Paclitaxel normalizes vessel function in preclinical models by increasing vascular perfusion and vascular permeability (Moschetta et al. 2012). Finally, the effects of paclitaxel lead to increased drug uptake in the tumor tissue. Another mechanism is that taxanes can decompress tumor

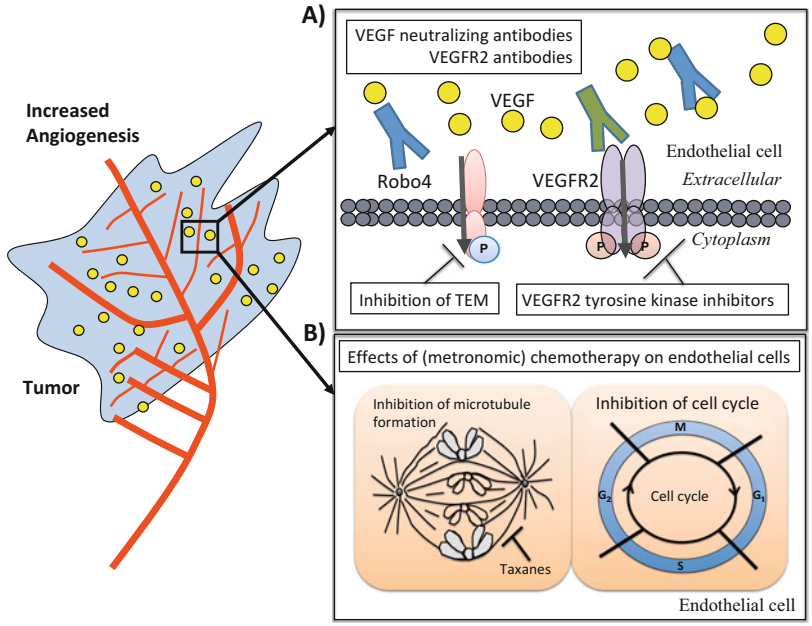

Fig. 1 Inhibition of tumor angiogenesis with anti-angiogenics and cytotoxics. Tumors show increased angiogenesis and form chaotic vascular networks (depicted on the *left side*). Furthermore, hypoxia leads to the upregulation of angiogenic growth factors (*yellow dots*). (**a**) Concept of traditional anti-angiogenic drugs by inhibition of vascular endothelial growth factor (VEGF) signaling. Monoclonal antibodies either neutralize VEGF extracellularly or inhibit the binding of the VEGFR2. Furthermore, small molecules can block the intracellular VEGFR2 signaling pathway. Inhibition of tumor endothelial markers (TEM) is an experimental approach to target tumor endothelail cells (TEC). (**b**) Standard chemotherapies given as metronomic treatment inhibit tumor angiogenesis either via blocking endothelial cell (EC) cytoskeleton formation (inhibition of microtubule formation) or inhibition of the cell cycle

vessels and therefore increase vessel surface and open compressed tumor vessels (Cesca et al. 2013; Taghian et al. 2005). The increased vessel surface and also the lowered IFP can then lead to a better tumor perfusion, which was reflected by a significant increase in albumin extravasation (Bronstad et al. 2004).

Preclinical models revealed that the combination of taxanes and anti-angiogenic therapies is highly effective and synergistic. Therefore, compounds were developed combining both VEGFR2 inhibition and concomitant tubulin inhibition (Gangjee et al. 2014). One member is 3-HCl, which combines both potent VEGFR2 inhibitory activity and potent anti-tubulin activity. In vivo, 3-HCl reduced tumor size and vascularity in xenograft models and in orthotopic tumor models. Furthermore, the activity of 3-HCl was superior to those of other standard drugs used for the tested tumor subtypes as temozolomide, docetaxel, and also sunitinib (Gangjee et al. 2014).

Metronomic chemotherapy has been shown to be a valid treatment option in a variety of malignant tumors (Romiti et al. 2013). It acts against cancer cells through immune-mediated effects and direct inhibition of tumor growth, as well as through the inhibition of vasculogenesis and angiogenesis (inhibition of endothelial cell proliferation, suppression of HIF-1, upregulation of TSP-1, inhibition of endothelial progenitor cells (EPC) mobilization, and homing) (Kareva et al. 2015; Gnoni et al. 2015). It shows promising tumor control rates and a lower incidence of adverse effects with considerable improvement in quality of life compared to conventional chemotherapy. Unfortunately, metronomic schedules (and its combinations) are very heterogeneous and results of randomized trials are scarce (Lien et al. 2013; Romiti et al. 2013). Metronomic chemotherapy can be administered as a primary treatment, as maintenance therapy after conventional, full-dose chemotherapy, or as a combination partner for anti-angiogenic compounds, targeted therapies, immunomodulatory strategies and anti-hormonal therapy, e.g., in breast cancer. Most commonly the following conventional drugs are in use for oral metronomic chemotherapy (Table 1):

Cyclophosphamide 50–100 mg/day: Cyclophosphamide was the most frequently used drug in preclinical studies (Penel et al. 2012). Metronomic cyclophosphamide as an alternative to conventionally scheduled chemotherapy (with an approximately tenfold higher dose) in advanced/metastatic breast cancer is commonly administered in combination with either methotrexate or capecitabine and vinorelbine-based schedules. Recent clinical studies provide evidence of efficacy and tolerability of metronomic oral cyclophosphamide and methotrexate as a maintenance therapy in patients with advanced triple-negative breast cancer (phase-III study) (Nasr et al. 2015) and ovarian cancer after clinical response to platinum and paclitaxel chemotherapy (El-Husseiny et al. 2016). In hormone receptor-negative early breast cancer, the International Breast Cancer Study Group Trial 22–00, a randomized phase III trial, found metronomic oral cyclophosphamide and methotrexate not to produce a significant reduction in DFS events. However, a benefit was again found in triple-negative, node-positive disease (Colleoni et al. 2016). In a randomized phase-II trial using letrozole (an aromatase inhibitor used for antihormonal treatment of breast cancer) with or without metronomic cyclophosphamide at a dose of 50 mg daily for 6 months in 114 elderly women with breast cancer, the addition of metronomic cyclophosphamide improved the response rate from 72% to 88% (Bottini et al. 2006).

Phase I/II trials and case reports on low-dose cyclophosphamide schedules are also available for hematological malignancies, particularly multiple myeloma (Zhou et al. 2014; Steiner et al. 2015a; Rueda et al. 2015; Suvannasankha et al. 2007; de Waal et al. 2015) and several solid tumors (Launay et al. 2016; Revannasiddaiah et al. 2015; Wang et al. 2015; Bandyopadhyay et al. 2014; Barroso-Sousa et al. 2015; Bojko et al. 2012; Mir et al. 2011; Kelley et al. 2013).

Trofosfamide is an orally available prodrug that is predominantly metabolized to ifosfamide. Usually it is given at doses of 50–400 mg/day. The standard administration schedule of trofosfamide per se follows the metronomic concept. Trofosfamide 50 mg/day with or without

Table 1 Summary of most common used drugs for oral metronomic chemotherapy

Treatment	Patient population	Number of points	Response rates	Response duration	Reference/type of study
Cyclophosphamide 50 mg/day and methotrexate 2.5 mg twice/day and twice a week	Maintenance after adjuvant chemotherapy in triple negative breast cancer	158	Overall distant metastasis recurrence rate 26%	OS 37 m, DFS 28 m	Nasr et al. (2015). Phase III
Cyclophosphamide 50 mg/day and methotrexate 2.5 mg twice/day twice weekly	Maintenance after adjuvant chemotherapy in hormone receptor negative breast cancer	1086	_	5-year DFS 78.1%; 5-year DFS in triple negative, node-positive subgroup 72.5%	Colleoni et al. (2016). International Breast Cancer Study Group Trial 22–00, Phase III
Cyclophosphamide 50 mg/day with or without letrozole	First-line treatment in elderly women	114	ORR 88% with cyclophosphamide vs. 72% with letrozole alone	5-year OS 65%	Bottini et al. (2006). Randomized phase-II trial
Cyclophosphamide 50 mg and methotrexate 2.5 mg	Maintenance in advanced ovarian cancer after platinum and paclitaxel	60	_	DFS 18 m	El-Husseiny et al. (2016). Prospective study
Low-dose oral cyclophosphamide and prednisone	Relapsed/refractory myeloma with cardiac insufficiency	56	CBR 63%	OS 8 m, PFS 6 m	Zhou et al. (2014). Prospective study
Cyclophosphamide 50 mg twice/day, thalidomide 200 mg/day and prednisone 50 mg every other day	Relapsed/refractory myeloma	37	ORR 62.9%	TTP 13.2 m	Suvannasankha et al. (2007). Phase II
Bortezomib twice weekly, cyclophosphamide 50 mg/day and dexamethasone	Relapsed/refractory myeloma	59	ORR 71%	OS 28.1 m, PFS 18.4 m	de Waal et al. (2015). Prospective study
Lenalidomide 20 mg/day and cyclophosphamide 50 mg/day	Relapsed/refractory Hodgkin lymphoma	46	ORR 38%, CBR 62%	OS 19 m, PFS 7 m	Rueda et al. 2015. Phase II
Lenalidomide 25 mg/day and cyclophosphamide 50 mg/day	Pretreated metastatic castration-resistant prostate cancer	19 (phase I) 6 (phase II)	>/= 50% reduction PSA: 31.7%. PR 17%, SD 68%	_	Wang et al. (2015). Phase I–II
Temozolomide 75 mg/m^2 per day from the first to the last day of radiotherapy	First-line in glioblastoma	573	_	OS 14.6 m	Stupp et al. (2005).
Temozolomide 50 mg/m^2/day	Recurrent glioblastoma	37	CBR (CR, PR, SD) 36%	OS 7 m, PFS 2 m. 1-year-OS 35%, 6-m PFS 19%	Omuro et al. (2013). Phase II

(*continued*)

Table 1 (continued)

Treatment	Patient population	Number of points	Response rates	Response duration	Reference/type of study
Sorafenib 400 mg twice/day and temozolomide 40 mg/m^2/day	Recurrent glioblastoma	43	PR 12%, SD 43%	OS 7.4 m, 6-m PFS 26%, TTP 3.2 m	Zustovich et al. (2013). Phase II
6-Mercaptopurine 75 mg/m^2/day	Palliative treatment in elderly acute myeloid leukemia	32	_	OS 6 m	Kapoor et al. (2016). Prospective study.
Capecitabine 650 mg/m^2 twice/day	First-line in advanced breast cancer	323	_	OS 22 m, PFS 6 m	Stockler et al. (2011). Prospective study
Capecitabine 500 mg twice daily	First-line in advanced and pretreated hepatocellular carcinoma	59 (not pretreated) 31 (pretreated)	_	OS 14.5 m, PFS 6 m (not treated), OS 9.8 m, PFS 3.3 m (pretreated)	Brandi et al. (2013). Phase II
Tegafur (S-1) 80–120 mg/m^2/day and leucovorin 25 mg/day in a 1-week-on/1-week-off schedule, and bevacizumab 5 mg/kg on day 1 of every 2-week	Refractory metastatic colorectal cancer	31	ORR 7%, DCR65%	OS 9.9, PFS 5.3 m	Yamaguchi et al. (2015). Phase II
Tegafur (S1) 200 mg/m^2/day and leucovorin 30 mg/m^2/day with biweekly oxaliplatin/infusional fluorouracil/leucovorin	Refractory metastatic colorectal cancer	28	PR 35.7%	OS 13.4 m, TTP 5.2 m	Lin et al. (2007). Prospective study
Irinotecan 60 mg/m^2 on days 1, 8, and 15 every 4 weeks and tegafur (S1) 80 mg/m^2 on days 3–7, 10–14, & 17–21 every 4 weeks	Pretreated metastatic breast cancer	40	ORR (CR, PR) 47%, SD 50%	OS 26 m, PFS 14 m. 1-year-OS 79.3%	Otsuka et al. (2015). Phase II
Sorafenib 400 mg twice/day &tegafur (S1) 125 mg/m^2 twice/day	First-line in advanced hepatocellular carcinoma	53	PR 8%, SD 49%	OS 7.4 m, PFS 3.7 m	Hsu et al. (2010). Phase II
Vinorelbine 70 mg/m^2, fractionated on days 1, 3, and 5, for 3 weeks on & 1-week off	Elderly metastatic breast cancer	34	CR 6%, PR 32%	OS 15.9 m, PFS 7.7 m	Addeo et al. (2010). Phase II
Capecitabine 500 mg thrice/day continuously and vinorelbine 40 mg thrice/week	Pretreated metastatic breast cancer	32	CBR (CR, PR, SD) 58.1%	_	Cazzaniga et al. (2014). Phase I–II

Vinorelbine 80 mg/m^2 on days 1, 8, 15 and trastuzumab 6 mg/kg on day 1 every 3 weeks or 4 mg/kg weekly	First-line in advanced HER2+ breast cancer	26	ORR 56%, CR 12%, PR 44%, SD 32%, CBR 88%	OS 27.9 m, PFS 6.7 m, DOR 7.1 m	Farhat et al. (2016). Phase II
Vinorelbine 60 mg/week, 90 mg/week, or 120 mg/week, respectively, and sorafenib at 200 mg–800 mg twice/day for 4 weeks	Advanced non-small cell lung cancer	48	PR 8.9%	_	Tan et al. (2015). Prospective study
Cisplatin 80 mg/m^2 and vinorelbine 60 mg every other day	First-line in advanced non-small cell lung cancer	41	PR 37.1%, SD 28.6%	OS 12.0 m, PFS 4.2 m. 1-year-OS 52.6%	Katsaounis et al. (2015). Phase II
Etoposide 25 mg twice/day and prednisone 5 mg twice/day	Pretreated metastatic prostate cancer	39	_	PFS 5.9 m	Zhu et al. (2014). Phase II
Ketoconazole 200 mg thrice/day and cyclophosphamide 50 mg twice/day for sevenconsecutive days during weeks 1, 3, and 5, and etoposide 50 mg twice/day in combination with estramustine 140 mg twice/day administered on alternate weeks plus prednisone 10 mg/day	Chemo naïve metastatic prostate cancer	17	>/= 50% reduction, PSA: 59%	_	Jellvert et al. (2011). Prospective study
Cisplatin 30 mg/m^2, days 1–3, etoposide 50 mg days 1–15 and bevacizumab 5 mg/kg, day 3	Advanced non-small cell lung cancer	45	PR 68.8%, SD 17.8%	PFS 9.5 m	Correale et al. (2011). Phase II
Continuous oral celecoxib, thalidomide, and fenofibrate, with alternating 21-day cycles of low-dose cyclophosphamide and etoposide	High-grade glioma, ependymoma, low-grade glioma, bone tumors, medulloblastoma/primitive neuroectodermal tumor, leukemia, neuroblastoma, and miscellaneous tumors	101	CR 1%, PR 12%, SD 35%	_	Robison et al. (2014). Phase II

OS overall survival, *PFS* progression free survival, *DFS* disease free survival, *CBR* clinical benefit rate, *DCR* disease control rate, *DOR* duration of response/remission, *TTP* time to progression, *EFS* event free survival, *m* month, *ORR* overall response rate, *PSA* prostate specific antigen, *CR* complete response, *PR* partial response, *SD* stable disease, *MR* minor response, *m* months, – not reported

docetaxel showed activity and good tolerability as second-line therapy in patients with metastatic non-small cell lung carcinoma (NSCLC) (Gorn et al. 2008; Reissig and Walther 2013) and 100 mg/day were found to demonstrate a durable activity in a patient with metastatic castration-resistant prostate cancer after docetaxel therapy (Greiner et al. 2010). Low-dose trofosfamide treatment showed promising results in heavily pretreated women with ovarian cancer (Gunsilius et al. 2001).

Temozolomide, an analog of dacarbazine is given continuously at doses of 50–75 mg/m^2/day. The addition of metronomic temozolomide to radiotherapy resulted in a significant survival benefit with minimal additional toxicity in newly diagnosed glioblastoma in a randomized study (Stupp et al. 2005). Even patients with glioblastoma, not eligible for standard treatment (Kerschbaumer et al. 2015) and patients with recurrent malignant glioma benefit from low-dose temozolomide only or combined with celecoxib (an nonsteroidal anti-inflammatory drug) or sorafenib (a multi-kinase inhibitor) (Omuro et al. 2013; Zustovich et al. 2013). In a reported case of a patient with metastatic poorly differentiated pancreatic neuroendocrine carcinoma, palliative temozolomide 75 mg/m^2/day/one-week-on/on-week-off led to remission and significant clinical benefit (De Divitiis et al. 2016).

Methotrexate inhibits DNA and RNA synthesis by its binding to the enzyme dihydrofolate reductase. Conventionally given in doses of several grams per square meter body surface area, in metronomic schedules only 2.5–5 mg twice weekly are given (Colleoni et al. 2016; Gebbia et al. 2012), usually in combination with low-dose cyclophosphamide and shows substantial effects in recent phase-III studies as maintenance therapy for advanced metastatic breast and ovarian cancer (Nasr et al. 2015; El-Husseiny et al. 2016). Using another antimetabolite, 6-mercaptopurine at 75 mg/m^2/day, Kapoor et al. found it an attractive metronomic treatment option in elderly acute myeloid leukemia patients who are not suitable for aggressive chemotherapy (Kapoor et al. 2016).

Capecitabine is a prodrug that is activated by tumor cells to 5-fluorouraciland is frequently used in second or higher line of treatment in breast cancer, but also it is an option for first-line treatment in selected patients (Banys-Paluchowski et al. 2016). The standard administration of capecitabine is an oral regimen consisting of daily capecitabine at a dose of 2500 mg/m^{2}over two weeks followed by one week of rest. The optimal dose for the metronomic use of capecitabine has not been defined yet. Stockler et al. found no significant differences with respect to survival, tumor response, and toxicity between standard dose and metronomic (1300 mg/m^2/day without breaks) capecitabine in first-line treatment of patients with advanced breast cancer. Survival and safety in both cohorts were better than in a classical schedule containing cyclophosphamide, fluorouracil and methotrexate (Stockler et al. 2011). As metronomic maintenance therapy, capecitabine in combination with cisplatin showed encouraging anti-tumor activity in anthracycline- and taxane-pretreated HER-2 negative metastatic breast carcinoma patients that usually have a dismal prognosis (Ozdemir et al. 2013). In retrospective analyses and phase-I studies, capecitabine salvage chemotherapy (1500–1700 mg/day) for upper gastrointestinal tract cancer and recurrent colorectal cancer was effective and well tolerated (Roberto et al. 2016; Deenen et al. 2015; Romiti et al. 2015), and 500–1000 mg/day showed anti-tumor activity in a subgroup of patients with hepatocellular carcinoma pretreated with sorafenib (Granito et al. 2015; Brandi et al. 2013).

Tegafur is another oral applicable prodrug of 5-fluorouracil that has been developed as a replacement for infusional 5-fluorouracil therapy. The standard regimen of its administration is 300 mg/m^2/day over four weeks followed by a one-week rest in metastatic colorectal carcinoma. Metronomic regimes with tegafur are heterogeneous in doses and indication. Phase I/II studies and retrospective analyses with tegafur doses between 80–250 mg/m^2/day, combined with other agents, showed promising results for advanced/metastatic colorectal carcinoma (Yamaguchi et al. 2015; Lin et al. 2007) and advanced hepatocellular carcinoma (Hsu et al. 2010), as well as for other solid tumors as oral

squamous cell carcinoma, gastric cancer, and advanced breast cancer (Zhong et al. 2015; Otsuka et al. 2015; Lin et al. 2015).

Vinorelbine is the only orally available microtubule-targeting agent and has emerged to a promising metronomic treatment (first-line and maintenance therapy) in elderly or previously treated patients with advanced metastatic breast cancer (Addeo et al. 2010; Cazzaniga et al. 2014; De Iuliis et al. 2015; Farhat et al. 2016) and advanced metastatic non-small-cell lung cancer (Tan et al. 2015; Sutiman et al. 2016; Elharrar et al. 2016; Katsaounis et al. 2015).

Etoposide is a topoisomerase-II inhibitor and low-dose treatment regimen with cyclophosphamide and etoposide has been shown to be an effective strategy in heavily pretreated patients with metastatic breast cancer and recurrent advanced and platinum-resistant ovarian cancer with an 18% rate of partial response and an impressive progression free survival of ten months (Mutlu et al. 2015; Uysal et al. 2014; Kucukoner et al. 2012). Moreover, oral etoposide shows activity in hormone-resistant prostate cancer and advanced non-small cell lung cancer (Zhu et al. 2014; Jellvert et al. 2011; Correale et al. 2011). Even in advanced malignancies in children, metronomic chemotherapy plays an important role as it can produce responses without significant toxicities (Robison et al. 2014; Felgenhauer et al. 2013; Zapletalova et al. 2012).

Although the oral administration of drugs is far more convenient for the patients, some potent cytotoxic drugs are only available for intravenous treatment, of which the following commonly can be used for metronomic schedules (Table 2):

Cisplatin: Metronomic schedules of cisplatin and its combinations were tested mostly in phase I/II non-randomized trials. Combination chemotherapy with 5-fluorouracil and metronomic cisplatin (5 mg/m^2/day on days 1–4 and 8–11 every 3 weeks) was well tolerated and showed activity in advanced gastric cancer with malignant bowel obstruction. Before treatment 69% of the patients could not eat due the bowel obstruction and after three cycles of treatment this was the case in only 15%, indicating a substantial palliative potential of this regimen (Yang et al. 2016). In advanced hepatocellular carcinoma, a metronomic schedule with cisplatin (15 mg/m^2) and 5-fluorouracil (50 mg/m^2) every week for 3 weeks followed by a one-week rest, resulting in a 20% rate of partial responses in this hard to treat malignancy (Woo et al. 2012). Metronomic schedules of cisplatin are effective also in other malignancies, see Table 2 (Gupta et al. 2016; Caro et al. 2016; Kumar et al. 2015).

The standard administration of **gemcitabine**, which is used in the treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer, and breast cancer is 1000–1250 mg/m^2 intravenously once a week. Demirci et al. found gemcitabine 50 mg/m^2 once weekly and concomitant radiotherapy a valuable treatment option with a low toxicity profile for patients with muscle invasive bladder cancer not eligible for surgery (Demirci et al. 2015). In phase-II studies, gemcitabine 250 mg/m^2 combined with cisplatin was given as a metronomic schedule for malignant pleural mesothelioma and this regimen produced a complete response in 5% of the patients and partial response in impressive 45%. The median overall survival was 17 months which is comparable to that of conventional aggressive regimens (Kovac et al. 2012). The same dose of gemcitabine with concurrent radiotherapy was given in advanced pancreatic cancer and resulted in a partial response of the tumor in 27% of the patients (Shibuya et al. 2011).

Taxanes show a dual role either by their anti-angiogenic activity and their cytotoxicity against tumor cells (Cesca et al. 2013). Treatment with weekly paclitaxel and carboplatin was found to be safe and efficacious in women with ovarian cancer who are ineligible for standard dose paclitaxel and carboplatin chemotherapy schedules and resulted in a response rate of 100%, here in a neoadjuvant setting (Dessai et al. 2016). In the treatment of metastatic breast cancer, weekly low-dose paclitaxel or three-weekly docetaxel are among the cornerstones of treatment (Smyth et al. 2016; Biganzoli et al. 2016). Metronomic paclitaxel has shown activity also in previously untreated advanced non-small cell lung cancer (Takeshita et al. 2014), and as a second-line agent in relapsed small cell lung cancer (Noronha et al. 2016).

Table 2 Summary of most common used drugs for intravenous metronomic chemotherapy

Treatment	Patient population	Number of points	Response rates	Response duration	Reference/type of study
5-Fluorouracil 300 mg/m^2/day on days 1–5 and 8–12 and cisplatin 5 mg/m^2/day on days 1–4 and 8–11 every 3 weeks	Advanced gastric cancer with inoperable malignant bowel obstruction	26	_	OS 182 days, DOR from malignant bowel obstruction 105 days. 3-m-OS 69.2%, 6-m-OS 53.8%	Yang et al. (2016). Prospective study
Epirubicin (to hepatic artery) 30 mg/m^2 every 4 weeks and cisplatin 15 mg/m^2 and 5-fluorouracil 50 mg/m^2 every week	Palliative treatment in advanced HCC with major portal vein tumor thrombosis	30	PR 20.0%, SD 20.0%	OS 162 days, TTP 63 days	Woo et al. (2012). Prospective study
Cisplatin 35 mg/m^2 once/week or 6 mg/m^2/day (on an outpatient basis) with radiotherapy	Advanced head and neck cancer (squamous cell carcinoma)	52	5-year-locoregional control 25%	5-year-OS 31%	Gupta et al. (2016). Prospective study
Short-course radiotherapy with cisplatin, 40 mg/m^2/week	Palliative treatment in advanced head and neck cancer (squamous cell carcinoma)	57	PR 50%, 6-m-locoregional control (CR+PR) 32.1%	OS 10.1 m, PFS 6.2 m	Kumar et al. (2015). Phase II
Gemcitabine 250 mg/m^2 prolonged infusion on days 1, 8 and cisplatin at 75 mg/m^2 on day 2 of a 3-week cycle	Malignant pleural mesothelioma	78	ORR 50%, CR 5.1%, PR 44.9% MR/SD 44.9%	OS 17.7 m, PFS 8 m. 1-year-OS 67.3, 2-year-OS 32.7, 3-year-OS 19.8%	Kovac et al. (2012). Phase II
Gemcitabine 250 mg/m^2/week with radiotherapy	Advanced unresectable pancreatic cancer	21	PR 28.6%, SD 71.4%	OS 16.6 m, 1-year-OS 74%	Shibuya et al. (2011). Phase II
Alternating cycles of ifosfamide-etoposide and vincristine, doxorubicin, cyclophosphamide with concomitant vinblastine and celecoxib	Newly diagnosed metastatic Ewing sarcoma, combination with standard multiagent chemotherapy	35	_	24-m EFS 35%; 71% for pts. with isolated pulmonary metastases, 26% for all others	Felgenhauer et al. (2013). Phase II

Paclitaxel 80 mg/m^2/week, trastuzumab and pertuzumab	First and second line in HER2+ metastatic breast cancer	51 (not treated) 18 (pretreated)	_	OS 44 m, PFS 25.7 m, 6-m-PFS 89% (not treated) OS 37.5 m, PFS 16.9 m, 6-m-PFS 78% (pretreated) 6-m-OS 98%	Smyth et al. (2016). Phase II
Paclitaxel 80 mg/m^2/week	Relapsed small cell lung cancer	57	ORR 9.1%, CBR 52.7%	OS 168 days, PFS 145 days	Noronha et al. (2016) Prospective study
Docetaxel 20 mg/m^2 on days 1, 8, and 15 every 4 weeks and oral estramustine phosphate 280 mg and prednisolone 5 mg every day	Castration-resistant prostate cancer	39	>/= 50% reduction PSA: 33%	OS 16.7 m	Nakano et al. (2016). Prospective study
Weekly low-dose docetaxel	Neoadjuvant hyperthermo-chemoradiotherapy in advanced esophageal squamous cell carcinoma	24	ORR 41.7%, CR 17.6%	3-year-OS 56.3%, 5-year-OS 50%	Nakajima et al. (2015). Prospective study

OS overall survival, *PFS* progression free survival, *DOR* duration of response/remission, *TTP* time to progression, *EFS* event free survival, *m* month, *ORR* overall response rate, *PSA* prostate specific antigen, *CR* complete response, *PR* partial response, *SD* stable disease, *MR* minor response, *m* months

Several schedules using **docetaxel**, a second generation semi-synthetic taxane, are existing, thereof its administration in a three-weekly manner as neoadjuvant chemotherapy in operable breast cancer and 50 mg/m^2 docetaxel once every 3–4 weeks in castration-resistant prostate cancer (Zhang et al. 2016; Nakano et al. 2016; Miura et al. 2015), in weekly doses for advanced esophageal carcinomas (Nakajima et al. 2015), and of 30 mg/m^2 on days 1 and 8 every 3 weeks in pretreated advanced non-small cell lung cancer (Chung et al. 2011).

Metronomic chemotherapy can be combined with anti-angiogenic compounds. In several clinical trials, low-dose continuous chemotherapy was combined with bevacizumab. Bevacizumab combined with octreotide and metronomic capecitabine in a prospective phase-II trial was well tolerated and effective in patients with metastatic neuroendocrine tumors, i.e., 18% of the patients achieved a partial response (Berruti et al. 2014). Adding low-dose cisplatin and oral daily etoposide to bevacizumab has shown a significant decline in tumor perfusion and substantial clinical activity in patients with advanced non-small cell lung cancer (Correale et al. 2010). In women with ovarian cancer pretreated with platinum-based regimens, the combination of bevacizumab and low-dose metronomic cyclophosphamide resulted in a 24% partial response rate (Garcia et al. 2008).

The addition of metronomic etoposide or temozolomide to bevacizumab in patients with glioblastoma refractory to bevacizumab alone, however, had no effect (Reardon et al. 2011). These data show that adding metronomic chemotherapy to bevacizumab in patients with selected advanced cancer can be beneficial. However, randomized clinical trials are rare.

Resistance to Metronomic Chemotherapy

As metronomic chemotherapy preferentially targets the tumor vasculature, it is not very likely that resistances occur, as endothelial cells have a pronounced genetic stability. However, there is evidence for the development of evasive resistance mechanisms. Tumors may acquire reduced vascular dependence by growing under nutrient and oxygen deprived conditions. As anti-angiogenic treatment can induce central necrosis in the tumor, a vascularized rim may remain after treatment and this can be a cause of recurrence and resistance (Liang et al. 2016). Another mode of evasive resistance is the recruitment of EPC from the bone marrow, which in the following form new blood vessels (vasculogenesis) in the tumor tissue (Liang et al. 2016).

Future Directions

Metronomic chemotherapy is shifting the target of cytotoxic agents from the tumor cells to the microenvironment, especially to the tumor supporting vasculature with the objective to overcome drug resistance of malignant cells. Despite encouraging results in advanced malignancies of various organs, well-controlled randomized trials are still lacking and it has become clear that, as we know from classical chemotherapeutic regimens, also in metronomic therapy there is no "one drug fits all," i.e., the treatment must be tailored to the tumor type and the condition of the patients and there is some evidence that metronomic chemotherapy adjusted to the pharmacokinetics of the respective cytotoxic drugs might be a way to optimize response. Unfortunately, biomarkers that can predict the response of a distinct tumor to a given drug are widely lacking. Also, the time point for the treatment can be crucial. For example, vinblastine produces high response rates and approximately 50% long-term survivors in pediatric patients with relapsed or refractory anaplastic lymphoma, but when given as a maintenance treatment in such patients, no survival benefit was found in a randomized trial (Brugieres et al. 2009; Le Deley et al. 2010).

Nevertheless, for patients in low-income countries with underdeveloped health-care infrastructure, cytotoxic drugs that are orally available for metronomic scheduling and that are often

low-priced because off-patent and have limited toxicity offer an opportunity for the treatment of their cancer.

In high-income countries, metronomic chemotherapy is an attractive option for patients who are fragile due to their age, comorbidities, or multiple treatment lines of anticancer therapy.

Several strategies may extend our armamentarium of compounds for metronomic therapy in combination with drugs that target different pathways. Drug repositioning, the application of known drug to new indications is one way. For example, tricyclic anti-depressants can inhibit autocrine signals in lung cancer that mediate tumor cell survival (Jahchan et al. 2013) and thus might be combined with metronomic chemotherapy. Furthermore, novel cytotoxic agents are under investigation regarding their anti-angiogenic activities. A relative new sources of anticancer agents are creatures from the deep sea (e.g., Aplidin™, Yondelis™, and Zalypsis™). We have shown that such marine compounds have also anti-angiogenic activities in vitro and in vivo in addition to their cytotoxic mode of action (Steiner et al. 2015b; Borjan et al. 2015) and thus might be used at lower dosage for metronomic treatment.

Metronomic treatment meets more and more the concept of "personalized" combination cancer therapy, i.e., to give a specific treatment to an individual patient with his unique tumor characterized by specific molecular targets.

Ultimately, only well-controlled clinical trials that prove equivalent efficacy to conventional chemotherapeutic regimens can pave the way for metronomic therapy to eventually become a widely used treatment strategy against cancer.

Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Anti-angiogenics and Radiation Therapy

Daniel H. Schanne, Anca-L. Grosu, and Dan G. Duda

Contents

Introduction 350
Rationale for Combination of Radiotherapy with Anti-angiogenic Therapy 350
Effects of Radiation on Angiogenesis 351
Preclinical Evidence of Combined Anti-angiogenesis and Radiotherapy 351

Clinical Experience 352
Clinical Efficacy of Anti-angiogenic Therapy in Combination with Radiotherapy 352
Toxicity Profile 355

Summary 355

Cross-References 355

References 356

Abstract

Investigation of the combined effects of radiation and anti-angiogenic therapy has yielded intriguing preclinical and clinical results. The cytotoxic effects of radiation on cancer cells are critically dependent on the formation of free radicals and therefore an adequate supply of oxygen by blood vessels. Most tumors, however, are characterized by irregular angiogenesis and marked hypoxia. Anti-angiogenic therapy could contribute to a normalization of blood vessels to improve blood flow, alleviate hypoxia, and subsequently increase the effectiveness of radiotherapy. Clinical evidence in glioblastoma and other tumor entities has shown encouraging outcomes and warrants further characterization of the synergism between these therapies. Care should be taken with respect to the toxicity profiles of both

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entities, especially where side effects overlap, for example, damage to organs such as the liver, kidney, or the lung; ischemic complications; organ perforation; and the impairment of bone marrow.

Keywords
Bleeding · Ischemia · Parenchymal damage · Perforation · Pneumonitis · Fistula · DNA damage · Double-strand break · Ionizing radiation · Hypoxia · Oxygen-enhancement ratio · Radiation · Angiogenesis · Endothelial cells · Macrophages · HIF-1α · MMP-9 · Preclinical · Xenograft · DC-101 · Oxygenation · Glioblastoma · Bevacizumab · Colorectal cancer · Cediranib · Sunitinib · Pazopanib · Vandetanib

Introduction

Rationale for Combination of Radiotherapy with Anti-angiogenic Therapy

The rationale to combine radiotherapy (RT) with anti-angiogenic therapy is based on the interplay of ionizing radiation with oxygen. The biologic effect of high-energy photons or other particles (neutrons, protons, heavy ions) is mainly caused by damage to DNA in the form of single-strand breaks (SSB) or double-strand breaks (DSB). DSB are the primary cause of cell death and are generated at a rate of approximately 25 DSB per Gray per cell (Stenerlöw et al. 2003). The repair mechanisms that are activated by damages to DNA and other cellular structures are often impaired in neoplastic cells, which is one of the main reasons for the differential effect of radiation to tumor and healthy tissues (Spitz et al. 1996; Abbott et al. 1998, 1999; Collis et al. 2003; Parshad et al. 1983). When therapeutic levels of radiation are administered to cells, the resulting responses take shape mainly as apoptosis, senescence, and mitotic catastrophe(Vakifahmetoglu et al. 2008; Sabin and Anderson 2011; Watters 1999; Eriksson and Stigbrand 2010).

Radiation-induced DNA damage can be mediated by two different pathways, (1) the direct impact and energy transfer to the DNA, leading to physical damage and breakage of the double strand. This effect becomes dominant with heavy particles and high irradiation doses. The other way is (2) the indirect path, wherein the incoming radiation cleaves water molecules and generates free OH radicals. These radicals can subsequently damage the DNA in a chemical reaction and thereby disturb cellular functions and survival. It is estimated that ca. 50–70% of radiation-induced DNA damage in cells is caused by the indirect effect under normoxia (Michaels and Hunt 1978). However, the range of this number depends strongly on the concentration of oxygen because the presence of O_2 can contribute significantly to the generation of radicals. This increases the number of potential reactions and thus the DNA damage. The oxygen enhancement ratio (OER) is a number that quantifies this influence of O_2 on radiation effects. Specifically, it describes the relationship of oxygen concentration and ability of ionizing radiation to achieve a certain effect. For survival of tumor cells in vitro, the OER is reported to be between 1.5 and 4, meaning a 50–400% increase of cell killing by increasing the O_2 concentration (Palcic et al. 1982; Drew et al. 1972; Ling et al. 1985). This makes oxygen one of the most critical determinants of biologic radiation effects, and there is ample clinical evidence that lower O_2 makes tumors more resistant to radiotherapy and decreases survival (Amberger-Murphy 2009; Nordsmark et al. 2001, 2005; Fyles et al. 1998; Gatenby et al. 1988).

To the disadvantage of radiotherapy, most tumors exhibit a hypoxic microenvironment that is not homogenously distributed but rather produces areas of differing O_2 gradients that renders some cell populations sensitive to radiation while making others profoundly radioresistant. The cause of this hypoxia is irregular angiogenesis which results in leaky blood vessels that are not able to properly supply all areas of the tumor with oxygen. Additionally, solid pressure within the tumor compresses these vessels and thereby

contributes to the hypoxic environment and resistance to radio- and chemotherapy (Trédan et al. 2007; Moeller et al. 2004).

Effects of Radiation on Angiogenesis

Radiation itself influences angiogenesis, although contradictory results have been observed. In the context of low-dose radiation, in vitro data shows that radiation can lead to a pro-angiogenic response via the release of vascular endothelial growth factor (VEGF) 2 and altered angiogenesis-related regulation of transcription (Vincenti et al. 2011; Vala et al. 2010). Moreover, vascular progenitor cells demonstrate increased migration without impairment of proliferation or survival upon irradiation with doses <0.8 Gy (Vala et al. 2010). Administration of 0.2–1 Gy also has been shown to enhance the formation of capillary-like structures in human umbilical vein endothelial cells (Vala et al. 2010). These results have been confirmed in vivo in mice and zebrafish, where enhanced angiogenesis was observed at 0.3–0.5 Gy (Lee et al. 2011). Additionally, this study showed accelerated tumor growth and metastasis in a murine tumor model, depending on VEGF receptor inhibition. On the other hand, high doses of radiation seem to have a contrary effect. Doses of 10 Gy were able to suppress expression of the pro-angiogenic factors VEGF, Ang-1 (Angiopoietin 1), Tie-2, and Ang-2 in irradiated rat brain and lead to apoptosis and suppressed proliferation of endothelial cells (Hlushchuk et al. 2008). In murine mammary tumor xenografts, 12 Gy administered over 4 days decreased the blood vessel density, predominantly damaging immature blood vessels while mature ones stayed intact. There was also a shift from sprouting to intussusceptive angiogenesis which the authors interpreted as an adaptive mechanism during the tissue recovery process (Geng et al. 2001). Tumor entity may also play a role in angiogenic response, as one study showed in subcutaneous xenograft models that melanoma responds with increased vascular length density at doses of 2–3 Gy whereas 6 Gy leads to a decrease. In contrast, the glioblastoma model in the same study already showed a reduction starting at 3 Gy (Meng et al. 2010).

But not only cancer cells contribute to the state of angiogenesis in tumors but also other cell types in the tumor microenvironment. Macrophages have been shown to secrete VEGF after irradiation and convey radioresistance. Inhibition of the macrophage-activating TNF-α could reverse this effect as well as targeting the VEGF pathway (Iyer et al. 1998; Semenza 2003). Macrophages also release nitric oxide (NO) in response to irradiation which in turn leads to activation of hypoxia-induced factor 1 alpha (HIF-1α). HIF-1α is a master regulator of hypoxia response and angiogenesis and among others upregulates expression of VEGF (Ahn and Brown 2008). Bone marrow-derived cells that are recruited to the tumor after irradiation also may play a role in increased angiogenesis through expression of matrix metalloprotease 9 (MMP-9) and subsequent angiogenesis (Fenton et al. 2004).

Preclinical Evidence of Combined Anti-angiogenesis and Radiotherapy

A multitude of studies have been conducted to test the synergistic potential of angiogenesis and radiotherapy. These studies usually make use of anti-angiogenic antibodies (e.g., bevacizumab) or VEGF receptor-blocking small molecules. Direct measurements of oxygen concentrations within the tumor have been equivocal; while O_2 levels in a study of murine breast cancer models were decreased (Winkler et al. 2004; Lee et al. 2000), an increase was found in two xenograft glioma models (Kozin et al. 2001) and no change in a study of glioma and small cell lung cancer (Landuyt et al. 2001). On the other hand, in vivo tumor control has generally been described as being improved by combining radiation and anti-angiogenic therapy. Landuyt et al., using 8 Gy single-fraction RT and a combination of two small molecule anti-angiogenic agents (combretastatin A-4 phosphate, TNP-470) in rhabdomyosarcoma, saw a delay in tumor growth,

particularly in large tumors (Lund et al. 2000). TNP-470 was also used in a glioma study where again it leads to delayed tumor growth when administered concurrently with a single fraction of 10 Gy RT (Li et al. 2005). Similarly, using the murine anti-VEGFR-2 antibody DC-101, long-term control could be achieved in squamous cell carcinoma cell lines as described by (Li et al. 2005; Kozin et al. 2001). In this study, RT was administered twice per week in 3 Gy fractions for 3.5 weeks, while DC-101 was also injected twice per week but only for 3 weeks. Kozin et al. also used DC-101 with six injections, every 3 days in small cell lung cancer and glioma xenografts. They used five RT fractions of 5–24 Gy, delivered every day and could show tumor growth delays of 17 and 7 days for the two tumor types, respectively (Jain 2005).

Among the factors that influence efficacy of RT and anti-angiogenic treatment, the time frame of administration seems to play a particularly important role. The concept of vascular normalization was introduced to describe a time window after anti-angiogenic treatment where the function and structure of the irregular blood vessels in the tumor are improved and subsequently lead to a better delivery of drugs and oxygen through the circulating blood (Winkler et al. 2004). Indeed, studies in glioma xenograft have confirmed this idea when anti-angiogenic therapy lead to a decrease in tumor hypoxia from days 1 to 5 but increased again thereafter (Dings et al. 2007). Similarly, a study of breast cancer, melanoma, and ovarian carcinoma could demonstrate tumor growth delay in the RT and anti-angiogenics (bevacizumab + anginex) group but found increased intratumor oxygen levels only between days 2 and 5 (Matsumoto et al. 2011). This was confirmed in a model of squamous cell carcinoma, where oxygenation was improved (compared to control) for several days but then declined when implanted tumors where irradiated with a single fraction of 10 Gy and then treated with sunitinib (Kennecke et al. 2012).

In summary, these results argue for a context-sensitive benefit of combining RT and anti-angiogenic therapy in a preclinical setting. The timing of radiation and anti-angiogenic therapy seems to be of special importance and should be given thorough consideration when designing clinical trials.

Clinical Experience

Clinical Efficacy of Anti-angiogenic Therapy in Combination with Radiotherapy

Combinations of radiotherapy (RT) with anti-angiogenic regimens have been tested in a wide array of cancers, including tumors of the colon (Crane et al. 2010), rectum (Bendell et al. 2012), esophagus (Crane et al. 2006), pancreas (Fury et al. 2012), head and neck (Canter et al. 2014), sarcoma (Chinot et al. 2014; Stupp et al. 2005), and others (Fig. 1). The largest share of

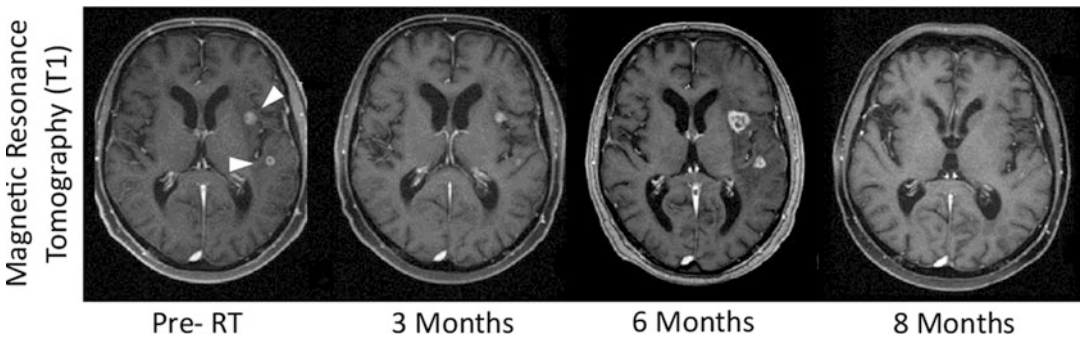

Fig. 1 Contrast-enhanced MRI (T1 sequenced of a patient treated with radiosurgery for cerebral brain metastases from colorectal carcinoma. Six months after RT, MRI shows potential signs of progressive disease and radiation necrosis. Treatment with bevacizumab is initiated and leads to complete remission on MRI 8 months after RT

clinical evidence, however, has been gathered in glioblastoma multiforme (Table 1) where both radiotherapy and the anti-vascular endothelial growth factor A (VEGF-A) antibody bevacizumab (BVZ) are standard of care (Chinot et al. 2014; Gilbert et al. 2014). Among anti-angiogenic treatments, BVZ is the most extensively studied agent in glioblastoma (GBM). Two large randomized, placebo-controlled studies have shown clear evidence of clinical benefit by addition of BVZ to chemo- and radiotherapy in newly diagnosed disease (Koukourakis et al. 2009). While both studies could not show an increase in long-term overall survival, Gilbert et al. could demonstrate a 3.4-month benefit in progression-free survival (PFS), whereas Chinot et al. reported a 4.4 months PFS gain in the BVZ group. Overall, there are currently more than 30 active clinical trials in the USA and Europe that investigate the role of BVZ in GBM, several of them in combination with radiotherapy that will further specify the role of these treatments.

Bevacizumab + RT has also been explored in multiple settings of colorectal cancer. In the case of locally advanced, inoperable disease, one trial saw 68.5% complete and 21.1% partial clinical response as well as downregulation of DNA repair and proliferation markers by BVZ in tumor biopsies (Crane et al. 2010). Side effects in this study were manageable with moist perineal desquamation in 9%, diarrhea grade 2/3 in 23%, and severe proctalgia in 9% of patients, respectively. Robust clinical activity was also found in locally advanced rectal cancer by Crane et al. who treated 25 patients with T3N0 or T3N1 tumors neoadjuvantly with 50.4 Gy in 28 fractions, capecitabine twice daily on radiation days, and bevacizumab every 2 weeks. Upon subsequent tumor resection, 32% of patients had pathologic complete response (pCR), and another 24% were found with $<10\%$ viable tumor cells in the resected mass (Velenik et al. 2011). In a similarly designed study, pCR rates were lower at 13%, but the overall downstaging rate was 74% and a radical resection was possible in 95% of patients. Of note, this trial included tumors with higher TNM stages such as 36% T3N2 and 8% T4N2 lesions, which could account for the lower response rate. Dermatitis, leukocytopenia, and infection were the most common grade-3 toxicities, and the authors reported one vascular grade-4 toxicity (Gasparini et al. 2012). Similar results stem from a publication by Gasparini et al. who treated 43 patients with tumors ranging between T2N1M0 and T4N2M1 using an analogous treatment regimen and found 14% pCR and 95% resection rate with negative margins. The authors also correlated clinical outcomes to biomarkers and found that a higher CD34+ vessel density in pathologic specimens correlates inversely with residual tumor area, suggesting that these tumors respond better to neoadjuvant treatment (Resch et al. 2012). Conflicting results were reported by Resch et al. who terminated a phase II study in the same clinical setting based on adverse events $\geq$grade 3, mostly intestinal bleeding, anemia, diarrhea, and abdominal pain (Buren et al. 2013; Small et al. 2011; Crane et al. 2009). The authors concluded that their early-termination criteria may have been too restrictive. In pancreatic ductal adenocarcinoma, three phase II trials – one in unresectable disease, two with subsequent surgical removal if eligible – found a tolerable safety profile but none to marginal clinical benefits compared to historic cohorts treated without BVZ (Lee et al. 2012; Schefter et al. 2012; Yoon et al. 2011; Salama et al. 2011). Additional diagnoses for which limited clinical data about BVZ + RT exist are cervical cancer, nasopharyngeal carcinomas, soft tissue sarcomas, and head and neck tumors. While results for the first three were generally encouraging, there is conflicting evidence in head and neck cancer (Salama et al. 2011; Seiwert et al. 2008). Seiwert et al. reported feasibility and activity of the regimen whereas Salama et al. terminated their study because of unexpected progression of four out of five BVZ + RT-treated T4N0-1 tumors (Batchelor et al. 2013).

Alternative anti-angiogenic treatment strategies most frequently involve small molecule tyrosine kinase inhibitors (TKI) that target the family of vascular endothelial growth factor receptors (VEGFR). As small molecules, these agents offer the advantages of oral bioavailability and a better penetration into tissues and cells. However, they usually also target a much broader spectrum

Table 1 Selection of clinical trials combining anti-angiogenic therapy and radiation in glioma patients

Anti-angiogenic agent	Mechanism	Concurrent with RT?	Other agents	RT dose	RT no. of fractions	Phase	Disease type	No. of Pat.	PFS (months)	PFS6 (%)	Median OS (months)	Pub. year	Reference
Bevacizumab	Anti-VEGF-A	Yes		60	30	2	nGBM	10	8.9			2008	Lai et al. (2008)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	60	30	2	nGBM	70	13.6		19.6	2011	Lai et al. (2011)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	59.4	33	2	nGBM	75	14.2		21.2	2011	Vredenburgh et al. (2011)
Bevacizumab	Anti-VEGF-A	Yes	TMZ, everolimus	60	30	2	nGBM	68	11.3	73	13.9	2012	Hainsworth et al. (2012))
Bevacizumab	Anti-VEGF-A	Yes	TMZ	59.4	33	2	nGBM	51	13	85.1	23	2012	Narayana et al. (2012)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	60	30	3	nGBM	458	10.6		16.8	2014	Chinot et al. (2014)
Bevacizumab	Anti-VEGF-A	Yes	IRI, TMZ	60	30	2	nGBM	120	7.1 (IRI/BVZ)/5.2 (control)	62 (IRI)/ 42 (control)	11.1 (both arms)	2014	Chauffert et al. (2014)
Bevacizumab	Anti-VEGF-A	No	Erlotinib, TMZ	59.4/ 60	33/30	2	nGBM	74	13.5		19.8	2014	Clarke et al. (2014)
Bevacizumab	Anti-VEGF-A	Yes	IRI, TMZ	60	30	2	nGBM	63	7.3 (IRI)/7.7 (TMZ)	52 (IRI)/ 53 (TMZ)	15.1 (IRI)/ 11.8 (TMZ)	2014	Hofland et al. (2014)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	36	6	2	nGBM	40	10		19	2014	Omuro et al. (2014)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	60	10	2	nGBM	30	14.3	90	16.3	2015	Ney et al. (2015)
Bevacizumab	Anti-VEGF-A	No	TMZ, IRI	NA	NA	2	nGBM	41	8.6		12	2015	Peters et al. (2015)
Bevacizumab	Anti-VEGF-A	Yes	TMZ	60	30	2	nGBM	19	9.6		16	2015	Van Linde et al. (2015)
Vandetanib	VEGFR-2/EGFR/ RET proto-oncogene	Yes	TMZ	59.4/ 60	33/30	1	nGBM	13	8		11	2010	Drappatz et al. (2010)
Vatalanib	Anti-VEGFR 1–3/ c-kit/PDGFR	Yes	TMZ	60	30	1/2	nGBM	20		63.2	17.3	2010	Brandes et al. (2010)
Vatalanib	Anti-VEGFR 1–3/ c-kit/PDGFR	Yes	TMZ	NA	NA	1	nGBM	15	7.2		16.2	2011	Gerstner et al. (2011)

BVZ bevacizumab, *IRI* irinotecan, *MG* malignant glioma, *nGBM* newly diagnosed glioblastorna, *OS* overall survival, *PDGFR* platelet-derived growth factor receptor, *PFS* progression-free survival, *PFS6* 6-month PFS, *PIGF* placental growth factor, *rGBM*, *TMZ* temozolomide, recurrent glioblastoma, *VEGF-A* vascular endothelial growth factor A, *VEGFR-1/2/3* vascular endothelial growth factor receptor 1/2/3. This table was adapted with permission from Lu-Emerson et al. (2015)

of molecular binding partners such as Flt-3, c-kit, or PDGFR, and this lower specificity opens up the possibility for a higher rate of potentially severe side effects. Combinations of TKI with radiotherapy have been evaluated in several oncological settings, but most data again stem from gliomas. Cediranib, an inhibitor of multiple VEGF receptors and the PDGFR pathway, has shown increased tumor oxygenation and survival when combined with radiochemotherapy in patients with newly diagnosed GBM when (Kreisl et al. 2012; Iwamoto et al. 2010; Neyns et al. 2011; Pan et al. 2012). This benefit only applied to patients with radiographic improvement of tumor perfusion but in turn leads to an improvement of overall survival by 9.3 months (17.0 vs. 26.3 months). Several other substance candidates such as sunitinib, pazopanib, and vandetanib have been evaluated in gliomas but generally failed to demonstrate an improvement in PFS or overall survival (OS), although individual clinical markers like radiographic response suggested biological activity in some cases (Lewin et al. 2014). Sunitinib plus radiotherapy was also tested in a neoadjuvant setting of soft tissue sarcoma (STS) but leads to dose-limiting toxicities in more than half of patients and moreover was associated with a higher rate of local failure compared to the control group (Canter et al. 2014; Haas et al. 2015). In contrast, the authors of two analogous studies using pazopanib and sorafenib, respectively, conclude clinical activity of the treatment regimen, although of note, these were phase I trials and more compelling evidence is needed to support this data (Lordick et al. 2006).

Toxicity Profile

An important issue with any combination therapy is toxicity. As encouraging as some of the clinical results with anti-angiogenics are, severe side effects have been reported. Adverse events cluster predominantly in the cardiovascular system, with bleeding, hypertension, thromboembolic events, and delayed wound healing as the most frequent problems. However, additional risks such as hepatotoxicity, nephrotoxicity, perforation of the gastrointestinal (GI) tract, severe diarrhea and abdominal pain, and hand and foot syndrome have also been reported. In a meta-analysis of >12,000 patients treated with BVZ, Hapani et al. reported an overall risk of 0.6–1.1% for GI perforations, depending on the diagnosis and administered BVZ dose, that was associated with 21.7% risk of mortality. Wherever these potential side effects coincide with the administration of radiation, the risk of severe events is consequentially potentiated. While radiotherapy has a rather low potential for short-term cardiovascular events, there is an evident risk of toxicity to parenchymal organs such as the liver and kidney, for perforations in the GI tract with potential subsequent bleeding and cytopenia if a substantial fraction of the bone marrow is irradiated. This is confirmed by reports of an increase in the risk of ischemic bowel complications during treatment with bevacizumab after pelvic irradiation (Spigel et al. 2009). Furthermore, three trials combining RT and BVZ in non-small cell lung cancer were terminated early, two because of tracheoesophageal fistulas and one because of radiation pneumonitis (Lai et al. 2008). Combinations of anti-angiogenics with RT should therefore be evaluated rigorously in clinical studies before routine application in clinical practice.

Summary

The combination of anti-angiogenic therapy and radiation has been a field of active research during the recent years. The data that have been gathered in tumors of the central nervous system make up the largest share of clinical experience and have shown a reasonable safety profile as well as improved clinical outcomes. However, more clinical evidence from intracranial malignancies and tumors in other sites is needed to fully understand the risks and benefits of this treatment combination.

Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Anti-angiogenics in Gastroesophageal Cancer
- ▶ Inhibition of Tumor Angiogenesis in the Treatment of Lung Cancer

- Mechanisms of Anti-angiogenic Therapy
- Pathology of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- The Value of Anti-Angiogenics in Head and Neck Cancer Therapy
- The Value of Anti-angiogenics in Prostate Cancer Therapy
- The Value of Anti-angiogenics in Soft Tissue Sarcoma Therapy

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Combination of Anti-angiogenics and Other Targeted Therapies

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Contents

Introduction 360

Synergy Between Anti-angiogenic Therapies and EGFR Inhibition 360
Combining Bevacizumab (VEGF) and Cetuximab (EGFR) 362
Combining Bevacizumab (VEGF) and Panitumumab (EGFR) 363
Combining Bevacizumab (VEGF) and Erlotinib (EGFR) 364
Combining Bevacizumab (VEGF) and HER2-Directed Therapy 365
Tyrosine Kinase Inhibitors Blocking Both VEGFR and EGFR 366

Synergy Between Anti-angiogenics and Immune Cell Therapies 366
Combining VEGF and CTLA4 Blockade 368
Combining VEGF and PDL1/PD1 Blockade 368

Synergy Between VEGF Blockade and Temsirolimus 369

Synergy of Three Targeted Agents Including VEGF Blockade 370

Combined Blockade of VEGF and Ang2 Signaling: Humanized Tri-specific Nanobody 371

A Novel VEGFR2 Targeted Antibody-Based Fusion Protein (mAb04-MICA) ... 372

Conclusion 372

Cross-References 373

References 373

Abstract

Angiogenesis is a hallmark of tumor development and metastasis and is now a validated target for cancer treatment. However, the overall benefits of anti-angiogenic drugs from the perspective of impacting survival have left much to desire, endorsing a need for developing more effective therapeutic regimens, e.g., combining anti-angiogenic drugs with established chemotherapeutic drugs. In this review, we discuss progress in the synergistic design of

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anti-angiogenic agents in combination with targeted therapies. Targeted cancer therapies include monoclonal antibodies and small-molecule inhibitors that have significantly changed the treatment of cancer over the past years. We focus on anti-angiogenic agents combined with targeted therapies inhibiting the epidermal growth factor receptor (EGFR) pathway and the PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B)/mTOR (mammalian target of rapamycin) pathway and inhibiting immune checkpoint receptors, such as CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) and PD1/PDL1 (programmed cell death protein 1/PD1 ligand). Of note, not always, encouraging preclinical data particularly of VEGF and EGFR inhibitor combinations did translate into the clinics. In addition, we highlight the rapidly developing field of VEGF-based humanized tri-specific nanobodies and novel VEGFR2-targeted antibody-based fusion proteins, potentially providing a new inspiration for antitumor treatment.

Keywords
Angiogenesis · VEGF · Angiogenesis inhibitor · Monoclonal antibodies · Bevacizumab · Cetuximab · Panitumumab · Targeted therapy · Preclinical studies · Clinical trials · Cancer · Tyrosine kinase inhibitors

Introduction

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in the survival of cancer cells, in local tumor growth, and in the development of distant metastases (Folkman 1971). Therefore, anti-angiogenic treatment in tumors is a highly promising therapeutic approach. The increasing understanding of the biological mechanisms of tumor-induced angiogenesis has stimulated the development of agents able to interfere with the molecules involved in this process (Folkman 1995). Two main approaches have been proposed for blocking vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation and subsequent tumor angiogenesis:

- Monoclonal antibodies directed against specific proangiogenic growth factors and/or their receptors.
- Small molecule tyrosine kinase inhibitors (TKIs) of multiple proangiogenic growth factor receptors. Of note, anti-angiogenic TKIs often inhibit multiple tyrosine kinases because of the structural similarities between VEGFR and other receptor tyrosine kinases, thus often providing tumor growth inhibition by several independent mechanisms.

Beside these, a plethora of agents are proposed to indirectly inhibit angiogenesis through mechanisms not completely understood. These include bortezomib and thalidomide.

However, a given tumor is unlikely to be dependent on only one receptor or signaling pathway for its growth and survival. This is due to the significant level of compensatory cross talk among receptors within a signaling network as well as heterologous receptor systems. Therefore, the survival benefits of anti-angiogenic drugs have, thus far, been rather modest and, subsequently, combining drugs inhibiting different signaling pathways is currently an important strategy to achieve synergy or overcome resistance.

Synergy Between Anti-angiogenic Therapies and EGFR Inhibition

The synergy between the VEGF and epidermal growth factor receptor (EGFR) pathways lies in their close relationship and sharing common downstream signaling pathways as well as their extensive cross talk (Herbst et al. 2005). Activation of EGFR signaling in tumor cells stimulates the production of angiogenic factors such as VEGF, causing endothelial cells to proliferate and migrate, suggesting that the oncogenic properties of the EGFR-driven pathway may, at least in part, be mediated by the stimulation of tumor angiogenesis (Tabernero 2007; Larsen et al.

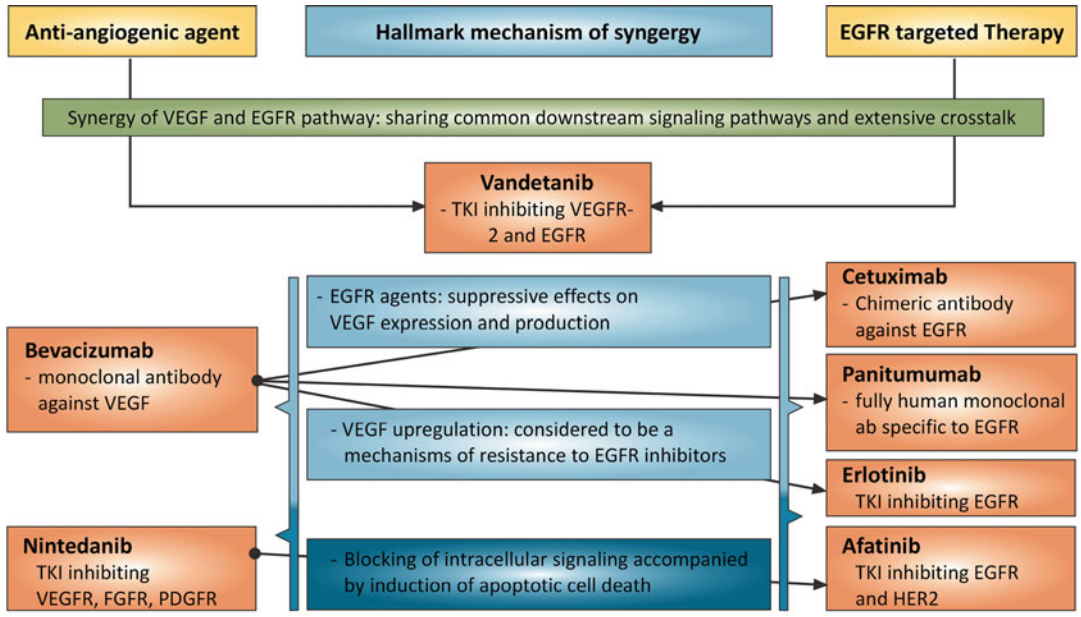

Fig. 1 Schematic representation of the main mechanisms of action postulated to mediate synergistic effects of anti-angiogenics and EGFR-targeted therapy

2011a). Accordingly, EGFR inhibitors have a suppressive effect on VEGF expression (Prewett et al. 1998). In addition, several studies have shown the role of VEGF-A upregulation in the acquired resistance to EGFR treatment in initially EGFR inhibitor-sensitive cancer cells (Viloria-Petit et al. 2001; Ciardiello et al. 2004). Therefore, targeting both these pathways could provide a better anti-cancer therapeutic strategy, especially for overcoming the acquired resistance of cancer cells to EGFR blockade (Tortora et al. 2008). An increased level of VEGF was paralleled with an increase in both angiogenic potential in vitro and tumor angiogenesis in vivo. In addition, elevated expression of VEGF in variants of the human epidermoid carcinoma cell line A431 obtained by gene transfection rendered the cells significantly resistant to anti-EGFR antibodies in vivo. The mechanism responsible for the elevated VEGF levels detected in the anti-EGFR-resistant tumor xenografts is not fully understood. The authors hypothesize that the activation of several oncogenes such as *ras, src,* and *erbB2/neu* or the inactivation/mutation of certain tumor suppressor genes such as *p53, VHL*, or *PTEN*, respectively, may account for this finding (Kerbel et al. 1998; Yen et al. 2000; Zhong et al. 2000). Thus, elevated VEGF levels may be the result of the selection of cells possessing one or more such genetic changes during the EGFR antibody-mediated therapy. Alternatively, aberrations in signaling pathways downstream of EGFR activation that are known to effect VEGF expression could conceivably be involved. Such changes, for example, could include phosphatidylinositol 3′-kinase (PI3 kinase), and/or SRC kinase overactivation, and/or ras mutation (Kerbel et al. 1998; Maity et al. 2000; Rak et al. 2000; Sato et al. 2000; Zhong et al. 2000). However, since VEGF upregulation in tumor cells is considered to be a mechanism of resistance to EGFR inhibitors, dual inhibition of both EGFR and VEGF may exert a synergistic effect (Fig. 1).

At least in preclinical studies, combinations of VEGF and EGFR inhibitors have shown synergy in antitumor activities in lung cancer and colorectal cancer (Ciardiello et al. 2000; Martinelli et al. 2010).

However, promising preclinical data of VEGF and EGFR inhibitor combinations did not translate into the clinical practice.

One potential explanation for the lack of activity might be that dual-pathway targeting with the EGFR inhibitor panitumumab and the VEGF inhibitor bevacizumab may have caused enhanced toxicity, leading to dose reductions or dose delays (Hecht et al. 2009), although this was not observed in other studies (Tol et al. 2009). Also, pharmacokinetic interactions might have occurred between the antibodies, as was suggested by a decrease in the incidence of bevacizumab-induced hypertension in the group receiving both VEGF and EGFR inhibitor treatment (Tol et al. 2009). Furthermore, bevacizumab alters tumor vascularity of subcutaneous human xenografts in mice, thereby limiting the delivery of cetuximab to the tumor leading to reduced therapeutic efficacy (Heskamp et al. 2013). In addition, interactions may have occurred between the downstream signaling pathways, e.g., EGFR-mediated changes in downstream targets may be necessary for the antitumor activity of bevacizumab or chemotherapy (Hecht et al. 2009). In mice, it was shown that cetuximab could also hamper the delivery of bevacizumab to the tumor, potentially resulting in reduced therapeutic efficacy (Heskamp et al. 2014).

Another mechanistic reason for the clinical failure might be that strategies to block VEGF or EGFR signaling by inhibition of extracellular ligands or receptors, as is the case for the monoclonal antibodies, may only prevent part of the oncogenic signaling accompanied with limited activity on intracellular signaling events. In contrast, the combination of EGFR- and VEGF(R)-targeted small-molecule tyrosine kinase inhibitors (TKIs) such as nintedanib (targeting VEGFR) and afatinib (targeting EGFR) block intracellular EGFR and VEGFR signaling, which is accompanied by the induction of apoptotic cell death (Poindessous et al. 2011). These findings provide a rationale for clinical trials combining TKIs.

All of the abovementioned reasons might, at least partly, explain the unfavorable results in some clinical studies.

Combining Bevacizumab (VEGF) and Cetuximab (EGFR)

The encouraging preclinical data of VEGF and EGFR inhibitor combinations did not translate into the clinics. To evaluate the combination of bevacizumab and cetuximab in patients with previously untreated, metastatic colorectal cancer, a large clinical trial was conducted among 755 patients, who were assigned in either the treatment group with chemotherapy (consisting of a combination of capecitabine and oxaliplatin) plus bevacizumab or the treatment group with chemotherapy plus bevacizumab plus cetuximab. Unexpectedly, the results indicated that the combination of bevacizumab and cetuximab resulted in shortened progression-free survival and worsened quality of life. Progression-free survival was 10.7 months among patients treated with chemotherapy plus bevacizumab and 9.4 months among patients treated with chemotherapy plus bevacizumab plus cetuximab (Tol et al. 2009). These data need to be put into perspective regarding the analysis of KRAS mutations. Among patients without KRAS mutations, survival was similar in the two treatment groups. Among patients with KRAS mutations, however, treatment with the combination of bevacizumab and cetuximab significantly worsened both progression-free and overall survival. Since cetuximab later on was only approved for patients without KRAS and NRAS mutations, and also other publications have found an inferior outcome of EGFR inhibition in RAS-mutated patients (Douillard et al. 2013), the conclusion from the clinical trial is that at least there is no benefit in the combined therapy.

There is no robust explanation given why the combination failed. The authors only state that the results of the trial might be due to a negative interaction between cetuximab and bevacizumab. Further they point out that hypertension, a common side effect of bevacizumab treatment, recently shown to correlate with clinical outcome in patients with colorectal cancer (Scartozzi et al. 2009), was less frequent in the patient group receiving capecitabine, oxaliplatin, and bevacizumab plus cetuximab, potentially

suggesting decreased efficacy of bevacizumab when administered in combination with cetuximab.

Also in other studies, the addition of cetuximab to bevacizumab plus FOLFOX in metastatic colorectal carcinoma did not result in better efficacy. Even increased toxicity was observed (Ocean et al. 2010; Saltz et al. 2012). Another clinical trial was prematurely terminated after other studies reported inferior outcomes with dual antibody treatment and although terminated early, the study supports the detrimental effect of combining VEGF and EGFR inhibition in metastatic colorectal cancer (Dotan et al. 2012).

Also in a xenograft mouse model with head and neck squamous cell carcinoma, the combination of anti-EGFR (cetuximab), VEGF antibodies (bevacizumab), and cisplatin appeared less effective than bevacizumab and cisplatin alone. In this study, the triple therapy resulted in less delay in tumor growth and worse survival compared to bevacizumab and cisplatin alone. This study, therefore, also argues against the combination of the two monoclonal antibodies (Wang et al. 2010).

In contrast, as forth-line treatment, the combination of VEGF and EGFR inhibitors appears to be safe and effective (Larsen et al. 2011b). Patients with metastatic colorectal cancer who had progressed on therapy with 5-FU, oxaliplatin, and irinotecan in the first- and second-line setting and with irinotecan and cetuximab as third-line therapy independent of their KRAS mutation status received irinotecan and cetuximab combined with bevacizumab. The triple combination was well tolerated and induced a high rate of disease control in heavily pretreated patients with metastatic colorectal cancer with a progression-free survival of 8.3 months and a median overall survival of 12 months (Larsen et al. 2011b). A possible explanation for this discrepancy in response between first- or fourth-line therapy might be that monoclonal antibodies could act differently in patients that are heavily pretreated compared to patients that are chemotherapy naïve. Previous chemotherapy could induce adaptive changes in tumor cells that increase the sensitivity for EGFR- and VEGF-directed monoclonal antibodies.

Combining Bevacizumab (VEGF) and Panitumumab (EGFR)

The replacement of the EGFR inhibitor cetuximab by panitumumab provided similar results when combined with bevacizumab in patients with colorectal cancer. A study by Hecht et al. (2009) showed that the addition of panitumumab to treatment with bevacizumab and chemotherapy (oxaliplatin and irinotecan based) for first-line mCRC resulted in an inferior median overall survival (19.4 months) compared with the control group receiving bevacizumab and chemotherapy only (25.4 months). Furthermore, toxicity was increased in the group receiving the combination of antibodies; therefore, treatment was discontinued early after an interim analysis (Hecht et al. 2009). While the exact explanation for these results is unknown, the authors speculated that pharmacokinetic and pharmacodynamic interactions might be responsible for the lack of activity. Since toxicity was exacerbated by dual-pathway inhibition in combination with chemotherapy, dose delays and reductions as well as decreases in dose intensity likely might explain the similar response rates observed with worse results of time-dependent end-points. In addition, potentially, a pharmacodynamic interaction induced by EGFR inhibition could explain the lack of activity of bevacizumab and/or chemotherapy. Possible mechanisms include EGFR-mediated alterations of downstream targets required for the activity of bevacizumab and/or chemotherapy or the induction of EGFR-mediated cell-cycle arrest leading to resistance to cytotoxics.

Interestingly, in two other studies addition of panitumumab and bevacizumab to chemotherapy (FOLFIRI) as second-line treatment resulted in improvement of progression-free survival and overall survival compared to FOLFIRI alone (Xie et al. 2014b; Liu et al. 2015).

However, in a recent meta-analysis of randomized controlled trials of patients with metastatic colorectal cancer, it was concluded that addition of bevacizumab to cetuximab- or panitumumab-based therapy did not improve progression-free survival and overall survival (Lv et al. 2015).

Thus the combined therapy of bevacizumab with cetuximab or panitumumab is not recommended for the treatment of metastatic colorectal cancer.

In contrast, recently a case report showed a dramatic response to panitumumab and bevacizumab in metastatic gallbladder carcinoma (Riley and Carloss 2011). In cholangiocarcinoma, EGFR expression is significantly associated with poor prognosis (Yoshikawa et al. 2008). In addition, genomic and genetic characterization of cholangiocarcinoma identified a subgroup of patients with poor overall survival and early recurrence that was characterized by multiple aberrantly regulated oncogenic pathways, including activation of HER2 and EGFR signaling (Andersen et al. 2012). In addition, several studies have revealed overexpression of VEGF in cholangiocarcinoma (ranging from 31 to 75%), and VEGF expression has been shown to be significantly associated with intrahepatic metastasis (Yoshikawa et al. 2008). Although there is the rationale for combining EGFR and VEGF inhibitors in cholangiocarcinoma and Riley and Carloss reported a single case of a patient with metastatic gallbladder carcinoma with an important response to treatment with panitumumab and bevacizumab (Riley and Carloss 2011), further clinical studies including targeted anti-EGFR and anti-VEGF(R) therapies are warranted in this entity.

Combining Bevacizumab (VEGF) and Erlotinib (EGFR)

Recent studies have demonstrated that since the oral EGFR inhibitor erlotinib and bevacizumab act on two different pathways critical to tumor growth and dissemination, administering these drugs concomitantly may confer additional clinical benefits to cancer patients with advanced disease. The combination of bevacizumab and erlotinib has been studied in phase I and II trials in metastatic breast (Dickler et al. 2008), lung (Tanaka et al. 2011), renal (Bukowski et al. 2007), and hepatocellular cancers (Thomas et al. 2009). No pharmacokinetic interaction between the two agents was demonstrated (Thomas et al. 2009). In vitro and in murine models, EGFR agents downregulate VEGF production; the combination of bevacizumab and erlotinib is likely to be synergistic in this regard (Fig. 1).

In biliary tract cancers, VEGF and EGFR have been identified as overexpressed, and VEGF has been suggested as a potential prognostic marker and correlated with poor outcome (Park et al. 2006). Therefore, a phase II trial testing the combination of bi-weekly bevacizumab and daily erlotinib in patients with unresectable biliary cancer has been conducted. The biologic-only combination showed clinical activity with an overall response rate of 64% (31 of 49 patients) with infrequent grade 3 and 4 adverse effects. The molecular analyses performed in this study suggest that patients whose tumors show mutations in EGFR vIII or have non-wild-type KRAS may be less likely to respond to erlotinib therapy (Lubner et al. 2010). These findings are consistent with trials in lung cancer and colon cancer relative to KRAS mutants and EGFR-based biologic therapy (Karapetis et al. 2008; Zhu et al. 2008). Shortcomings of this combination (bevacizumab and erlotinib) include a lack of demonstrable improvement in overall survival compared with that of historical controls, however a problem plaguing many trials in biliary tract cancers.

In patients with advanced non-squamous non-small lung cancer harboring EGFR mutations, the combination of bevacizumab and erlotinib in the first-line setting resulted in increased PFS compared to the erlotinib monotherapy group (16 months versus 9,7 months, $p = 0.0015$) (Seto et al. 2014). Results from a retrospective study in Japan showed that the serum concentrations of EGF, hepatocyte growth factor (HGF), and VEGF in patients with NSCLC who received EGFR-TKI were significantly higher among patients with progressive disease (PD) than among those with stable disease (SD) or partial response (PR) (Kasahara et al. 2010). Furthermore, the higher concentrations of HGF and VEGF were significantly associated with shorter PFS and OS. The study suggested that the serum concentration of VEGF might be an independent prognostic factor in NSCLC.

Since excessive angiogenesis is also associated with resistance to EGFR-TKI, several preclinical studies to overcome the resistance have suggested that a combination of an EGFR-TKI and anti-VEGF therapy could enhance antitumor activity in NSCLC cells harboring an EGFR mutation, especially in cells that express high levels of VEGF. Several mechanisms of antitumor activity of the combination therapy have been found. Tumor blood vessels are structurally and functionally abnormal because abnormal tumor vessels are hyperpermeable; the pressure gradient may be insufficient to ensure effective flow of drug from the vessel lumen to the tumor cells. Bevacizumab blocks angiogenesis by decreasing VEGF levels, and EGFR-TKI blocks synthesis of VEGF and TGF (transforming growth factor); they normalize tumor vessels transiently. The normalized vessels improve tumor oxygenations and restore delivery of drug into tumor by decreasing interstitial fluid pressure. In addition, EGFR plays a role in the regulation of cell proliferation. Partial normalization of tumor vessels by bevacizumab causes proliferation of the tumor cells, which make them more sensitive to EGFR-TKI.

In contrast to the abovementioned study with a remarkable efficacy of the erlotinib and bevacizumab combination with an increase in median PFS of 6.3 months compared to the erlotinib monotherapy group (16 months versus 9.7 months), in a small, single-arm study of 25 unselected patients who were elderly or had a performance status of 2, the bevacizumab/erlotinib combination was not encouraging with a median time to progression of 3.4 months and an overall survival rate of 5.1 months (Riggs et al. 2013). Additionally, in the TASK study, 124 patients with advanced or recurrent stage IIIB/IV NSCLC were randomized to bevacizumab plus chemotherapy versus bevacizumab plus erlotinib, and no benefit in PFS was observed for the bevacizumab/erlotinib arm at the time of interim analysis; thus the study was terminated (Ciuleanu et al. 2013). Based on these findings, the erlotinib plus bevacizumab combination is not currently recommended for first-line NSCLC. However, further results from studies currently evaluating the combination of anti-angiogenic inhibitors, such as bevacizumab and ramucirumab, in combination with targeted therapies in the EGFR mutation-positive patient population are expected within the next 5 years.

Combining Bevacizumab (VEGF) and HER2-Directed Therapy

Human epidermal growth factor receptor 2 (HER2) is a protein in the epidermal growth factor receptor (EGFR) family. Overexpression of HER2 promotes neoplastic transformation of cells making it a popular therapeutic target. Inhibition of HER2 is an established therapy for HER2-positive breast and gastric cancer. Trastuzumab is a monoclonal antibody that is FDA approved for HER2 overexpressed breast cancer and gastric or gastroesophageal (GE) junction patients, binding to the extracellular domain of the HER2/neu protein and inhibiting the proliferation of human tumor cells that overexpress HER2 (Baselga et al. 1996). While trastuzumab improves overall survival and response rate, resistance has been shown to develop in metastatic breast cancer patients (Tripathy et al. 2004). Therefore, the need to inhibit HER2 via alternate pathways exists. Lapatinib, also FDA approved for breast cancer patients, is a TKI of both EGFR and HER2R. Combining lapatinib and trastuzumab provides the opportunity to treat two members of the HER subfamily simultaneously and both the extracellular and intracellular domains.

Overexpression of HER2 has been associated with upregulation of VEGF in breast and lung cancer cell lines (Yen et al. 2000; Konecny et al. 2004). Preclinical data have shown that combining HER2 inhibition therapy and anti-VEGF therapy, bevacizumab, may bypass resistance to trastuzumab (du Manoir et al. 2006) (Fig. 2).

Clinically, two different phase II studies have shown responses in advanced HER2-positive breast cancer patients combining trastuzumab and bevacizumab (Drooger et al. 2016) and combining

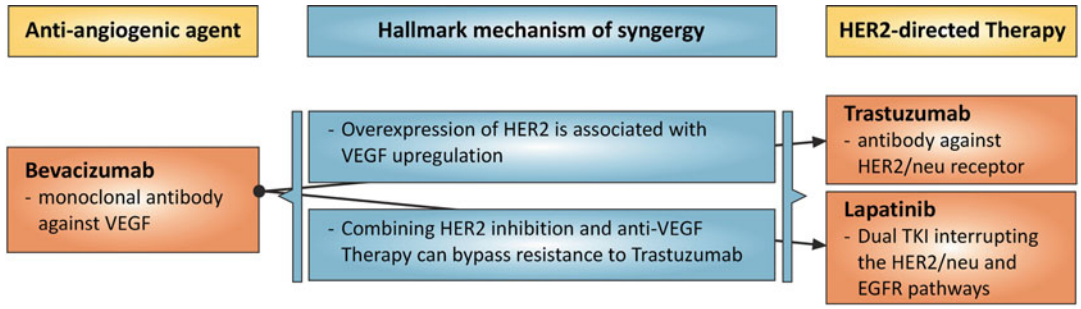

Fig. 2 Schematic representation of the main mechanisms of action postulated to mediate synergistic effects of anti-angiogenics and HER2-directed therapy

lapatinib and bevacizumab (Rugo et al. 2012). Recently, a phase I trial combined trastuzumab, lapatinib, and bevacizumab in patients with advanced cancer (Falchook et al. 2015). The combination was well tolerated with successful escalation to the FDA-approved doses of all three drugs without reaching a maximum tolerated dose (MTD). In addition, the combination has demonstrated antitumor activity in heavily pretreated patients with advanced malignancies with an overall response rate of 25% (SD > 6 months/PR/CR = 23/94 (25%). A Response (SD > 6 months/PR/CR) was achieved in 50% of heavily pretreated breast cancer patients in this study. These patients had all received prior trastuzumab and the majority prior lapatinib. Despite failing prior concurrent or sequential trastuzumab and lapatinib treatment, these patients continued to achieve SD > 6 months/PR/CR with the addition of bevacizumab to the treatment combination. Overcoming resistance to prior concurrent trastuzumab and lapatinib and achieving longer treatment duration with combining trastuzumab, lapatinib, and bevacizumab suggest that bevacizumab contributes to this HER2 treatment combination (Falchook et al. 2015). Other disease categories also achieved SD > 6 months/PR including a patient with non-small cell lung cancer harboring a HER2 mutation at exon 20, a patient with HER2-positive salivary duct cancer, and patients with HER2-negative breast and pancreatic cancer (N = 1 of each). Based on these observations, further evaluation of this combination of dual HER inhibition plus VEGF inhibition is warranted.

Tyrosine Kinase Inhibitors Blocking Both VEGFR and EGFR

Vandetanib is a tyrosine kinase inhibitor of both VEGFR-2 and EGFR, and preclinical studies have confirmed its antitumor effects in a range of cancer types. A randomized phase III trial demonstrated that vandetanib treatment is effective in patients with metastatic symptomatic or progressive medullary thyroid cancer (Wells et al. 2012), leading to the approval by the US Food and Drug Administration (FDA) in April 2011, followed by the European Medicines Agency (EMA) in 2012. This approval was based on a statistically significant and clinically meaningful improvement in progression-free survival. However, toxicity of vandetanib was worse than that of other kinase inhibitors, including abdominal pain and diarrhea, rashes, prolonged QT interval, hypertension, headache, and fatigue. The drug underwent clinical trials as a potential targeted treatment for non-small cell lung cancer; however, EU regulatory submissions for vandetanib were withdrawn in October 2009 after trials showed no benefit when the drug was administered along with chemotherapy.

Synergy Between Anti-angiogenics and Immune Cell Therapies

Immunotherapy has now been clinically validated as an effective treatment for many cancers. There is tremendous potential for synergistic combinations of immunotherapy agents and for

combining immunotherapy agents with conventional cancer treatments.

Emerging data indicate that abnormal tumor vasculature, resulting from the prevalence of pro- versus anti-angiogenic signals, fosters an immunosuppressive tumor microenvironment that enables the tumor to evade host immunosurveillance.

VEGF is a potent angiogenic factor that regulates angiogenesis while increasing the proliferation, migration, and metastasis of tumor cells. In addition to its proangiogenic function, mounting evidence shows that VEGF also plays a major role in the immunosuppression of innate and adaptive immune system cells (Soto-Ortiz 2016). VEGF suppresses their antitumor function due to the capability of these cells of expressing VEGF receptors once they have been activated and have migrated to the tumor site (Soto-Ortiz 2016). VEGF has immune-modulating properties, which include decreasing the influx of lymphocytes and dendritic cells (DCs) into the tumor while increasing the intratumoral frequencies of regulatory T cells (TREGs) and myeloid-derived suppressor cells (MDSCs). MDSCs have been recently identified as a further major component of the microenvironment, inversely linked with outcome, representing a heterogeneous population of myeloid progenitors and precursors of granulocytes, macrophages, and dendritic cells. MDSCs can inhibit T-cell responses limiting immune therapeutic approaches and are induced by various factors, such as VEGF, expressed or secreted in states of cancer, inflammation, or trauma. Importantly, this systemic immunosuppression induced by excess VEGF can be reversed by the blockade of VEGF/VEGFR2 signaling pathway (Gabrilovich et al. 1999). Therefore, VEGF inhibition suggests synergism of immunotherapeutic effector mechanisms.

In addition to its ability to promote an immunosuppressive local tumor microenvironment, VEGF has profound effects on immune regulatory cell function, specifically inhibiting dendritic cell maturation and antigen presentation contributing to the suppression of antitumor immune responses (Oyama et al. 1998). In patients with colorectal cancer, bevacizumab has been shown to improve the antigen-presenting capacity of circulating dendritic cells (Osada et al. 2008). Furthermore, treating mice with recombinant VEGF at concentrations similar to those observed in patients with advanced-stage cancer induced T-cell defects via inhibition of Delta ligand signaling through Notch.

Furthermore there is evidence that E-selectin expression induced by bevacizumab facilitates lymphocyte adhesion and rolling. In addition, CD31 influences adhesive and signaling functions for vascular cellular extravasation. These results are consistent with previous observations of anti-VEGF treatment increasing lymphocyte tumor infiltrates in adoptive therapy models. Further evidence for immunologic changes resulting from bevacizumab was demonstrated in the peripheral blood through increasing circulating memory T cells (Hodi et al. 2014), providing a definite role for bevacizumab in effecting broad changes in the circulating immune composition.

Thus, the concept of antagonizing VEGF accompanied by immune-modulating properties could provide an attractive approach for enhancing immune responses (Fig. 3).

Indeed, anti-angiogenic agents have the potential to modulate the tumor microenvironment and improve immunotherapy, but often they are used at high doses in the clinic to prune tumor vessels and paradoxically may compromise various therapies. Recently Huang et al. demonstrated that targeting tumor vasculature with lower vascular-normalizing doses, but not high antivascular/anti-angiogenic doses, of an anti-VEGF receptor 2 (VEGFR2) antibody results in a more homogeneous distribution of functional tumor vessels (Huang et al. 2012). In addition, lower doses are superior to the high doses in polarizing tumor-associated macrophages from an immune inhibitory M2-like phenotype toward an immune stimulatory M1-like phenotype and in facilitating CD4+ and CD8+ T-cell-dependent manner in both immune-tolerant and immunogenic murine breast cancer models. These findings indicate that vascular-normalizing lower doses of anti-VEGFR2 antibody can reprogram the immunosuppressive tumor microenvironment in a manner that augments anticancer vaccine therapy.

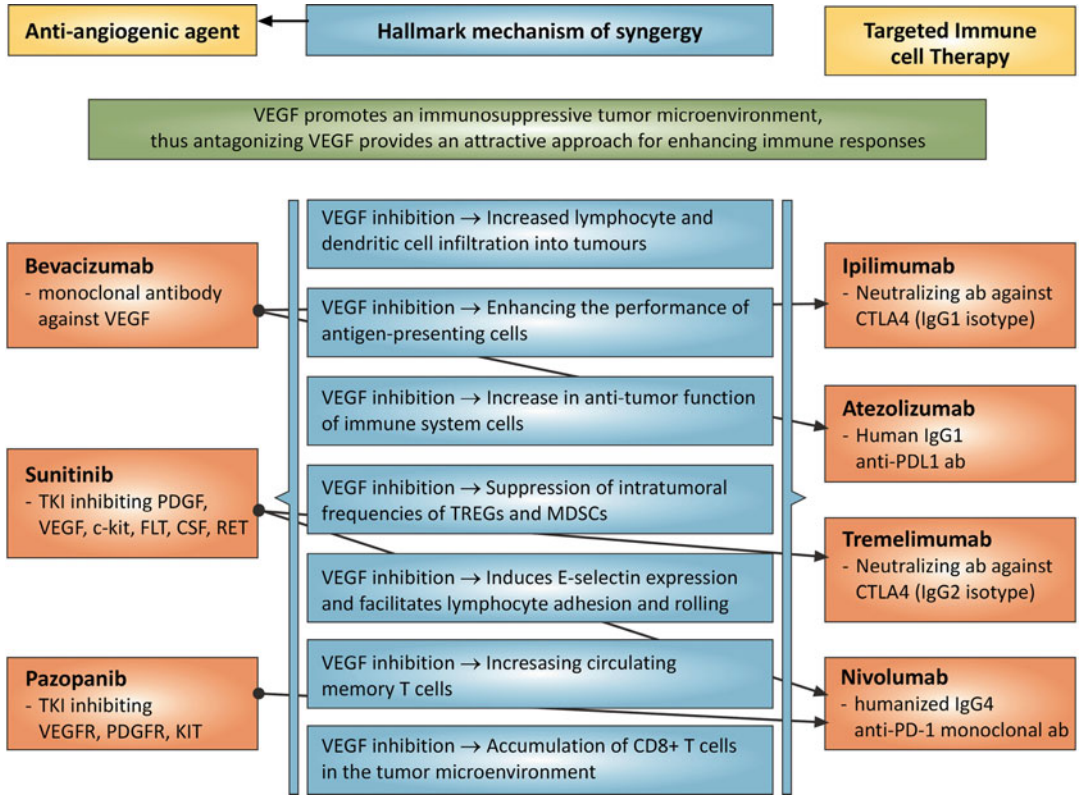

Fig. 3 Schematic representation of the main mechanisms of action postulated to mediate synergistic effects of anti-angiogenics and targeted immune cell therapy

Combining VEGF and CTLA4 Blockade

The VEGF inhibitor bevacizumab has recently been combined with ipilimumab, a monoclonal antibody that inhibits the checkpoint receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA4) for advanced-stage melanoma. A total of 46 patients with metastatic melanoma were treated with this combination, and the efficacy was remarkably good, resulting in a median overall survival of more than 2 years (Hodi et al. 2014). High-grade toxicity was more common than expected for either drug alone, but it was manageable and included inflammatory events such as hypophysitis, temporal arteritis, dermatitis, hepatitis, and uveitis. Interestingly, the combination led to an accumulation of CD8+ T cells and DCs in the tumor microenvironment – suggesting synergism of immunotherapeutic effector mechanisms – and warrants further investigation of this combination.

The anti-CTLA-4 mAb tremelimumab administered with the VEGFR TKI sunitinib produced partial remissions in 9/21 evaluable patients with renal cell carcinoma but was associated with acute renal toxicity, which the authors proposed might be immune related (Rini et al. 2011).

Further investigation is needed to evaluate the mechanistic basis of bevacizumab activity and the full impact of clinical activity. Continued development of immune checkpoint and anti-angiogenic combination therapies are warranted for the treatment of melanoma and other cancers.

Combining VEGF and PDL1/PD1 Blockade

The VEGFR TKIs sunitinib and pazopanib are standard of care in the treatment of patients with metastatic renal cell carcinoma; however their antitumor effects are not durable. As it was

hypothesized that anti-VEGF strategies suppress regulatory T cells to attenuate tumor-induced immunosuppression and might sensitize tumors to immunotherapy when used in combination, nivolumab has been combined with either sunitinib or pazopanib in patients with metastatic renal cell carcinoma. Nivolumab is a fully human monoclonal antibody inhibiting the programmed death-1 immune checkpoint receptor to restore T-cell antitumor immune responses. It also demonstrated clinical activity in metastatic renal cell carcinoma (mRCC) (Motzer et al. 2015). The median progression-free survival was 48.9 versus 31.4 weeks for sunitinib plus nivolumab and pazobanib plus nivolumab, respectively. The authors concluded that combination therapy with sunitinib plus nivolumab showed encouraging antitumor activity and was associated with a manageable safety profile in patients with mRCC. They also noted that the combination therapy resulted in responses that were higher than previously reported for monotherapy of either agent. However, the combination of pazopanib plus nivolumab is not a feasible treatment option at the dose and schedule studied here, because of dose-limiting toxicities, including liver enzyme elevations and fatigue.

Atezolizumab is a human anti-PD-L1 monoclonal antibody preventing PD-L1 binding to the inhibitory receptors PD-1 and B7.1 on activated T cells and has demonstrated clinical activity in various cancers including metastatic renal cell carcinoma (McDermott et al. 2016). In April 2016, the FDA granted priority review to atezolizumab for patients with locally advanced or metastatic non-small cell lung cancer who express PD-L1 and have progressed after a platinum-containing regimen. In May 2016 it was approved by the FDA for the second-line treatment of advanced bladder cancer.

As bevacizumab has been proposed to enhance the antitumor effects of atezolizumab by blocking VEGF-related suppressive effects on immune function and lymphocyte traffic, a multicenter phase Ib study was conducted to determine the safety and activity of atezolizumab plus bevacizumab in a cohort of metastatic renal cell carcinoma patients. The combination of atezolizumab and bevacizumab showed strong antitumor activity with an overall response rate of 40% (in 4 of 10 patients). In addition, increases in tumor-infiltrating CD8+ T cells were observed on-treatment and the combination was well tolerated (Sznol et al. 2015). A phase II trial of atezolizumab +/− bevacizumab versus sunitinib in patients with previously untreated metastatic renal cell carcinoma is currently ongoing.

Interestingly, the anti-PD-L1 antibody atezolizumab was also investigated in colorectal cancer in an open-label, multicenter phase Ib study. Patients were either treated with atezolizumab plus bevacizumab in refractory metastatic colorectal cancer (Arm A) or with atezolizumab plus bevacizumab plus chemotherapy FOLFOX in oxaliplatin-naïve metastatic colorectal cancer (arm B). Both treatment combinations were well tolerated with no unexpected toxicities, and in both arms clinical activity was observed with an unconfirmed overall response rate of 8% (1/13) in arm A and 36% (9/25) in arm B (Bendell et al. 2015). Longer follow-up and randomized studies will be needed to estimate the potential benefit of adding atezolizumab to bevacizumab and chemotherapy.

Synergy Between VEGF Blockade and Temsirolimus

Temsirolimus is a mTOR (mammalian target of rapamycin) inhibitor that inhibits the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mTOR pathway, which is involved in protein synthesis, cellular proliferation, and tumor angiogenesis. mTOR inhibitors inhibit endothelial cell VEGF expression as well as VEGF-induced endothelial cell proliferation (Dormond et al. 2007) and are an important class of anti-angiogenic agents. Temsirolimus has been approved by the FDA to treat renal cell carcinoma.

One mechanism of tumor resistance to anti-angiogenic therapy, e.g., bevacizumab is upregulation of hypoxia-inducible factor 1α (HIF-1α), which mediates adaptive responses to hypoxic conditions (Zhong et al. 1999). HIF-1α inhibition in combination with anti-angiogenic therapy is a promising strategy for targeting

tumor resistance. Temsirolimus has been shown to inhibit the activity of mTOR and has resulted in reduced levels of HIF-1α, HIF-2α, and VEGF (Zhong et al. 1999). The discovery of the HIF-1α inhibition properties of temsirolimus makes it an ideal candidate for combination with bevacizumab.

However, in treatment-naïve patients with mRCC, the combination of a VEGF pathway and a mTOR inhibitor was associated with toxicity and no apparent antitumor synergy. Some postulated that not only was the benefit of combining VEGFR TKI and mTOR inhibitors over VEGFR TKI alone affected by dose reductions required for toxicity but also that the dose reductions may negatively affect the benefit expected from first-line VEGFR TKI therapy. Also in mRCC patients previously treated with VEGFR TKI, combining bevacizumab and temsirolimus required significant dose reductions and discontinuations and even applying this combination at full doses of each drug resulted in modest activity overall and would not be recommended for routine clinical use (Mahoney et al. 2016).

In contrast, in a phase I clinical study of 41 heavily pretreated patients with gynecological malignancies, after all 37% of the patients achieved disease control (Piha-Paul et al. 2014). Of note, in this study, the combination of bevacizumab and temsirolimus showed excellent tolerance without dose-limiting toxicity even when the maximum FDA-approved dose of each drug was used in the combination. Further study of bevacizumab and temsirolimus in larger populations at least with gynecological cancers may be warranted.

Synergy of Three Targeted Agents Including VEGF Blockade

There are several compelling rationales for combining bevacizumab, temsirolimus, and cetuximab in treating advanced malignancies:

(i) Bevacizumab and cetuximab may be synergistic.
(ii) Temsirolimus inhibits mTOR and the PI3 kinase/AKT/mTOR pathway as well as CYP2A, which may be a resistance mechanism for cetuximab.
(iii) Temsirolimus attenuates upregulation of HIF-1α levels, which may be a resistance mechanism for bevacizumab.
(iv) The three agents have non-overlapping toxicities.

Liu et al. investigated safety and responses in 21 patients with advanced solid tumors treated with these combined three targeted agents (Liu et al. 2016). The authors conclude that the combination of bevacizumab, temsirolimus, and cetuximab demonstrated promising activity with an overall response rate of 33% with 11% (2/18) partial responses and 22% (4/18) stable diseases but at the expense of toxicity. Overall, 11/21 (52%) of patients treated on the trial developed grade 3 to 4 toxicities including among others hyperglycemia, hypophosphatemia, headache, fatigue, leukopenia, and anemia, respectively. This reflects synergistic toxicity that could limit further development of this combination.

Unlike these findings, the combination of cetuximab, erlotinib, and bevacizumab that was investigated in a phase I trial of 34 patients with non-small cell lung cancer (NSCLC) was well tolerated (Falchook et al. 2013). Of the NSCLC patients in this trial, the most common treatment-related grade > 2 adverse events were rash (41%), hypomagnesemia (27%), and fatigue (15%). The overall response rate in these heavily pretreated patients was 32% (11/34) and thus comparable to results of the abovementioned trial applying the triple combination of bevacizumab, temsirolimus, and cetuximab.

In another phase I trial, 32 patients with different types of solid tumors received the combination of everolimus, bevacizumab, and panitumumab (Vlahovic et al. 2012). This trial was also well tolerated and appeared to have only moderate clinical activity in refractory tumors.

In summary, the results of combined three targeted agents including VEGF inhibitors fail to come up to expectations.

Combined Blockade of VEGF and Ang2 Signaling: Humanized Tri-specific Nanobody

As already mentioned above, therapies targeting single antigens with monospecific antibodies have shown limited efficacy in patients with cancer. Advances in antibody engineering technologies have enabled strategies that simultaneously target multiple receptors to circumvent the limitations of conventional monospecific therapies and achieve enhanced therapeutic efficacy.

Besides VEGF, angiopoietin2 (Ang2) is an important player in angiogenesis. Ang2, primarily expressed by endothelial cells, is a ligand of the Tie2 receptor, and Ang2/Tie2 signaling regulates tumor vessel plasticity, allowing vessels to respond to other angiogenic factors (Fig. 4). Its in vivo inhibition results in tumor growth inhibition and vasculature changes. The inhibition of Ang2 is currently being tested in phase II/III trials of the peptibody trebananib in ovarian cancer. In a randomized phase III trial in patients with recurrent ovarian cancer, trebananib was tested in combination with paclitaxel compared with chemotherapy alone and demonstrated improvement in progression-free survival (7.2 month vs 5.4 months, HR 0.66, $p < 0.0001$) (Monk et al. 2014).

Both proangiogenic pathways (VEGF/VEGFR and Ang2/Tie-2) have been reported to synergize and to cross talk with Ang2 enhancing VEGF signaling and VEGF upregulating Ang2 expression on endothelial cells. Thus, combined inhibition of VEGF and Ang2 might well result in modulation of tumor angiogenesis and reduced tumor growth rate with improved clinical efficacy compared to VEGF pathway blockade alone.

Limited clinical experience of dual blockade is available. Recently, phase I data of the bispecific human anti-Ang2/anti-VEGF-A antibody RG7221 were reported. The maximum tolerated dose (MTD) was not reached with only one dose-limiting toxicity (DLT) reported (fatal pulmonary hemorrhage). Hypertension was the most common observed adverse event. Previous clinical experience with nanobodies in different disease showed acceptable safety profile with no specific side effect related to this technology.

Recently, the humanized tri-specific nanobody BI 836880 comprising two single variable domains blocking VEGF and Ang2, and an additional module for half-life extension in vivo has been generated. This VEGF/Ang2 blocking nanobody was highly potent and showed in vivo monotherapy efficacy (tumor growth inhibition) in several tumor xenograft models representing colon cancer, non-small cell lung cancer,

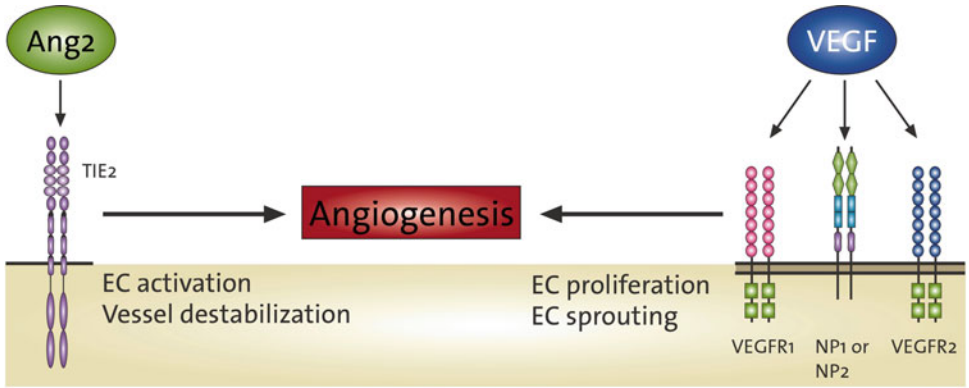

Fig. 4 Mode of action of BI 836880. BI 836880 binds the soluble ligands VEGF-A and angiopoietin Ang2 and inhibits proangiogenic signaling by their receptors, VEGFR2 and Tie2, respectively. Preclinical data demonstrate cross talk between the VEGF and Ang2 pathways, where inhibition of Ang2 increases VEGF expression, providing additional rationale for dual target inhibition

mammary cancer, ovarian cancer, pancreatic cancer, and renal cell cancer. In addition, the nanobody was found to inhibit signaling downstream of VEGF and Ang2, leading to a decrease of endothelial cell proliferation. Combined blockade of VEGF and Ang2 signaling pathways was found superior to inhibition of the individual pathways in patient-derived xenograft studies. The molecule was well tolerated in cynomolgus monkeys.

This novel VEGF/Ang2 blocking nanobody showed promising properties in vitro and in vivo, which strongly support the evaluation of this molecule in the clinic.

At present, a first-in human phase I, nonrandomized, open-label, multicenter dose escalation trial of the VEGF/Ang2 blocking nanobody BI 836880 administered by repeated intravenous infusions in patients with solid tumors is under way.

A Novel VEGFR2 Targeted Antibody-Based Fusion Protein (mAb04-MICA)

Very recently, a novel human IgG1 antibody (mAb04) specific for VEGFR2 was generated. This antibody had high affinity to VEGFR2 and exhibited anti-angiogenic activity both in vitro and in vivo (Xie et al. 2014a). To enhance the immunostimulatory activity of mAb04, this antibody was fused to MHC class I-related chain A (MICA). MICA is one of the major ligands for the NKG2D (natural killer (NK) cell receptor NK group 2, member D) which represents an activating receptor expressed on NK cells, the major effectors of antibody-dependent cellular cytotoxicity (ADCC). Thus, binding of MICA to NKG2D is thought critical for activating NK-mediated immunosurveillance.

In humans, MICA is often overexpressed in many tumor tissues from patients with epithelial tumors and some primary leukemia cells. However, since the tumors progressed despite the expression of MICA, it appeared that the MICA-NKG2D system was functionally compromised in these patients (Wu et al. 2004). Studies found that tumor cells avoid the response of NKG2D through shedding MICA from the cell surface, and this soluble MICA hinders recognition of the MICA-expressing tumor cells, thereby impairing the antitumor immune response.

Therefore, mAb04-MICA was designed and produced with the goal of reinforcing the immune surveillance activity of NK cells while retaining the anti-angiogenic and antineoplastic activity of mAb04. Indeed, mAb04-MICA localized in tumor lesions via the recognition of mAb04 to tumor cell surface VEGFR2 and attracted NK cells to the tumor lesions through the associated MICA. In human breast tumor-bearing nude mice, the antibody-based fusion protein mAb04-MICA demonstrated superior antitumor efficacy compared to combination therapy of mAb04 plus docetaxel or bevacizumab plus docetaxel, highlighting the immunostimulatory effect of MICA.

In conclusion, this novel VEGFR2 targeted antibody-based fusion protein mAb04-MICA provides a new inspiration for antitumor treatment and might have prospects for clinical application.

Conclusion

Abnormal vessel growth and function are hallmarks of cancer, and they contribute to disease progression. Therapeutic approaches to block vascular supply have reached the clinic, but limited efficacy and fast development of resistance pose unresolved challenges. A question of high priority is whether the approved anti-angiogenic regimes are optimally used in terms of dosing, duration, and combination therapy. Clinicians should acknowledge that the ability to predict which combinations are best suited for which malignant indications or clinical scenarios currently still lacks sophistication.

However, the field is developing rapidly, and the goal is to move from an era of empirical combinations to one of rational design by considering the compatibility of mechanisms that interacts synergistically, either to mediate antitumor efficacy or to reduce on-target side effects. A very promising combination approach involves delivering anti-angiogenics and targeted therapy – a newer type of cancer treatment that interferes with specific molecules involved in cancer cell growth and survival. Targeting of VEGF(R) combined with EGFR inhibition resulted in encouraging preclinical

results. However, these results did not translate into clinics, at least in patients with previously untreated, metastatic colorectal cancer, where the combined therapy of bevacizumab with cetuximab or panitumumab failed to improve progression-free survival or overall survival due to reasons that are not fully understood.

In contrast, since VEGF promotes an immunosuppressive tumor microenvironment, antagonizing VEGF provides a very attractive approach for enhancing immune responses, and thus VEGF inhibition is a very promising combination partner for targeted immunotherapy. Combining VEGF with CTLA4 blockade as well as with PDL1/PD1 blockade provided clinical activity in advanced-stage melanoma. This strategy is currently tested in clinical trials investigating nivolumab or pembrolizumab and bevacizumab in, e.g., metastatic renal cell carcinoma, high-grade glioma, or glioblastoma (clinicaltrials.gov). The combined blockade of VEGF and angiopoietin2 signaling with a humanized tri-specific nanobody and novel VEGFR2 targeted antibody-based fusion proteins are other emerging directions for the medical treatment targeting angiogenesis. In conclusion, angiogenesis-based drug combinations may provide novel, selective, safe, and reasonable future treatment options.

Cross-References

- Inhibition of Tumor Angiogenesis in GIST Therapy
- Inhibition of Tumor Angiogenesis in the Treatment of Lung Cancer
- Mechanisms of Anti-angiogenic Therapy
- Mechanisms of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- The Value of Anti-angiogenics in Breast Cancer Therapy
- The Value of Anti-angiogenics in Head and Neck Cancer Therapy
- The Value of Anti-angiogenics in Prostate Cancer Therapy

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Part X

Anti-angiogenics in Gastrointestinal Cancers

Anti-angiogenic Targeting in Metastatic Colorectal Cancer Therapy

Thomas Zander, Valentin Goede, and Michael Hallek

Contents

Introduction 380
Colorectal Cancer 380
The Role of Tumor Angiogenesis in Colorectal Cancer 380

Anti-angiogenic Treatment 381
Approved Monoclonal Antibodies and Derived Constructs 381
Approved Tyrosine Kinase Inhibitor Regorafenib 387
Anti-angiogenic Drugs in Development in mCRC 388

Summary 390

Cross-References 391

References 391

Abstract

Angiogenesis has been identified as a hallmark of cancer. Thus, anti-angiogenic targeting has been evaluated in cancer. Colorectal cancer is one of the entities where this therapeutic principal has been most successfully introduced into the daily care of patients. Today, several anti-angiogenic drugs are approved in all lines of metastasized colorectal cancer (mCRC). In adjuvant settings, anti-angiogenic treatment did not show any benefit. In summary, still overall the benefit from anti-angiogenic treatment is modest and the identification of specific patient subgroups benefiting from this treatment is missing. Several new drugs are in development to further improve the treatment of patients with mCRC. In addition, large efforts were made to identify predictive biomarker, but so far, none of these has entered the clinical routine. Here we present the current status of anti-angiogenic drugs in mCRC and the new drugs in development for this clinical entity.

Keywords

Colorectal cancer · Metastatic · Chemotherapy · Clinical Trial · Bevacizumab · Aflibercept · Ramucirumab · Regorafenib · VEGF · VEGFR

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Introduction

Colorectal Cancer

Colorectal cancer is one of the leading cancer health-care problems worldwide. It is estimated that 1.3 million people were newly diagnosed with colorectal cancer in 2012 and 690,000 died from this disease. Over the last years, the incidence of colorectal cancer has been rising particularly in those countries where this disease traditionally had a low incidence. In several developed countries with traditional high incidence, mortality of colorectal cancer has decreased during the last decade. This is most likely due to the introduction of effective screening and the development of new treatments for metastatic colorectal cancer (Torre et al. 2015). At initial diagnosis, about 25% of patients present with hematogenous metastasis and additional 25% of patients develop metastases subsequently (Ferlay et al. 2013; Siegel et al. 2014). In the last 20 years, the median overall survival has significantly improved for patients with metastasized colorectal cancer from 12 months when 5-fluorouracil (5-FU) single agent was given (Van Cutsem et al. 2001) to about 30 months as of today with combined chemotherapy and biological agents (Cremolini et al. 2015b). In detail, the first step of treatment improvement was the introduction of the two cytostatic agents oxaliplatin and irinotecan, which allowed for the doublet chemotherapy regimens FOLFOX (5-FU, folinic acid, oxaliplatin) (de Gramont et al. 2000) and FOLFIRI (5-FU, folinic acid, irinotecan) (Douillard et al. 2000), respectively. Both regimens were safe and showed superior activity compared to 5-FU monotherapy. Even the triplet chemotherapy FOLFOXIRI exploiting the synergistic activity of the three chemotherapeutic agents could be applied safely (Falcone et al. 2007). In addition to these chemotherapeutic agents, subsequently biological agents were introduced into the therapy of mCRC, namely, bevacizumab, aflibercept, ramucirumab, regorafenib, cetuximab, and panitumumab, further widening the therapeutic armamentarium for the treatment of mCRC. Along with constantly improving methods of local treatment, multiple options are now available to treat patients with mCRC.

In this chapter, we will focus on the role of anti-angiogenic agents in the treatment of mCRC. In this context, we will discuss approved drugs as well as further anti-angiogenic drugs in development.

The Role of Tumor Angiogenesis in Colorectal Cancer

Tumor angiogenesis is a prerequisite of neoplastic growth and represents a hallmark of cancer (Hanahan and Weinberg 2000). Vascular endothelial growth factor (VEGF) is one of the key regulators of tumor angiogenesis. The VEGF family consists of different member (VEGF A, VEGF B, VEGF C, VEGF D, and placental-growth factor, PlGF). VEGF-A is a survival factor for endothelial cells (EC) and induces proliferation, thus playing a central role in the process of sprouting angiogenesis (Tung et al. 2012). In solid tumors, VEGF is expressed by different cells of the tumor stroma as well as by tumor cells. The role of VEGF expression levels as a prognostic marker in colorectal cancer patients, however, is controversial. Morphologically, in contrast to "normal," physiological angiogenesis, tumor angiogenesis results in an abnormal blood vessel network. Structurally, vessels within a tumor are often dilated, tortuous, and in an immature state (i.e., EC are not covered by pericytes). In addition, there is marked heterogeneity of distribution of vessels with both hypovascular and hypervascular areas, resulting in an increase in the interstitial fluid pressure (IFP) within tumors. It was demonstrated in several models that an increased IFP decreases the accessibility of chemotherapeutic compounds to the tumor (Carmeliet and Jain 2011). Inhibition of the VEGF pathway was shown to impair sprouting angiogenesis and to normalize the chaotic vascular structure by pruning immature vessels in a number of preclinical models and in patient with rectal cancer treated with the

anti-VEGF antibody bevacizumab (Willett et al. 2004). Vascular normalization results in an improvement of the efficacy of chemotherapy (Goel et al. 2012) or irradiation therapy in preclinical models (Winkler et al. 2004). These data in part may serve as an explanation for the fact that in colorectal cancer combinations of anti-angiogenic compounds plus chemotherapy are most effective, while anti-angiogenic monotherapy alone exerts only limited efficacy.

The platelet-derived growth factor (PDGF)-receptor (PDGFR) pathway and the Angiopoietin-Tie-2-system represent other key signaling pathways involved in tumor angiogenesis. Specifically, pericytes (PC) are dependent on PDGF produced by endothelial cells to support their interaction with EC, thus playing an important role during angiogenesis and vascular maturation (Heldin 2013). Interestingly, in colorectal cancer patients, high tumor expression of the ligand PDGF-BB was associated with significantly poorer survival compared to low PDGF-BB expression (Nakamura et al. 2008).

With respect to the angiopoietin-Tie-2-system, the ligands angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) bind to the Tie-2 receptor. Binding of Ang-1 to the Tie-2 receptor induces signal transduction leading to a stabilization of the EC/PC interaction. In contrast, Ang-2 binds to the Tie-2 receptor without inducing a signal, thereby inhibiting the activity of Ang-1. Consequently, overexpression of Ang-2, as can be found in different solid tumor types including colorectal cancer, results in a more immature vascular state (Maisonpierre et al. 1997). Based on these key angiogenic pathways, a number of compounds have been developed to target tumor angiogenesis: (i) Monoclonal antibodies targeting the VEGF-pathway (i.e., bevacizumab, ramucirumab); (ii) a fusion construct targeting VEGF A, VEGF B, and placental growth factor (PlGF) (i.e., aflibercept); and (iii) a number of different tyrosine kinase inhibitors (TKI) targeting the VEGF pathway and other pathways involved in tumor angiogenesis.

Anti-angiogenic Treatment

Approved Monoclonal Antibodies and Derived Constructs

Bevacizumab

Bevacizumab is a recombinant humanized monoclonal antibody targeting VEGF-A.

First-Line Treatment

Chemotherapy Doublets ± Bevacizumab

In the pivotal phase III randomized AVF2107 trial, initially three therapeutic regimens, 5-FU/folinic acid plus bevacizumab, 5-FU, folinic acid and irinotecan (IFL), and IFL plus bevacizumab, were compared in 923 mCRC patients in the first-line setting (Hurwitz et al. 2004). Since the toxicity profile of IFL plus bevacizumab was acceptable at an interim analysis after 300 patients, overall 813 patients were recruited to be treated in the two primary comparison arms IFL +/− bevacizumab. The primary endpoint was overall survival (OS). Progression-free survival (PFS) and overall response rate (RR) were secondary endpoints. Addition of bevacizumab to IFL leads to a significant increase in median OS of almost 5 months from 15.6 to 20.3 months, Hazard Ratio (HR) 0.66;$p < 0.001$. Similarly, progression-free survival time was significantly increased from 6.2 to 10.6 months, HR 0.54;$p < 0.001$ as well as overall response rate (34.8–44.8%; $p < 0.001$). The main grade 3/4 toxicity conferred by bevacizumab was hypertension. However, this side effect was easily manageable. The importance of this trial should be underlined as it represents the first randomized phase III trial formally proofing the hypothesis that blockade of VEGF by a monoclonal antibody is active in cancer patients improving overall survival when combined with chemotherapy.

In the phase III NO16966 trial following a 2x2 factorial design FOLFOX or XELOX ± bevacizumab was compared in 1401 mCRC patients. The primary endpoint of this study was PFS. The addition of bevacizumab- to oxaliplatin-based first-line chemotherapy significantly improved median PFS from 8.0 months to 9.4

months, HR 0.83; p = 0.0023. Interestingly, the improvement in median PFS "*on-treatment*" was even more pronounced (10.4 months in the bevacizumab arm vs. 7.9 months in the placebo arm, HR 0.63; p = 0.0001). In contrast, no statistically significant improvement in median OS was found (i.e., 21.3 months in the bevacizumab arms vs. 19.9 months in the placebo arm; HR 0.89; p = 0.08). Response rates, were identical in both arms (38%, HR 1.00; p = 0.99). The study confirmed the toxicity profile of bevacizumab as no new or unexpected adverse events occurred (Saltz et al. 2008).

The results regarding the secondary endpoint of PFS "*on-treatment*" were extensively discussed. Specifically, median duration of treatment in both arms of the NO16966 study was approximately 6 months. This implicates that treatment was discontinued prior to progression in a relevant number of patients. Censoring of these patients as in the PFS "*on-treatment*" provided a significantly greater PFS benefit. This finding pointed to the hypothesis that bevacizumab should be continued until final progression of the therapy line, and this hypothesis indeed was later validated and extended in clinical trials investigating the role of bevacizumab continuation beyond progression (see 1.3.3).

As in the NO16966 study, a number of subsequent randomized clinical trials evaluating the addition of bevacizumab to first-line treatment in mCRC failed to show an overall survival benefit, while uniformly demonstrating improved progression-free survival. Interestingly, this effect was most pronounced in fluoropyrimidine monotherapy combinations (Tebbutt et al. 2010; Cunningham et al. 2013a), and this points to the role of bevacizumab in the context of less effective combination therapies (IFL) or fluoropyrimidine monotherapy.

5-FU-Based Chemotherapy ± Bevacizumab

Apart from studies examining the addition of bevacizumab to chemotherapy-doublets, randomized phase II studies have been conducted that combined 5-FU or capecitabine monotherapy +/− bevacizumab. In a small randomized phase II study, published by the group of Kabbinavar in 2003, 104 patients with previously untreated mCRC received first-line treatment with either bolus 5-FU/folinic acid (n = 36) or 5-FU/folinic acid with two doses of bevacizumab: 5 mg/kg, (n = 35) or 10 mg/kg, (n = 33) (Kabbinavar et al. 2003). The primary endpoints of time to disease progression and response rate were reached: Addition of bevacizumab increased the response rate in both arms: Control arm 17%, 5 mg/kg arm 40%, and 10 mg/kg arm 24%. The time to tumor progression was longer in the bevacizumab arms: Control arm, 2.0 months, 5 mg/kg arm 9.0 months and 10 mg/kg arm 7.2 months. OS was also improved: Control arm 13.8 months, 5 mg/kg arm 21.5 months and 10 mg/kg arm 17.3 months. Based on these data the authors recommended a dose of 5 mg/kg every 2 weeks.

Addition of bevacizumab to 5-FU/folinic acid was further evaluated in a randomized phase II trial (Kabbinavar et al. 2005) in elderly (≥65 years of age) or less fit patients not deemed to be candidates for IFL treatment. Endpoints of this trial were overall survival, progression-free survival, overall response rate, and duration of response along with safety. A total of 209 patients were randomized in this trial. Also in this poor prognostic patient population, addition of bevacizumab improved median overall survival by 3.7 months (12.9 vs. 16.6 month, HR 0.79; p = 0.16), progression-free survival by 3.7 months (5.5 vs. 9.2 months, HR 0.5; p = 0.0002), and response rate from 15.2% to 26%.

The MAX study is the largest trial investigating the addition of bevacizumab to capecitabine (Tebbutt et al. 2010). In this first-line three-arm randomized study, a 1: 1: 1 randomization was applied to compare the following treatment arms: Capecitabine at the standard dose of 2500 mg/m^2 +/− bevacizumab 7.5 mg/kg every 3 weeks. In a third arm, capecitabine + bevacizumab + mitomycin C was tested. Overall 471 patients were enrolled. Bevacizumab significantly improved PFS compared to capecitabine alone (HR 0.63; p = 0.001) and compared to the mitomycin-containing combination (HR 0.59; p = 0.001). It was demonstrated that the benefit for the addition of bevacizumab was preserved in the subgroup of

patients >75 years of age compared to the younger patients and that toxicities were not different (Price et al. 2012). Interestingly, many of the patients enrolled had only received a starting dose of 2.000 mg/m^2 capecitabine, which did not translate in an inferior clinical outcome, while the bevacizumab effect on PFS was fully preserved.

In the APEX trial, patients aged 70 years and older and not deemed candidates for oxaliplatin or irinotecan combination chemotherapy were randomized to receive capecitabine ± bevacizumab and a total of 280 patients were included (Cunningham et al. 2013a). PFS was significantly improved with bevacizumab/capecitabine compared to capecitabine (median 9.1 months vs. 5.1 months; HR 0.53; $p < 0.0001$). Treatment-related adverse events of ≥ grade 3 occurred in 40% of patients in the combination group and 22% in the capecitabine group. The most common ≥ grade 3 adverse events related to bevacizumab or chemotherapy were hand-foot syndrome (16% vs. 7%), diarrhea (7% in both arms), and venous thromboembolic events (8% vs. 4%).

Chemotherapy Triplet ± Bevacizumab

The finding that the use of all available chemotherapeutic drugs (i.e., 5-FU, oxaliplatin, and irinotecan) during the entire treatment course is correlated with improved survival compared to doublets or a monotherapy in mCRC patients led to the development of the triplet chemotherapy regime (FOLFOXIRI) consisting of all three drugs. Initially, a single-group phase 2 study of FOLFOXIRI plus bevacizumab was conducted by the Italian GONO group and safety and activity data of the combination were promising (Masi et al. 2015). This concept was further investigated by the same group in a phase III trial. Specifically, in the TRIBE study a combination of FOLFOXIRI/bevacizumab was compared with FOLFIRI/bevacizumab in 508 patients. The median follow-up was 48.1 months and the median overall survival was 29.8 months in the FOLFOXIRI plus bevacizumab group vs. 25.8 months in the FOLFIRI plus bevacizumab group (HR 0.80; $p = 0.03$). As expected, a better OS and PFS was found in patients with wild-type Ras tumors as compared to patients with mutated Ras tumors. Patients with mutated B-Raf tumors carried the most adverse prognosis. The efficiency of FOLFOXIRI/Bevacizumab, however, was preserved in all molecular groups. Interestingly, in a small subgroup of patients with mutated B-Raf tumors, FOLFOXIRI/bevacizumab compared to FOLFIRI/bevacizumab even resulted in a more pronounced improvement in OS (HR 0.54; 95% CI 0.24–1.20) pointing to a role for the combination of FOLFOXIRI/bevacizumab in patients with mutated B-Raf tumors, see Table 1 (Loupakis et al. 2014; Cremolini et al. 2015a).

Use of Bevacizumab for Conversion Therapy Based on the fact that patients with initially unresectable liver metastasis, who show a response to systemic chemotherapy, allowing complete resection of metastases, have a far better long-term outcome compared with patients treated with chemotherapy alone, conversion therapy is an approach that aims at rendering technically irresectable metastasis resectable. (Van Cutsem et al. 2016a). There is a large body of evidence for the use of EGFR-antibodies in wild-type Ras mCRC in combination with chemotherapy in this setting and triple chemotherapy regimen have also been shown to increase response as well as resection rates compared with chemotherapy doublets (Van Cutsem et al. 2016a). Table 2 gives an overview on clinical trials having tested bevacizumab combinations with respect to response and resection rates in Ras-unselected patients.

Second-Line Therapy

Bevacizumab was also tested in the second-line treatment of mCRC. Patients pretreated with 5-FU and irinotecan were randomized in a phase III trial to receive FOLFOX4 or FOLFOX4 + bevacizumab (10 mg/kg) or bevacizumab (10 mg/kg) alone as a second-line treatment. A total of 291 patients were randomized to FOLFOX4, 286 to FOLFOX4 + bevacizumab, and 243 to bevacizumab alone. Addition of bevacizumab to chemotherapy significantly increased median overall survival from 10.8 to

Table 1 Approved anti-angiogenic drugs in mCRC a key clinical trial

Trial	Line	N	Treatment	OS	PFS
AVF2017	1	813	IFL+ (A)/−(B) Bevacizumab	20.3 (A)vs. 15.6 (B) $p < 0.001$	10.6 (A) vs. 6.2 (B) $p < 0.001$
NO16996	1	1401	XELOX/FOLFOX + (A)/− (B) Bevacizumab	21.3 (A) vs. 19.9 (B) $p = 0.077$	9.4 (a) vs. 8 (B) $p = 0.0023$
ITACA	1	376	FOLFOX/FOLFIRI + (A)/− (B) Bevacizumab	20.6 (A) vs. 20.6 (A) $p = 0.278$	9.2 (A) vs. 8.4 (B) $p = 0.265$
AVEX	1	280	Capecitabine +(A)/− (B) Bevacizumab	20.7 (A) vs. 16.8 (B) $p = 0.180$	9.1 (A) vs. 5.1 (B) $p < 0.001$
CAIRO3	Maintenance	558	CAPOX + Cap/Beva maintenance (A) vs. CAPOX	21.6 (A) vs. 18.1 $p = 0.27$	8.5 (A) vs. 4.1 (B) $p < 0.001$
E3200	2	829	FOLFOX4 + (A) vs. –(B) Bevacizumab	12.9 (A) vs. 10.8 (B) $p = 0.001$	7.3 (A) vs. 4.7 (B) $p < 0.001$
ML18147	2	820	FOLFOX or FOLFIRI + (A) vs. –(B) Bevacizumab	11.2 (A) vs. 9.8 (B) $p = 0.0062$	5.7 (A) vs. 4.1 (B) $p < 0.001$
VELOUR	2	1226	FOLFIRI + (A) vs. –(B) Aflibercept	13.5 (A) vs. 12.06 (B) $p = 0.0032$	6.9 (A) vs. 4.7 (B) $p < 0.001$
RAISE	2	1072	FOLFIRI + (A) vs. –(B) Ramucirumab	13.3 (A) vs. 11.7 (B) $p = 0.021$	5.7 (A) vs. 4.5 (B) $p < 0.001$
CORRECT	3	760	BSC + (A) vs. –(B) Regorafenib	6.4 (A) vs. 5 (B) $p = 0.0052$	1.9 (A) vs. 1.7 (B) $p < 0.001$

AVF2017 (Hurwitz et al. 2004)**; NO16966** (Saltz et al. 2000); ITACA (Passardi et al. 2015); AVEX (Cunningham et al. 2013a); CAIRO3 (Simkens et al. 2015); E3200 (Giantonio et al. 2007); ML18417 (Bennouna et al. 2013); VELOUR (Van Cutsem et al. 2016b); RAISE (Tabernero et al. 2015); CORRECT (Grothey et al. 2013)

Table 2 Conversion chemotherapy approach in patients with liver-limited disease. Overview on studies including bevacizumab treatment

Study	Regimen	Patient number (n)	Response rates [%]	Liver resection rates [%]	Inclusion criteria
GONO	FOLFOXIRI + bevacizumab	30	80	40	metastatic colorectal cancer (mCRC)
TRIBE	FOLFIRI + bevacizumab FOLFOXIRI + bevacizumab	508	65 vs. 53	15 vs. 12 (R0) n.s.	mCRC
BOXER	CAPOX + bevacizumab	45	78	40	Primarily irresectable liver only metastasis
OLIVIA	FOLFOXIRI + bevacizumab vs. FOLFOX6 + bevacizumab	80	81 vs. 62	54 vs. 36 (R0)	Primarily irresectable liver only metastasis

GONO (Masi et al. 2010)**; TRIBE** (Loupakis et al. 2014)**; BOXER** (Wong et al. 2011)**; OLIVIA** (Gruenberger et al. 2015)

12.9 months ($p = 0.0011$), median progression-free survival from 4.7 to 7.3 month ($p < 0.0001$), and overall response rate from 8.6% to 22.7%. The bevacizumab mono arm was inferior to the chemotherapy alone arm (OS 10.2 months, PFS 2.7 months, and overall response rate 3.3%) (Giantonio 2007).

In another more recent second-line trial, the dose of bevacizumab (5 or 10 mg/kg) does not seem to have a major effect. In a randomized phase III trial (Iwamoto et al. 2015), patients were treated with FOLFIRI +5 or 10 mg/kg bevacizumab after progression during an oxaliplatin-based chemotherapy as differences in

progression-free survival or overall survival were observed (Table 1).

Maintenance Therapy

A potential role for maintenance therapy became evident for the first time, when the results of the NO16966 study demonstrated an improved PFS in the subgroup of patients receiving chemotherapy only arm until disease progression.

The Concept Trial in part fits into the concept of maintenance therapy as it compared intermittent versus continuous treatment. Specifically, patients either received FOLFOX7 and bevacizumab 5 mg/kg every 2 weeks (CO-arm) continuously or intermittent oxaliplatin (IO). This IO-arm consisted of 8 cycles of FOLFOX4/bevacizumab followed by 8 cycles without oxaliplatin (i.e., 5-FU and bevacizumab) and so on. The study had to be terminated prematurely. The time to failure of treatment strategy (TTF) was longer in the IO arm: TTF reached a median of 25 weeks in the IO-arm compared to 18 weeks in CO-arm, HR 0.58; p = 0.0025 (Hochster et al. 2014).

Later, the MACRO trial (Díaz-Rubio et al. 2012) examined a bevacizumab-based maintenance strategy applying a noninferiority design. Following induction chemotherapy of six cycles of XELOX/bevacizumab in the first-line treatment of mCRC, XELOX/bevacizumab was compared with bevacizumab monotherapy given until disease progression. The primary endpoint was PFS, and secondary endpoints were OS, objective RR, time to response, duration of response, and safety. A total of n = 480 patients were included. After a median follow-up of 29 months, no significant differences in PFS and OS were found between the arms. The median PFS was 10.4 months in the continuous XELOX/bevacizumab arm and 9.7 months in the bevacizumab mono arm (HR 1.10; 95% CI 0.89–1.35, ns). Median survival was 23.2 months in XELOX/bevacizumab arm and 20.0 months in the bevacizumab-mono arm (HR 1.05; p = 0.65). Importantly, second-line therapies were well balanced between the two arms (72% and 74% of patients received at least one second-line treatment). Thus, although the noninferiority of bevacizumab versus XELOX plus bevacizumab could not be formally confirmed statistically, a median PFS detriment >3 weeks could be excluded, pointing to a possible role of bevacizumab monotherapy as a maintenance concept. In this respect two further trials have recently broadened the data base.

In the AIO 0207 study and the CAIRO-3 study, the concept of maintenance therapy was further evaluated including treatment arms of bevacizumab monotherapy during maintenance (Simkens et al. 2015). The AIO 0207 trial was an open-label, noninferiority, randomized phase III trial. Following 24 weeks of induction therapy (fluorouracil/leucovorin, oxaliplatin plus bevacizumab or capecitabine, oxaliplatin plus bevacizumab), patients without disease progression during induction therapy were randomly assigned to the following treatment arms: fluoropyrimidine plus bevacizumab, bevacizumab alone, or no treatment at all. At first progression, re-induction with all drugs of the induction treatment was a planned part of the protocol. The primary endpoint was "time to failure of strategy," defined as time from randomization to second progression after maintenance (and if applicable re-induction), death, or initiation of further treatment including a new drug. Consequently, for patients who did not receive re-induction, time to failure of strategy was equivalent to time to first progression. A total of 472 patients were randomized. Median time to failure of strategy was 6.9 months for the fluoropyrimidine/bevacizumab arm, 6.1 months for the bevacizumab-mono arm, and 6.4 months for the no treatment arm. Bevacizumab monotherapy was noninferior to standard fluoropyrimidine/bevacizumab (HR 1.08;95% CI [0·85–1·37]; p = 0.53),whereas no treatment was not (HR 1.26 [0·99–1·60];p = 0·056). OS was similar in both arms in this trial (Hegewisch-Becker et al. 2015). The results of this study support the concept of maintenance therapy. Accordingly, discontinuation of oxaliplatin following induction phase and maintenance with a fluoropyrimidine and bevacizumab has evolved as a practical approach in the clinic to prevent oxaliplatin related neurotoxicity.

In the phase III CAIRO-3 study, induction treatment consisted of six 3-weekly cycles of capecitabine, oxaliplatin, and bevacizumab.

Patients without disease progression were then randomized to either maintenance treatment with capecitabine/bevacizumab or observation. On first progression (PFS1), patients in both groups were to receive the induction regimen again until second progression (PFS2), which was the study's primary endpoint. Median PFS2 was significantly improved in the maintenance group (11.7 months vs. 8.5 months in the observation group, HR 0.67; $p < 0.0001$). Maintenance therapy was well tolerated; however, the rate of hand foot skin reactions was increased (23% all grades). Maintenance treatment resulted in a nonsignificant absolute increase in median overall survival of 3.5 months (from 18.1 to 21.6 months).This study again underlined the role of maintenance therapy with a fluoropyrimidine and bevacizumab in the first-line treatment of mCRC patients.

Finally, the DREAM trial (Tournigand et al. 2015) conducted by the French GERCOR working group study evaluated the combination of erlotinib with bevacizumab as a maintenance therapy. In this randomized phase III study, patients received induction chemotherapy consisting of a chemotherapy doublet plus bevacizumab. If at least stable disease was reached, they were randomized between bevacizumab monotherapy (7.5 mg/kg every 3 weeks) or a combination of bevacizumab and erlotinib (150 mg/day). The primary endpoint was PFS during maintenance therapy. Of 694 patients who started induction therapy, 446 patients were randomized. Maintenance PFS was improved from 4.57 to 5.75 months (HR 0.72; $p = 0.005$). Even the PFS from registration was significantly improved (10.2 vs. 9.23 months, HR 0.73; $p = 0.0045$). As expected, diarrhea (all grades 58% versus 12% and skin rash 85% versus 8%) was the main side effect attributable to the addition of erlotinib. This concept, however, has not gained a role in clinical practice due to increased toxicities and the fact that erlotinib is not approved for the treatment of mCRC (Table 1).

Bevacizumab Treatment Beyond Progression

Retrospective analyses have indicated that patients without response to combination chemotherapy and bevacizumab treatment experienced an improvement in overall survival and progression-free survival by the addition of anti-angiogenic therapy to chemotherapy (Grothey et al. 2008). This provided the rational for maintaining bevacizumab treatment also in case of progression and switching the chemotherapeutic agents. Beneficial effects for the continuation of bevacizumab beyond progression were subsequently demonstrated in registries (Grothey et al. 2008; Bendell et al. 2012). In the ML18147 study, 820 patients treated with chemotherapy plus bevacizumab in first line were randomized to receive standard second-line chemotherapy with or without bevacizumab. Patients were eligible if they progressed within 3 months after the end of first-line treatment. The continuation of bevacizumab beyond progression significantly increased overall survival from 9.8 to 11.2 ($p = 0.0062$) months and progression-free survival from 4.1 to 5.7 months ($p < 0.0001$) (Bennouna et al. 2013). No new safety signals were observed in this trial. The gain in overall survival and progression-free survival was significant but modest. A similar trial was performed by the Italian group (BEBYP) which stopped recruitment prematurely due to the presentation of the ML18147 trial. Nevertheless, the analysis of the 185 recruited patients confirmed the statistical benefit of maintaining bevacizumab in second line with an improvement in progression-free survival from 5 to 6.8 months ($p = 0.010$) and in overall survival from 14.1 to 15.5 months ($p = 0.043$) (Table 1) (Masi et al. 2015).

Aflibercept

Aflibercept is a soluble decoy receptor fusion protein consisting of the second IgG-like domain of VEGFR1, the third IgG-like domain of VEGFR2, and the human IgG1 constant region. Due to this specificity, aflibercept is able to bind VEGF-A, VEGF-B, and PIGF. PIGF has been shown to be increasingly expressed during anti-angiogenic treatment (Cao 2009; Van de Veire et al. 2010; Yao et al. 2011). Antibodies against PIGF are able to block such an angiogenic rescue program (Giampieri et al. 2016). As

the decoy receptor aflibercept binds VEGF-A, VEGF-B, and PIGF, second-line treatment following a prior bevacizumab challenge appeared a promising strategy, thus defining the clinical setting in which this new drug could be preferentially evaluated. First early trials exploring aflibercept monotherapy as well as aflibercept, in combination with chemotherapy, confirmed the safety of this drug (Gaya and Tse 2012; Van Cutsem et al. 2013). In a subsequent phase II trial, 75 patients with refractory metastatic colorectal cancer were treated with single agent aflibercept at a dose of 4 mg/kg (Tang et al. 2012). In the bevacizumab-naive control group, the tumor control rate after 16 weeks was 20%, whereas in the bevacizumab-experienced group, the tumor control rate was only 11.8%, hence indicating limited activity of single agent aflibercept. At the same time, a randomized phase III trial was launched to investigate aflibercept in combination with irinotecan-based chemotherapy after failure of oxaliplatin-based chemotherapy (Van Cutsem et al. 2012). In this VELOUR trial, 1226 patients were randomized to FOLFIRI +/− aflibercept. Addition of aflibercept lead to an increase in median overall survival from 4.7 to 6.9 months (HR 0.758; $p < 0.0001$), median overall survival from 12.6 to 13.5 months (HR 0.817; $p = 0.0032$) and response rate of 11.1–19.8% ($p < 0.0001$) (Van Cutsem et al. 2012). Interestingly, the improvement in survival was independent of pretreatment with bevacizumab (Tabernero et al. 2014). Aflibercept treatment was not only associated with side effects typically attributed to anti-angiogenic drugs such as hypertension, hemorrhage, or thromboembolism but also side effects typically attributed to chemotherapy such as diarrhea and neutropenia. In contrast, addition of aflibercept to an oxaliplatin-based chemotherapy in first-line colorectal cancer did not improve patient outcome (Folprecht et al. 2016). In summary, aflibercept is a new anti-angiogenic drug available for the treatment of recurrent metastatic colorectal cancer that recently broadened the therapeutic armamentarium of this disease (Table 1).

Ramucirumab

In contrast to bevacizumab and aflibercept, ramucirumab is an antibody that specifically binds to VEGF receptor 2. Ramucirumab prevents binding of VEGF to its natural receptor expressed on target cells, thereby inhibiting the activation of the downstream signaling cascades. In preclinical models, ramucirumab demonstrated antitumor activity (Fontanella et al. 2014). In a phase I trial exploring ramucirumab in patients with solid tumors, a reduction of vascularity and perfusion was observed (Spratlin et al. 2010). To further evaluate the therapeutic potential of ramucirumab in colorectal cancer, a phase III randomized trial was launched in patients with recurrent metastatic disease. In this trial, ramucirumab (8 mg/kg) was added to a FOLFIRI-based chemotherapy. A total of 1072 patients were enrolled. Addition of ramucirumab increased median overall survival from 11.7 months to 13.3 months (HR0.84; $p = 0.0219$). Progression-free survival increased from 4.5 months to 5.7 months (HR0.79; $p = 0.0005$). The toxicity profile was typical of anti-angiogenic drugs with hypertension being a leading adverse event. The results of this trial lead to the recent approval of ramucirumab for second line treatment of colorectal cancer (Tabernero et al. 2015).

To date no direct comparison between the different anti-angiogenic drugs exist. In first-line treatment, only bevacizumab has proven beneficial, whereas in second-line treatment three different anti-angiogenic drugs are available. Of these, bevacizumab has also been shown to be beneficial when added to an oxaliplatin-based chemotherapy while for ramucirumab and aflibercept improved outcome has only been demonstrated so far for a combination with irinotecan-based chemotherapy (Table 1).

Approved Tyrosine Kinase Inhibitor Regorafenib

Regorafenib is a multityrosine kinase inhibitor that potently targets several kinases involved in tumor angiogenesis. As many patients with metastatic colorectal cancer remain in relatively good

performance status even after two lines of chemotherapy, regorafenib was evaluated in third-line treatment. In the CORRECT trial, 760 patients were randomly assigned to regorafenib or best supportive care. The primary endpoint was overall survival. Regorafenib increased the overall survival from 5 to 6.4 months (HR = 0.77; p = 0.0052). However, a significant number of patients experienced clinically meaningful side effects, mainly mucositis (Grothey et al. 2013). Nevertheless, on the basis of the available data, regorafenib was approved for the treatment of recurrent metastatic colorectal cancer.

The data were later validated in an Asian study cohort in the CONCUR trail with very similar results. Median OS was 8.8 months in the regorafenib arm compared to 6.3 month in the placebo arm (HR0.55; p = 0·00016). Again, considerable toxicity was seen (Table 1) (Li et al. 2015a).

Anti-angiogenic Drugs in Development in mCRC

Several new anti-angiogenic drugs were more recently developed clinically in colorectal cancer where we are still awaiting final clinical results (Tampellini et al. 2016).

Fruquintinib

Fruquintinib is a tyrosine kinase inhibitor that targets VEGFR1-3 and is administered orally. In early clinical trials in metastatic colorectal cancer, a dose with manageable toxicity profile was established. These trials showed also promising efficacy of this drug with a disease control rate of 83% and a progression-free survival at 16 weeks of 65% (Cao et al. 2016). A randomized phase II trial with fruquintinib was performed in metastatic colorectal cancer refractory to previous treatments. This study met its primary endpoint with an increase in progression-free survival from 1 to 4.7 months (HR 0.3; $p < 0.001$) and an increase in disease control rate from 20.8% to 68.1% (Li et al. 2015b). We are awaiting results from a randomized phase III trial that is still recruiting.

Nintedanib

Nintedanib is a multikinase inhibitor targeting VEGFR 1–3, FGFR 1–3, as well as PDGFA and PDGFRB. In a randomized phase I/II trial, nintedanib was compared with bevacizumab when added to FOLFOX in previously untreated metastasized colorectal cancer. In this study, bevacizumab was slightly superior concerning the primary endpoint of PFS rate at 9 months: 62.1% (95% CI 50.2–73.9) in the nintedanib group and 70.2% in the bevacizumab arm), although the response rate was higher in the nintedanib group (63.5% and 56.1%) (Van Cutsem et al. 2015). Currently, nintedanib is evaluated as a single agent in last line treatment of colorectal cancer in a randomized design with comparison to best supportive care (Van Cutsem et al. 2016c).

Famitinib

Famitinib is a multikinase receptor inhibitor targeting VEGFRs 2 and 3, KIT, PDGFRa, and RET. In a phase I trial, good tolerability was observed (Zhou et al. 2013). In a 2:1 randomized phase II trial in refractory metastatic colorectal cancer, an increase in median progression-free survival from 1.5 to 2.8 months (HR 0.58 p = 0.0034) and disease control rate from 30.9% to 57.5% (p = 0.0023) was observed. The safety profile was favorable (Xu et al. 2015).

Brivanib

Brivanib is an orally available tyrosine kinase inhibitor which blocks VEGFR-2 and FGFR-1/FGFR-2 signaling. After promising results in early clinical trials in colorectal cancer, brivanib was investigated in a randomized phase III trial in KRAS wild-type recurrent mCRC after prior treatment in combination with cetuximab (Siu et al. 2013). This trial did not meet its primary endpoint overall survival although progression-free survival was prolonged by adding brivanib. Importantly, because a higher rate of gastrointestinal and dermal toxicity as well as increased hypertension rates was observed, this concept was no longer pursued.

Cediranib

Cediranib is a tyrosine kinase inhibitor with high affinity to all VEGF receptors and additionally some activity to PDGF and KIT. In early trials, cediranib was combined with FOLFOX and demonstrated manageable toxicity profiles (Satoh et al. 2012). Subsequently, several randomized clinical trials were launched evaluating the addition of cediranib to oxaliplatin-based chemotherapy in first-line metastatic colorectal cancer. In the HORIZON I trial, different doses of cediranib added to FOLFOX were tested in a phase II design. No difference in progression-free survival was observed (Cunningham et al. 2013b). Further evaluation in a phase III trial recruiting 502 patients receiving FOLFOX/CAPOX with or without cediranib resulted in a minimal increase of progression-free survival of 0.3 months but no improvement in overall survival or response rate (Hoff et al. 2012). The HORIZON III trial was designed as a double-blind randomized phase III trial. FOLFOX + bevacizumab was compared with FOLFOX + cediranib in 1422 patients. No major difference was seen in the efficacy of these two treatments, but cediranib lead more often to delays of treatment (Schmoll et al. 2012). As of today, no further trials are planned to develop cediranib in colorectal cancer.

Lenvatinib

Lenvatinib is a multityrosine kinase inhibitor targeting VEGFRs 1–3, FGFRs 1–4, PDGFRA, KIT, and RET. The drug has been successfully applied in radio-iodine refractory metastasized thyroid cancer. No trial has specifically evaluated lenvatinib in metastasized colorectal cancer so far.

Linifanib

Linifanib is a tyrosine kinase inhibitor targeting VEGFRs 1–3 and PDGFRB. Linifanib showed single agent activity in a variety of solid tumors. Therefore, a randomized trial was performed in colorectal cancer, evaluating two doses of linifanib added to standard FOLFOX and compared to FOLFOX + bevacizumab. This trial did not meet its primary endpoint (PFS) nor did it demonstrate relevant differences in response rate or overall survival between treatment arms. Slightly increased toxicity was observed in the linifanib arm, leading to a higher rate of treatment discontinuation (O'Neil et al. 2014). Another phase II trial explored the response rate tolinifanib given as single agent in recurrent colorectal cancer. In 23 evaluated patients, the response rate was 0% (NCT01365910).

Motesanib

Motesanib is an orally available multikinase inhibitor targeting VEGFRs 1–3 and KIT together with several other kinases. When given at the maximum tolerated dose of 125 mg, some activity has been observed in advanced solid tumors. The toxicity profile of motesanib was tested in a larger phase I trial, in which it was combined either with FOLFOX or FOLFIRI plus panitumumab in a total number of 119 patients. Unfortunately, response rates were relatively low (24% in first line, 14% in second line). A substantial benefit of motesanib for this refractory patient population therefore seems unlikely (Tebbutt et al. 2015).

Tivozanib

Tivozanib also targets VEGFRs 1–3, KIT, and PDGFRB and is orally available. Safety of tivozanib in combination with FOLFOX was evaluated in a phase I clinical trial in metastasized colorectal cancer. In this early trial, some clinical activity was assumed as one of 30 patients experienced a complete remission and then a partial response (Oldenhuis et al. 2015). In a subsequent randomized phase II trial, tivozanib was compared with bevacizumab in patients with metastasized colorectal cancer receiving FOLFOX as the chemotherapy backbone. No significant difference in response rate or progression-free survival was observed (Benson et al. 2016). The safety profile of this agent appeared very similar to the other multikinase inhibitors described above.

Trebananib

Trebananib is an angiopoietin-1 and angiopoietin-2 neutralizing peptide. Angiopoietins play an important role in pathological vascular remodeling, thus making trebananib a promising agent for treatment of cancer. After defining a

maximum tolerated dose in a phase I trial in refractory solid tumors (Peeters et al. 2013). Trebananib was evaluated in a randomized phase II trial in combination with FOLFIRI in patients with recurrent colorectal cancer after oxaliplatin-based chemotherapy. This trial did not meet its primary endpoint with no significant difference in progression-free survival (3.5 vs. 5.2 months, $p = 0.33$). No greater grade III/IV toxicities were observed (Tampellini et al. 2016).

Vandetanib

Vandetanib is a multikinase inhibitor with primary affinity to EGFR and VEGFR2 and additionally to RET, EPH receptor, and SRC family members. After vandetanib had demonstrated preclinical activity in several tumor models including colorectal cancer, its toxicity profile in combination with chemotherapy was evaluated specifically in metastasized colorectal cancer. Toxicity of vandetanib in combination with FOLFOX and FOLFIRI was manageable, but efficacy of this combination in randomized phase II trials was not promising (Tampellini et al. 2016). The combination of vandetanib with cetuximab and irinotecan was feasible but demonstrated no favorable efficacy (Meyerhardt et al. 2012). In contrast, combination of vandetanib with bevacizumab and capecitabine with oxaliplatin demonstrated an unfavorable toxicity profile (Cabebe et al. 2012).

Vatalanib

Vatalanib targets VEGFRs as an orally available tyrosine kinase inhibitor. Combination with FOLFOX demonstrated a favorable toxicity profile, but efficacy was low in randomized phase II trials in refractory colorectal cancer. Therefore, this drug is not further developed in colorectal cancer (Hecht et al. 2011; Sobrero and Bruzzi 2011; Van Cutsem et al. 2011).

Additional New Anti-angiogenic Agents in Early Clinical Development

Additional new agents are in early clinical development and still await clinical evaluation in colorectal cancer. For example, cabozantinib as a multikinase inhibitor is approved for medullary thyroid cancer. A phase I trial is performed to evaluate the tolerability of cabozantinib in combination with panitumumab in colorectal cancer (NCT02008383). Sevacizumab is a new antibody against VEGF-A and currently evaluated in a phase I trial. This drug will be further explored specifically in metastatic colorectal cancer (NCT02453464). A new antibody against VEGF-C (VGX-100) is currently evaluated in phase I trials in combination with bevacizumab. Apatinib is another multikinase inhibitor which has shown clinical activity mainly in gastric cancer. It is now evaluated in metastatic colorectal cancer in an open label randomized phase II trial (NCT01531777). Vanucizumab is a bispecific antibody against angiopoietin-2 and VEGF-A. In an early phase I trial, a safe dose was established with mostly hypertension, headache, and asthenia occurring as side effects. Currently a phase II trial is performed in metastatic colorectal cancer comparing bevacizumab with vanucizumab when added to FOLFOX (NCT02141295).

Summary

Altogether these trials indicate that there is a benefit for the addition of anti-angiogenic drugs to standard chemotherapy in mCRC in first-line, maintenance, second-line treatment and for a treatment beyond progression. The beneficial effect is modest in an unselected population but the low toxicity profile warrants this anti-angiogenic treatment. Due to the successful introduction of anti-angiogenic drugs in the treatment of colorectal cancer, many companies have tried to establish new agents in this disease. However, the majority of these drugs have failed to show sufficient efficacy while exerting relevant toxicity or are still in clinical development

Today the differential role of the different anti-angiogenic drugs in mCRC remains to be established. In addition, there is still a major need for the identification of subgroups particularly sensitive to anti-angiogenetic targeting.

Cross-References

- Anti-angiogenics in Gastroesophageal Cancer
- Anti-angiogenics in Pancreatic Cancer Therapy
- Cytotoxics and Anti-angiogenics: Metronomic Therapies
- Inhibition of Tumor Angiogenesis in GIST Therapy
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- The Value of Anti-angiogenics in Head and Neck Cancer Therapy

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Anti-angiogenics in Gastroesophageal Cancer

Ulrich Hacker and Florian Lordick

Contents

Introduction 396

The Role of Angiogenesis in Gastroesophageal Cancer 396
Biological Background 396
Key Angiogenic Pathways in Gastric Cancer 397
Cellular Components of the Tumor Stroma that Drive Angiogenesis in Gastric Cancer 399

Potential Novel Targets and Drug Development 400

Angiogenesis-Related Biomarker Research and Gastric Cancer 402

Clinical Results on Angiogenesis Inhibitors in Gastroesophageal Cancer 403
Antibodies 403
Tyrosine Kinase Inhibitors 406

Future Directions 408

Cross-References 408

References 409

Abstract

Gastroesophageal cancer represents a leading cause of cancer death worldwide. Over the last decades, significant improvements have been made in the systemic chemotherapy of both locally advanced and metastatic gastroesophageal cancer, and human epidermal growth factor receptor 2 (HER2) has been implemented as an important target for molecular stratified treatment. Overall, however, the prognosis of advanced gastroesophageal cancer remains poor. Preclinical data clearly indicate that angiogenesis plays a pivotal role in gastroesophageal cancer driving progression and metastasis. Consequently, anti-angiogenic treatment strategies have been tested in a number of clinical trials. Currently, there is a growing body of evidence that anti-angiogenic treatment strategies result in improved clinical outcome in gastroesophageal

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cancer. Ramucirumab, a monoclonal antibody directed against vascular endothelial growth factor receptor 2 (VEGFR2), has proven efficacy in the second-line treatment of advanced gastric cancer, either given alone or in combination with paclitaxel. Combinations of platinum-based combination chemotherapy and ramucirumab in the first-line setting of advanced disease and in the perioperative setting of localized disease are under way. This chapter describes the role of angiogenesis in gastroesophageal cancer biology and gives a comprehensive overview on recent clinical trials with respect to anti-angiogenic treatment strategies.

Keywords
Gastric cancer · Angiogenesis · Ramucirumab · Bevacizumab · Apatinib · Chemotherapy

Introduction

Gastroesophageal cancer is a global health problem, with 1,417,000 newly diagnosed patients per year and 1,123,000 annual deaths from this diagnosis (Ferlay et al. 2015). The incidence and geographical distribution of gastroesophageal cancer vary: non-cardia gastric cancer is more prevalent in East Asia, East-Central Europe, Latin America, and Africa, whereas adenocarcinomas of the distal esophagus, the gastroesophageal junction (GEJ), and the proximal stomach are more prevalent in Western Europe, North America, and Australia (Colquhoun et al. 2015). Gastroesophageal cancers are clinically aggressive. In the Western hemisphere, most patients present with locally advanced or metastatic disease, which mandates the use of systemic chemotherapy, either perioperatively or in the palliative setting (Lordick and Janjigian 2016).

For patients with gastroesophageal cancer that is not amenable to complete resection owing to metastatic disease, palliative chemotherapy can prolong survival and improve symptoms and quality of life compared with best supportive care (BSC) alone (Wagner et al. 2006). Chemotherapy combinations comprising platinum compounds (e.g., oxaliplatin or cisplatin) and fluoropyrimidines (5-fluorouracil [5-FU], capecitabine, or S-1) are more effective than fluoropyrimidine monotherapy in the first-line setting (Lordick et al. 2014). The addition of a third chemotherapy agent – docetaxel or epirubicin – in patients with good functional and nutritional status and with uncompromised organ functions can improve disease control and tumor response rate, which translates to a modest overall survival (OS) benefit when compared with doublet therapy (Van Cutsem et al. 2006, 2015). In the second-line setting, cytotoxic monotherapy (irinotecan, docetaxel, or paclitaxel) has been established as a standard of care (Lordick 2012; Lordick et al. 2014).

Human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor receptor 2 (VEGF-R2) are clinically validated molecular targets in the treatment of advanced-stage gastroesophageal cancer. Trastuzumab (an HER2-targeting monoclonal antibody) and ramucirumab (an anti-VEGF-R2 antibody) are now considered standard-of-care treatments for metastatic gastroesophageal cancer (Bang et al. 2010; Fuchs et al. 2014; Wilke et al. 2014), but the availability of these drugs differs among countries. A recently proposed treatment algorithm, based on national and international guidelines and on our interpretation of the latest published data, is shown in Fig. 1 (Lordick 2015; Lordick and Janjigian 2016).

This algorithm underscores the emerging role for anti-angiogenic treatment of gastroesophageal cancer in clinical practice and highlights the need to understand the pathophysiological role of angiogenesis in this disease, mechanisms of response and resistance, and potential biomarkers.

The Role of Angiogenesis in Gastroesophageal Cancer

Biological Background

The term angiogenesis describes the formation of new blood vessels from preexisting vascular structures. In tumors, angiogenesis is driven by pro-angiogenic factors derived from tumor cells and from cellular components of the

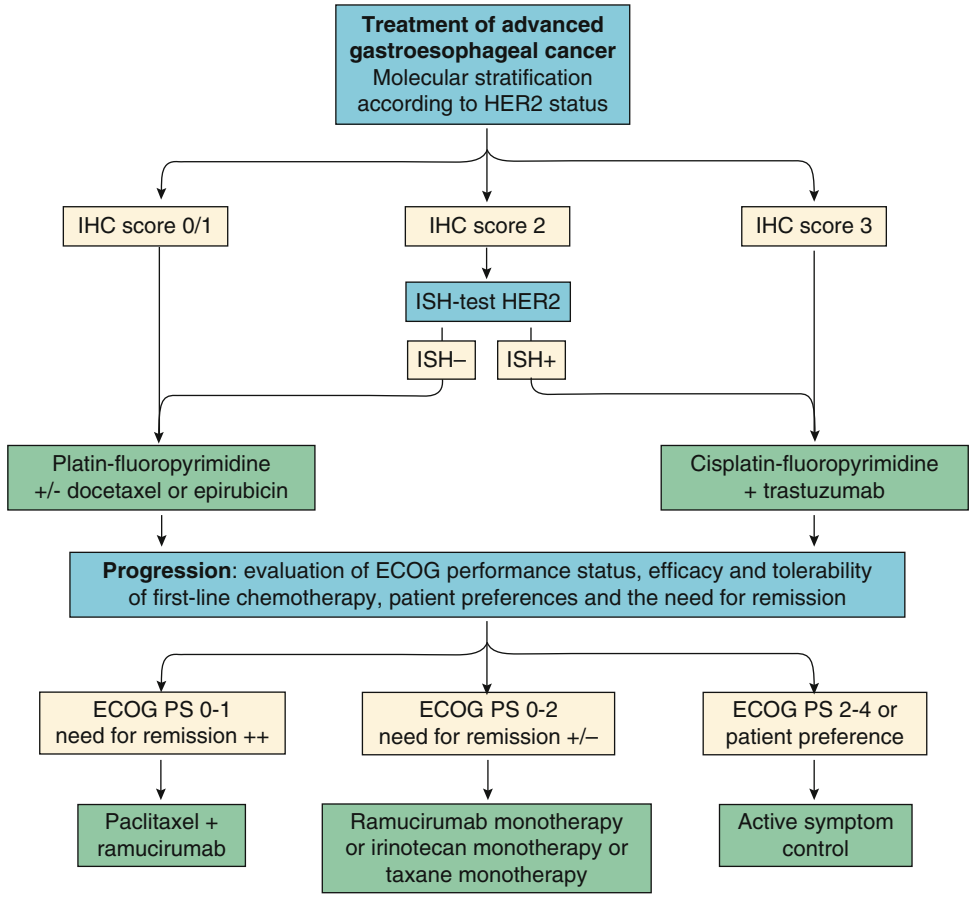

Fig. 1 Proposed treatment algorithm for advanced gastroesophageal cancer based on published recommendations. *ECOG* Eastern Cooperative Oncology Group, *IHC* immunohistochemistry, *FISH* fluorescence in situ hybridization (Adapted with permission © Lordick 2015; Lordick and Janjigian 2016)

surrounding tumor stroma. The process of angiogenesis has to be distinguished from vasculogenesis, which means the formation of primitive vascular structures during early embryonic development, driven by vascular precursor cells derived from the bone marrow. While angiogenesis clearly represents a hallmark of cancer and plays a prominent role in tumor progression and metastasis, the tumor-promoting role of vascular precursor cells is still controversial due to conflicting preclinical data and may only have a limited role in human solid tumors.

With respect to gastroesophageal cancer, scientific evidence suggests that angiogenesis is centrally involved in tumor growth and metastasis, as early results have indicated that tumor vascularization correlated with hematogenous metastasis and prognosis (Tanigawa et al. 1996).

Key Angiogenic Pathways in Gastric Cancer

Vascular Endothelial Growth Factor (VEGF)

The vascular endothelial growth factor (VEGF) family comprises of different members (i.e., VEGF-A to VEGF-D and placental growth factor [PlGF]). In addition, a number of VEGF-A isoforms have been described, which are generated by alternative splicing. They exert their biological activity through binding to VEGF receptors (VEGF-R1, VEGF-R2, and VEGF-R3). While

VEGF-R1 and VEGF-R2 are expressed on endothelial cells (EC) and play a central role in sprouting angiogenesis, VEGF-R3 is expressed on lymphatic vascular cells and has a role in lymphangiogenesis. VEGF is expressed by tumor cells and by different cell types of the tumor stroma, like cancer-associated fibroblasts. In contrast to "normal," physiological angiogenesis, tumor angiogenesis driven by proangiogenic factors results in the formation of a structurally abnormal blood vessel network, which causes an increase in interstitial fluid pressure within tumors and in turn a decrease in the accessibility of chemotherapeutic compounds.

In gastric cancer, high VEGF expression has predominantly been found in intestinal-type tumors correlating with vessel counts (Takahashi et al. 1996). Blood VEGF levels correlated with clinical stage and highest VEGF concentrations were found in metastatic gastric cancer patients (Yoshikawa et al. 2000). Preclinical data showed that invasion of gastric cancer cells was driven by VEGF in an αvβ6 integrin-dependent manner (Zhao et al. 2010). The expression of VEGF-C, which plays a role in lymphangiogenesis, was associated with intestinal-type cancer and correlated with lymphatic invasion and lymph node metastasis (Onogawa et al. 2005). Finally, VEGF has been described as a prognostic marker in gastric cancer patients with advanced or metastatic disease (Van Cutsem et al. 2012).

In esophageal cancer, tumor angiogenesis is also centrally involved.

VEGF expression and VEGF blood levels were increased both in squamous cell carcinoma and adenocarcinoma patients compared to healthy controls, and there was a correlation with vessel counts (Kitadai et al. 1998). Interestingly, VEGF-R1 expression in the tumor tissue was correlated with dissemination of tumor cells to the bone marrow in patients with esophageal cancer (42% adenocarcinoma) underlining a role of this pathway in metastatic progression (Schultze et al. 2012).

Platelet-Derived Growth Factor (PDGF)

Platelet-derived growth factor (PDGF) signaling is centrally involved in tumor angiogenesis. Four PDGF genes (PDGF-A to PDGF-D) are known, forming five dimeric PDGF isoforms (i.e., PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD). Signal transduction is mediated by PDGF-receptor α (PDGF-Rα) and PDGF-Rβ as well as heterodimeric PDGF-Rα/PDGF-Rβ complexes. While PDGF-AA, PDGF-AB, PDGF-BB, and PDGF-CC activate PDGF-Rα, PDGF-Rβ signaling is mediated through PDGF-BB and PDGF-DD. The heterodimeric PDGF-Rα/PDGF-Rβ complexes are activated by binding PDGF-AB, PDGF-BB, and PDGF-CC, respectively (Fredriksson et al. 2004).

PDGF-BB is abundantly expressed in fibroblasts, myofibroblasts, pericytes, and vascular smooth muscle cells (VSMC). PDGF-Rβ activation by PDGF-BB in stromal and vascular mural cells from the tumor microenvironment stimulates angiogenesis: PDGF-BB has marked effects on vascular remodeling, maturation, and stability by recruiting pericytes and VSMC to newly formed angiogenic vessels (Heldin 2012, 2013). It is believed that the responsiveness of mature vessels, which are characterized by a tight spatial interaction between EC and PC toward VEGF-targeting therapies, can be enhanced by blocking the PDGF-R-signaling cascade, thus disrupting the interaction between EC and PC, resulting in a more immature vascular state. Important interactions have been described linking VEGF-R2 activation with an inhibition of PDGF-Rβ signaling in perivascular cells. Similarly, an interaction between fibroblast growth factor-2 (FGF-2) and PDGF-BB-mediated signaling was described due to an upregulation of PDGF-Rβ upon stimulation with FGF-2 in perivascular cells (Cao et al. 2008). From this background it becomes clear that simultaneous blockade of two or more pathways represents a promising strategy to target tumor angiogenesis. In a series of 109 gastric adenocarcinoma cases, VEGF-A and PDGF-BB were simultaneously expressed at high levels in the tumor stroma of both intestinal- and diffuse-type gastric cancer, and phosphorylation of PDGF-Rβ was significantly associated with depth of invasion (Suzuki et al. 2010).

Angiopoietin: Tie2

The angiopoietin – tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-1 (Tie1) and Tie2 receptor/ligand system – is centrally involved in vessel formation and maturation. It is characterized by activities complementing the VEGF system, specifically during late stages of vessel development following sprouting angiogenesis, by promoting EC survival and vessel maturation and stabilization. The vascular state in tumors is often immature and instable, and this phenotype is related to the formation of metastasis. Consequently, it becomes clear that angiopoietin/Tie2 signaling plays a pivotal role in tumor angiogenesis and metastasis (Thurston and Daly 2012).

Ang-1, which is mainly derived from pericytes, plays a role in stabilizing vessels by interacting with the Tie2 receptor on endothelial cells (EC). In contrast, the activity of Ang-2 is related to vessel remodeling and to the generation of an immature vascular state. Consequently, a shift in the balance between Ang-1 and Ang-2 toward Ang-2 results in impaired pericyte coverage, vessel destabilization, and increased vascular permeability. Additionally, Ang-2 can directly induce sprouting angiogenesis by engaging αvβ5 integrins, and it is linked to the formation of metastases (Albini and Noonan 2012; Felcht et al. 2012; Rigamonti and De Palma 2013). Combined inhibition of VEGF and Ang-2 in a murine xenograft model resulted in improved vascular normalization. Interestingly, the effect was even better at lower doses of the anti-VEGF monoclonal antibody bevacizumab in combination with an anti-Ang-2 peptibody (L1–10) (Coutelle et al. 2015). These findings, among others, suggest that dosing of anti-angiogenic compounds is an important issue.

In the clinical setting, a significant correlation was found between Ang-2 mRNA expression in the tumor tissue of gastric cancer patients and an immature vascular state. In addition, the presence of Ang-2 and VEGF was associated with an upregulation of matrix metalloproteinases (MMP) in vitro. Finally, high Ang-2 expression was associated with shorter survival times in gastric cancer patients (Etoh et al. 2001) and there was a significantly higher expression of Ang-2 in advanced stage gastric cancer compared to early-stage disease (Sun et al. 2004). Preoperative serum Ang-2 levels were correlated with lymph node metastasis (Jo et al. 2009), and pre-therapeutic plasma Ang-2 levels were prognostic for overall survival in the AVAGAST trial (Hacker et al. 2016).

Integrins

Integrins are heterodimeric transmembrane receptors, centrally involved in the crosstalk between cancer cells as well as between cancer cells and other cellular and noncellular components of the tumor microenvironment. Integrin-signaling pathways in tumor cells are very similar to those observed in activated EC that in principle require the same functional properties to remodel during tumor angiogenesis. Sprouting EC are characterized by expression of a unique profile of integrins. Furthermore, integrin-mediated signaling occurs in tumor-associated fibroblasts and inflammatory cells that contribute to tumor angiogenesis. Finally, pericyte coverage of maturing blood vessels is influenced by integrin adhesion to ECM proteins within the tumor stroma, linking integrin function to vascular remodeling and maturation (Weis and Cheresh 2011). In a large cohort ($n = 482$) of gastric cancer patients, both αvβ3 and αvβ5 integrins were expressed in at least one tumor component (i.e., tumor or stroma). Both were expressed significantly more often in intestinal-type gastric cancer, and patients positive for expression of αvβ3 on endothelial cells showed a significantly longer survival. In addition, patients with intestinal-type gastric cancer negative for expression of αvβ5 on stroma cells had significantly longer survival (Boger et al. 2015).

Cellular Components of the Tumor Stroma that Drive Angiogenesis in Gastric Cancer

Progression of solid tumors is driven by a crosstalk of tumor cells with surrounding cells of the tumor stroma. Among them, fibroblasts

accumulate in the activated stroma. The so-called cancer associated-fibroblasts (CAF) were shown to play a pivotal role in promoting tumor growth, inflammation, angiogenesis, and metastasis in different solid tumors as well as in gastric cancer (Guo et al. 2008). In gastric cancer, the expression of galectin-1, an evolutionarily conserved glycan-binding protein with angiogenic potential, which is overexpressed in CAF, was demonstrated to be correlated with VEGF expression and CD31 expression from EC. Furthermore, high expression of galectin-1 in CAF increased proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVEC), as well as VEGF-R2 phosphorylation and enhanced VEGF expression in gastric cancer cells (Tang et al. 2016). In another translational study in gastric cancer, immunohistochemistry and mRNA expression of protein markers of CAF were used to determine their prevalence in the tumor tissue. There was a correlation with tumor size, depth of tumor invasion, lymph node metastasis, and liver or peritoneal metastasis. Importantly, CAF-specific proteins were identified that might serve as both prognostic markers and as novel targets for anticancer drugs (Zhi et al. 2010).

Macrophages represent another important cell type of the tumor stroma, involved in tumor progression and metastasis. Primarily, they play a central role in innate host defense. Precursor cells migrate to target tissues were they mature and acquire different phenotypes. Generally, macrophages have been divided into two major subtypes, M1 and M2, based on differences in cell surface receptors and gene expression data (Mantovani et al. 2005). Tumor-associated macrophages (TAM) were demonstrated to exhibit functions of M2 macrophages and can be characterized as M2d subtype according to a recent subclassification (Murray et al. 2014). M1 macrophages secrete pro-inflammatory cytokines and are involved in MHC class I- and II-mediated presentation of tumor antigens and in the stimulation of Th1 responses such as cytotoxic T-lymphocyte (CTL) generation. Therefore, they are assumed to exert antitumoral functions. In contrast, M2 macrophages secrete immunosuppressive cytokines, such as IL-10, CCL17, and CCL22, and produce pro-angiogenic and tissue remodeling factors such as VEGF, PlGF, and matrix metalloproteinase 9 (Mantovani and Sica 2010). Macrophage polarization has been demonstrated to play a crucial role in determining the maturation status of the tumor vasculature in murine models. More specifically, the soluble factor histidine-rich glycoprotein (HRG) when overexpressed in tumor cells induced vascular normalization associated with a shift toward M1 macrophages. Moreover, tumor growth and metastasis were inhibited. Mechanistically, HRG downregulated the expression of PlGF by TAM. Interestingly, HRG levels were found to be decreased in human cancer (Rolny et al. 2011). In addition, M2 macrophage-derived PlGF and VEGF-C was shown to play an important role in inducing resistance toward VEGF-targeting drugs, which are an integral part of standard treatment in many cancer entities (Fischer et al. 2007).

In gastric cancer, there is clear evidence for a tumor-promoting role of TAM. Recently, a correlation of the frequency of M2 polarized macrophages with overall survival was demonstrated in a cohort of 180 patients with gastric cancer (Zhang et al. 2015). In another study, intraperitoneal TAM in gastric cancer patients with peritoneal dissemination were found to be polarized to the M2 phenotype (Yamaguchi et al. 2015).

Potential Novel Targets and Drug Development

Targeting the VEGF axis is the most commonly used anti-angiogenic approach: the majority of currently used anti-angiogenic treatment strategies in different solid tumors like colorectal, breast, lung, ovarian, or cervical cancer rely on the blockade of this signaling pathway using monoclonal antibodies, bevacizumab (VEGF-A), ramucirumab (VEGF-R2), or the fusion construct aflibercept targeting VEGF-A, VEGF-B, and PlGF, respectively. Inhibition of the VEGF-pathway using tyrosin kinase inhibitors (TKI), targeting different VEGF-Rs, has proven to be efficient in renal cell cancer and thyroid cancer.

TKI can target different pathways, thus allowing combinations of targets that might result in improved anti-angiogenic treatment efficacy. The TKI nintedanib targets VEGF-R, PDGF-R, and fibroblast growth factor receptors. Based on the importance of these pathways for angiogenesis and tumor cell proliferation, nintedanib is a promising compound. Recently, nintedanib has been approved for second-line treatment of non-small cell lung cancer in combination with docetaxel (Reck et al. 2014) and is going to be tested in gastroesophageal cancer patients as well in the near future.

The angiopoietin/Tie2 receptor ligand system represents another attractive drug target in gastric cancer. Both a fully human species cross-reactive Ang-2 selective antibody (LC06) and a corresponding Ang-2/-1 cross-reactive antibody (LC08) have been developed. Preclinical data indicate an increased specificity of the Ang-2 selective antibody for tumor vasculature compared to the Ang-2/Ang-1 reactive antibody, which additionally induced regression of physiological vessels (i.e., in the trachea) (Thomas et al. 2013). Trebananib (formerly AMG 386) is a peptide-Fc fusion protein (i.e., peptibody) that inhibits angiogenesis by preventing the interaction of Ang-1 and Ang-2 with their receptor, Tie2 (Herbst et al. 2009). The compound is in late clinical development; however, a recent phase III study in ovarian cancer failed to demonstrate an overall survival benefit (Sheridan 2015), and in a phase II study in gastroesophageal cancer patients, there was no benefit for adding trebananib to chemotherapy (Eatock et al. 2013). These data might indicate that a combined inhibition of both Ang-1 and Ang-2 could be less effective than blocking Ang-2 alone. As an alternative combination of Ang-2/Tie2 inhibition with inhibitors of well-characterized targets like VEGF-R-signaling is attractive, since combined anti-angiogenic treatment can prevent primary or secondary resistance in preclinical models, which are mediated by the upregulation of alternative angiogenic pathways (Rigamonti et al. 2014).

In this respect, recent technological advances enabled the developed of bispecific antibodies and antibody constructs termed CovX-Bodies. These constructs consist of two different peptide pharmacophores covalently bound to the nucleophilic heavy chain lysine at position 93 located deep in the hydrophobic binding pockets on each of the two Fab arms of the scaffold antibody (Doppalapudi et al. 2010). While the antibody scaffold enables long half-life times and distribution properties very similar to IgG, the peptide pharmacophores of the CovX-Body are responsible for functional activities.

Different CovX-Bodies targeting Ang-2 were generated and extensively tested in murine models. Interestingly, these compounds were shown to reduce the frequency of pro-angiogenic TEM in the tumor stroma as well (Huang et al. 2011). The CovX-Body (CVX-060) showed good pharmacodynamic properties (half-life 110h) and efficacy. Currently this compound is being tested in early clinical trials in combination with angiogenesis-targeting TKI. Additionally, a CovX-Body that targets both Ang-1 and VEGF has been developed that showed favorable anti-angiogenic activity in murine models and is now further developed for clinical testing (Kienast et al. 2013; Thomas et al. 2012).

Although integrins play a key role in tumor cell-tumor cell and tumor cell-stroma cell interactions as well as in propagating tumor angiogenesis, recent efforts to target integrins have not been successful. In principle, integrin ligand binding can be mimicked with synthetic peptides containing the RGD sequence such as cyclic RGD peptides that competitively inhibit ligand binding to integrins, thus disrupting integrin-mediated signaling pathways. RGD-mimetic peptides or small molecules act as potent anti-angiogenic compounds by disrupting $\alpha\nu\beta3/\alpha\nu\beta5$ integrin-ligand interactions. Such compounds were shown to inhibit angiogenesis in preclinical models by blocking the proliferation of EC and by inducing apoptosis of EC. Cilengitide was the first small molecule cyclic peptide targeting the integrins $\alpha\nu\beta3$, $\alpha\nu\beta5$, and $\alpha5\beta1$ that was developed for clinical application (Mas-Moruno et al. 2010). There was a clear focus on the treatment of malignant highly vascularized brain tumors (i.e., glioblastoma). Recently, however, a

placebo-controlled randomized phase III clinical trial failed to demonstrate a benefit for the use of cilengitide combined with standard treatment in patients with glioblastoma (Tucci et al. 2014; Stupp et al. 2014; Kurozumi et al. 2012). Thus, it is unclear today, whether this class of drugs will indeed make its way to the clinic. In this respect dosing aspects again might play an important role, as low doses of cilengitide were shown to mediate pro-angiogenic activity. Another very interesting recent finding is that interventions improving angiogenesis with respect to increasing the number of functional blood vessels in tumors using a low-dose therapy regimen of cilengitide and verapamil enhanced the uptake of chemotherapy (gemcitabine), improved tumor metabolism, and resulted in reduced tumor growth and progression in murine models (Wong et al. 2015).

Besides targeting specific angiogenic pathways or pathway combinations, another attractive approach is to target cell types that are centrally involved in angiogenesis like TAM or CAF. Based on the fact that colony stimulating factor 1 (CSF-1) represents a major survival factor for macrophages in the tumor microenvironment, a monoclonal antibody against CSF-Receptor-1 (CSF-R1) was generated, which led to a depletion of TAM and showed antitumor activity in preclinical models and in patients with giant cell tumors (Ries et al. 2014). Another approach is to shift the polarization of TAM from angiogenic M2 type toward the antitumoral M1 type. However, while basic mechanisms of TAM polarization have been described in preclinical models (Rolny et al. 2011), no specific pharmacological approach is clinically available, yet.

With respect to CAF targeting, a number of immunological strategies (i.e., vaccines, T-cell therapies) have been tested (Kakarla et al. 2012). Another preclinical study demonstrated that inhibition of the PDGF-signaling axis resulted in suppression of pro-tumoral activities of CAF (Pietras et al. 2008). In this respect TKI targeting PDGF-R signaling together with other pathways like nilotinib (VEGF-R, PDGF-R, FGF-R) are promising.

Angiogenesis-Related Biomarker Research and Gastric Cancer

Over the last decade, intense efforts have been made to identify biomarkers predicting the efficacy of anti-angiogenic drugs. The largest database has been generated with respect to the anti-VEGF antibody bevacizumab, which was the first anti-angiogenic compound to enter clinical research focused on expression levels of VEGF in tumor tissue and on the measurement of VEGF in the blood (serum, plasma). Furthermore, single nucleotide polymorphisms (SNP) in angiogenesis-related genes (i.e., VEGF and VEGF receptors) were extensively studied in different cohorts. While in some studies predictive markers could be identified, overall, the results remained inconclusive and often could not be reproduced (Maru et al. 2013; Jubb and Harris 2010; Lambrechts et al. 2012).

In a cohort of patients with locally advanced or metastatic gastric cancer treated in the phase III AVAGAST trial (cisplatin/fluoropyrimidine±bevacizumab), patients with high baseline plasma VEGF-A levels showed a trend toward improved overall survival with the addition of bevacizumab (hazard ratio [HR], 0.72; 95% CI, 0.57–0.93) compared to patients with low VEGF-A levels (HR, 1.01; 95% CI, 0.77–1.31; interaction $p = 0.07$). This correlation was predominantly found in the non-Asian patient population (Van Cutsem et al. 2012).

Based on the preclinical findings that resistance toward VEGF-targeting therapy can be mediated by increased expression of Ang-2 (Rigamonti et al. 2014), the prognostic and predictive role of baseline plasma Ang-2 levels were studied in the AVAGAST trial cohort. Ang-2 did not predict efficacy of bevacizumab therapy, neither alone nor in combination with baseline plasma VEGF levels. However, Ang-2 was prognostic for overall survival, and a strong correlation was found with the occurrence of liver metastasis (Hacker et al. 2016).

Clinical Results on Angiogenesis Inhibitors in Gastroesophageal Cancer

Antibodies

Targeting VEGF with Bevacizumab

Bevacizumab is a humanized monoclonal antibody that inhibits angiogenesis by neutralizing circulating VEGF. It binds to VEGF A, preventing its binding with VEGF-R1 and VEGF-R2 on the surface of endothelial cells. Bevacizumab is currently approved for the treatment of colon, lung, breast, ovarian, endometrial, and clear cell renal carcinoma in a metastatic setting. Several phase II and three completed phase III clinical trials have investigated the efficacy of first-line bevacizumab combined with chemotherapy, in patients with locally advanced or metastatic gastroesophageal cancers.

A multicenter phase II study evaluated the efficacy and safety of the addition of bevacizumab and cisplatin-irinotecan in 47 patients with advanced gastroesophageal cancers. The overall response rate (ORR) was 65%, median time to progression (TTP) was 8.3 months, and the OS was 12.3 months.

Bevacizumab related grade 3/4 toxicity included arterial hypertension (28%), thromboembolic events (25%), gastric perforation (4.2%), and serious cardiovascular events (2.1%) (Shah et al. 2006).

In another phase II trial, bevacizumab was combined with a modified schedule of docetaxel, cisplatin, and fluorouracil in 44 patients with advanced gastroesophageal cancer. ORR was 67%, the median progression-free survival (PFS) was 12 months, and OS was 16.8 months. Although no evidence of increased chemotherapy-related toxicities with the addition of bevacizumab were shown, 39% of patients experienced venous thromboembolism (Shah et al. 2011).

Another phase II study with promising results was reported where bevacizumab was administered in combination with docetaxel and oxaliplatin in 38 patients with advanced gastroesophageal cancer. The disease control rate was 79% with a PFS of 6.6 months and an OS of 11.1 months (El-Rayes et al. 2010).

Finally, a phase II study investigated the combination of bevacizumab with capecitabine and oxaliplatin (ORR 51%, PFS 7.2 months, OS 10.8 months) (Uronis et al. 2013).

Based on these results, the international, randomized, double-blind, placebo-controlled phase III *Avastin for Advanced Gastric Cancer Trial* (AVAGAST) was conducted. AVAGAST investigated the combination of bevacizumab with cisplatin and capecitabine in previously untreated unresectable locally advanced or metastatic gastroesophageal cancers (Ohtsu et al. 2011). The primary endpoint of this study was OS. Seven hundred and seventy-four patients were enrolled and were 1:1 assigned to each treatment group. Median OS was 12.1 months in the bevacizumab arm and 10.1 months with placebo. However, this numerical difference did not meet the prespecified criteria for statistical significance (HR = 0.87, 95% CI, 0.73–1.03, $P = 0.1002$). In contrast, both PFS (median 6.7 vs. 5.3 months) and ORR (46.0% v 37.4%) were significantly improved with bevacizumab versus placebo. The incidence of grade $\geq$3 adverse events was not increased with the exception of arterial hypertension (6% vs. <1%). Interestingly, a preplanned subgroup analyses revealed regional differences in efficacy outcomes. The effectiveness of bevacizumab on all study outcomes was substantially higher among patients recruited in North and South America compared to patients recruited in Europe (intermediate effect) or in the Asia-Pacific region (no or limited effect). Different patient selection, clinical practice, and tumor and population genetics and the influence of second-line chemotherapy were discussed to explain these results (Roviello et al. 2016).

Subsequently, a smaller phase III study, with similar design as AVAGAST, was conducted in 202 Chinese patients. In the *bevacizumab plus capecitabine and cisplatin in Chinese patients with inoperable locally advanced or metastatic gastric or gastroesophageal junction cancer: randomized, double-blind, phase III study* (AVATAR) (Shen et al. 2015),

Table 1 Randomized phase III studies investigating bevacizumab in gastroesophageal cancer

Study	Indication	Patient number (n)	Regimen	Primary endpoint	Overall response rate	Progression-free survival (mon)	Overall survival (mon)
AVAGAST Ohtsu et al. (2011)	Stage IV 1°	674	CX+Bev CX +placebo	OS	46% versus 37.4% (HR = 8.61; 95% CI, 0.6–16.6; $P = 0.0315$)	6.7 versus 5.3 (HR = 0.80; 95% CI, 0.68–0.93; $P = 0.0037$)	12.1 versus 10.1 (HR = 0.87; 95% CI, 0.73–1.03; $P = 0.1002$)
AVATAR Shen et al. (2015)	Stage IV 1°	202	CX+Bev CX +placebo	OS	41% versus 34% (HR = 7.02; 95% CI, −8.3–22.4; $P = 0.34$)	6.3 versus 6.0 (HR = 0.89; 95% CI, 0.66–1.21; $P = 0.47$)	10.5 versus 11.4 (HR = 1.11; 95% CI, 0.79–1.56; $P = 0.56$)
STO3 Cunningham et al. (2015)	Stage Ib-III	1063	ECX+Bev ECX	OS	40% versus 42%; no statistical comparison	Not reported	34.5 versus 34.0 (HR 1.067; 95% CI 0.8911–1.279; $p = 0.4784$)

Bev bevacizumab, *CX* cisplatin+capecitabine, *ECX* epirubicin+cisplatin+capecitabine, *mon* months, *n* number, *OS* overall survival

the baseline patient clinical characteristics and the second line of treatments were more similar to the European-American subgroup of the AVAGAST trial. However, this study showed no significant differences between the two arms with regard to OS (10.5 months vs. 11.4 months) and PFS (6.3 months vs. 6.0 months).

Finally, ST03 was a multicenter, randomized, phase II/III study comparing perioperative epirubicin, cisplatin, and capecitabine with or without bevacizumab. The first 200 patients contributed to a phase II study powered to exclude unacceptable rates of gastrointestinal and cardiac adverse events. The incidence of cardiac complications was similar in both arms except for arterial thromboembolic events and asymptomatic drops in left ventricular ejection fraction with bevacizumab (Okines et al. 2013). Recently, survival results have been reported for STO3 in abstract form without showing any gain in OS by the addition of bevacizumab to perioperative chemotherapy (Cunningham et al. 2015).

Based on the reported results, bevacizumab is currently not an option for patients with locally advanced or metastatic gastroesophageal cancer. The results of phase III trials with bevacizumab in gastroesophageal cancer are summarized in Table 1.

Targeting VEGF-R2 with Ramucirumab

Ramucirumab, a human IgG1 monoclonal antibody directed against VEGF-R2, prevents binding of ligands to VEGF-R2 and receptor-mediated pathway activation in endothelial cells. It was shown that ramucirumab can be safely administered and that objective antitumor activity and anti-angiogenic effects are observed over a wide range of dose levels in different malignancies treated in phase I. Four (15%) of 27 patients with measurable disease had a partial response, and 11 (30%) of 37 patients had either a partial response or stable disease lasting at least 6 months. Tumor perfusion and vascularity decreased in 69% of evaluable patients. Ramucirumab 8 mg/kg intravenously given every 2 weeks was chosen as the preferred regimen for further investigation in phase III (Spratlin et al. 2010).

Anti-VEGF-R2 therapy is the first biological strategy in an unselected patient population to be associated with a survival benefit in patients with chemotherapy-refractory gastroesophageal cancer. Recently, two phase III clinical trials have showed that ramucirumab is a valuable therapeutic option in second line. Results are shown in Table 2.

Ramucirumab monotherapy for previously treated advanced gastric or gastroesophageal

Table 2 Randomized phase III studies investigating ramucirumab in gastroesophageal cancer

Study	Indication	Patient number (n)	Regimen	Primary endpoint	Overall response rate	Progression-free survival (mon)	Overall survival (mon)
REGARD Fuchs et al. (2014)	Stage IV 2°	355	Ram + BSC versus BSC	OS	3% versus 3% ($P = 0.76$)	2.1 versus 1.3 (HR = 0.483; 95% CI, 0.376–0.620; $P = 0.0001$)	5.2 versus 3.8 (HR = 0.776; 95% CI, 0.603–0.998; $P = 0.047$)
RAINBOW Wilke et al. (2014)	Stage IV 2°	665	Pac + Ram versus Pac + placebo	OS	28% versus 16% ($P = 0.0001$)	4.40 versus 2.86 (HR = 0.635; 95% CI, 0.536–0.752; $P = 0.0001$)	9.63 versus 7.26 (HR = 0.807; 95% CI, 0.678–0.962; $P = 0.0169$)

BSC best supportive care, *mon* months, *n* number, *OS* overall survival, *Pac* paclitaxel, *Ram* ramucirumab

junction adenocarcinoma (REGARD) is an international, randomized, double-blind, placebo-controlled, phase III trial. REGARD involved patients with advanced gastroesophageal adenocarcinoma and disease progression after first-line platinum- or fluoropyrimidine-containing chemotherapy (Fuchs et al. 2014). Patients were randomly assigned (2:1) to receive best supportive care plus either ramucirumab or placebo. The primary endpoint was OS. Three hundred and fifty-five patients were assigned to receive ramucirumab ($n = 238$) or placebo ($n = 117$). Median OS was 5.2 months in the ramucirumab group and 3.8 months in the placebo group. PFS with ramucirumab was 2.1 months versus 1.3 months with placebo (HR 0.483, $P < 0.0001$); the rate of disease control was significantly higher in patients given ramucirumab (49% vs. 23%, $P < 0.0001$). Ramucirumab was well tolerated. Grade 3/4 hypertension was more common in the ramucirumab group (16% vs. 8%), whereas other adverse events were similar between groups (94% vs. 88%). Performance status was maintained for a significantly longer time with ramucirumab. Patients who received at least four cycles of ramucirumab maintained their quality of life. Although no regional differences in the effects of ramucirumab were reported, the small number of Asian patients (16%) recruited for REGARD does not allow for any definitive conclusion.

Ramucirumab plus paclitaxel versus *placebo plus paclitaxel in patients with previously treated advanced gastric or gastroesophageal junction adenocarcinoma* (RAINBOW) is a randomized, placebo-controlled, double-blind, phase III trial. Patients had advanced gastroesophageal adenocarcinoma and disease progression on or within 4 months after first-line chemotherapy (platinum plus fluoropyrimidine with or without an anthracycline) (Wilke et al. 2014). The primary endpoint was OS. Six hundred and sixty-five patients were randomly assigned (1:1) to treatment with paclitaxel plus ramucirumab or placebo. OS was significantly longer in the ramucirumab plus paclitaxel group than in the placebo plus paclitaxel group (median 9.6 vs. 7.4 months). The study also met its secondary endpoint of PFS (2.9 vs. 4.4 months, HR = 0.635) and ORR (16% vs. 28%, $P = 0.0001$). The PFS rate was 36% vs. 17% at 6 months and 22% vs. 10% at 9 months. Subgroup analyses suggest that ramucirumab has similar activity in both Asian (33.5% of the study population) and non-Asian patients. Overall, ramucirumab was well tolerated, although adverse events of grade ≥ 3 were somewhat greater in the combination arm and included neutropenia (40.7% vs. 18.8%). But the incidence of febrile neutropenia was similar (3.1% vs. 2.4%). Arterial hypertension (14.1% vs. 2.4%) and fatigue (7.0% vs. 4.0%) were more common with ramucirumab (Wilke et al. 2014). A subsequent analysis showed that ramucirumab in combination with paclitaxel prolonged overall survival while maintaining patient quality of life with delayed symptom worsening and functional status deterioration (Al-Batran et al. 2016).

Based on these results, ramucirumab has been granted approval by the US Food and Drug Association (FDA) and the European Medical Agency (EMA) as second-line treatment in patients with advanced or metastatic gastroesophageal cancers who progressed on fluoropyrimidine- or platinum-containing first-line chemotherapy. However, based on economic considerations, ramucirumab is not refunded in all health systems. A biomarker-based selection of patients who have a greater benefit from anti-angiogenic treatment would probably help to convince authorities.

In contrast, ramucirumab failed to show superiority over chemotherapy alone in the first-line setting. In a double-blind phase II trial, 168 patients with previously untreated, unresectable locally advanced or metastatic gastroesophageal adenocarcinoma were randomized to receive modified FOLFOX6 plus ramucirumab or placebo (Yoon et al. 2014). PFS which was the primary endpoint and OS were similar in both arms (median PFS 6.4 vs. 6.7 months and median OS 11.7 vs. 11.5 months). Subgroup analyses suggest that the inclusion of patients with esophageal cancers (>45%) and a higher rate of treatment discontinuation before progression in the investigational arm (27% vs. 10%) may have negatively impacted on the results of the study.

The phase III *study of ramucirumab (LY3009806) in combination with capecitabine and cisplatin in participants with stomach cancer* (RAINFALL) is now recruiting 616 patients with metastatic HER2-negative gastric cancer to receive cisplatin/capecitabine chemotherapy with or without ramucirumab in first line (NCT trial number 02314117).

Tyrosine Kinase Inhibitors

Multitarget TKI represent another approach to block angiogenesis by simultaneously targeting VEGF-R and other signaling pathways.

Sunitinib

Sunitinib, an oral inhibitor of multiple kinases, has shown broad effects in different solid tumors, and its activity is mediated through platelet-derived growth factor receptor (PDGF-R), VEGF-R, KIT, Flt-3, and RET that impair tumor proliferation and angiogenesis (O'Farrell et al. 2003). In a phase II study, sunitinib at 50 mg/day for 4 weeks, followed by 2 weeks off treatment, was given to 78 patients with advanced gastroesophageal adenocarcinoma who had failed prior chemotherapy. Two patients (2.6%) had partial responses, and 25 patients (32.1%) maintained stable disease for at least 6 weeks. Median PFS was 2.3 months (95% CI, 1.6–2.6 months), and median OS was 6.8 months (95% CI, 4.4–9.6 months). Grade ≥3 thrombocytopenia and neutropenia were reported in 34.6% and 29.4% of patients, respectively, and the most common non-hematologic adverse events were fatigue, anorexia, nausea, diarrhea, and oral mucositis (Bang et al. 2011). Similar results were observed in another phase II study that enrolled 52 pretreated patients to receive sunitinib 50 mg/day for 4 weeks with 2 weeks rest until disease progression or unacceptable toxicity. The ORR was only 3.9%, median PFS was 1.3 months (95% CI, 1.18–1.90), and median OS was 5.8 months (95% CI, 3.48–12.32). Serious adverse events occurred in 26 patients, leading to 13 treatment-related deaths (Moehler et al. 2011). Due to the lack of clinically relevant activity of sunitinib as single agent in advanced gastric cancer, its role has been assessed in combination with chemotherapy. A Korean study randomized 107 patients with unresectable or metastatic gastric cancer to single-agent docetaxel (60 mg/m^2, every 3 weeks) or docetaxel (60 mg/m^{2}every 3 weeks) in combination with sunitinib (37.5 mg/day). The primary endpoint was TTP which was not significantly prolonged in the combination arm when compared with the chemotherapy alone arm: 3.9 months (95% CI 2.9–4.9) versus 2.6 months (95% CI 1.8–3.5), with an HR of 0.77 (95% CI 0.52–1.16, $p = 0.21$). Patients exposed to the combination experienced more stomatitis, diarrhea, and hand-foot syndrome (Yi et al. 2012). A phase I study evaluated the maximum tolerated dose (MTD), safety, pharmacokinetics, and antitumor activity of sunitinib plus S-1 and cisplatin in patients with metastatic gastric cancer. The oral angiogenic inhibitor was

administered on a continuous daily dosing or with a 2-weeks-on/2-weeks-off schedule (25–37.5 mg/day), plus S-1 (80–120 mg/day) and cisplatin (60 mg/m^2). MTD of sunitinib was 25 mg/day; this regimen had a manageable safety profile and promising antitumor activity. The most frequently reported ≥grade 3 adverse events were neutropenia (93.8%) and leucopenia (75.0%). The ORR was 37.5%; six additional patients did not have any disease progression for ≥24 weeks. Median PFS was 12.5 months. No pharmacokinetic interactions were observed between sunitinib and S-1 or cisplatin (Boku et al. 2014).

Sorafenib

Sorafenib is a TKI that targets VEGF-R2, PDGF-R, RET, FLT3, and RAF and interferes with tumor growth, progression, and angiogenesis. A phase I study demonstrated acceptable toxicity and preliminary efficacy when combining sorafenib (400 mg bid, days 1–35) with S-1 (40 mg/m^2 bid, days 1–21) and cisplatin (60 mg/m^2, day 8). Thirteen patients were enrolled and received at least one dose of the study treatment. No specific or serious adverse events were reported. Five patients had partial response, and eight had stable disease as best response (Yamada et al. 2014). In another dose-finding study of sorafenib in combination with capecitabine and cisplatin as first-line treatment in patients with advanced gastric cancer, sorafenib 400 mg twice daily, capecitabine 800 mg/m^2 twice daily (days 1–14), and cisplatin 60 mg/m^2 (day 1) were the recommended phase II doses found. An ORR of 62.5%, a median PFS of 10.0 months (95% CI, 7.4–13.8), and a median OS of 14.7 months (95% CI, 12.0–20.0) were reported (Kim et al. 2012). A phase II study was subsequently conducted to determine the activity and toxicity of the three-weekly combination of sorafenib (400 mg twice daily continuously), docetaxel (75 mg/m^2 day 1), and cisplatin (75 mg/m^2 on day 1) in 44 patients with advanced gastric cancer. Partial response, the primary endpoint, was reported in 41% (18 patients), and 32% (14 patients) achieved stable disease. A median PFS of 5.8 months and a median OS of 13.6 months were reported (Sun et al. 2010).

Regorafenib

Regorafenib, which targets several receptor tyrosine kinases, including VEGF-R2, also showed enhanced antitumor activity compared with placebo in a randomized phase II study in patients with gastroesophageal cancer after failure of first-line or second-line chemotherapy (Pavlakis et al. 2016). A total of 152 patients were randomly assigned, yielding 147 evaluable patients (regorafenib, $n = 97$; placebo, $n = 50$). Median PFS significantly differed between groups (regorafenib, 2.6 months; 95% CI, 1.8–3.1 and placebo, 0.9 months; 95% CI, 0.9–0.9; HR, 0.40; 95% CI, 0.28–0.59; $P < 0.001$). The effect was greater in South Korean patients compared with patients enrolled in Australia, New Zealand, and Canada (HR, 0.12 v 0.61; interaction $P < 0.001$) but was consistent across age, neutrophil-to-lymphocyte ratio, primary site, lines of chemotherapy, peritoneal metastasis presence, number of metastatic sites, and plasma vascular endothelial growth factor A. A survival trend in favor of regorafenib was seen. Twenty-nine patients assigned to placebo received open-label regorafenib after disease progression. Regorafenib toxicity was similar to that previously reported (Pavlakis et al. 2016). The next step for the investigators will be to consider the design for a phase III trial and seek funds for this.

Apatinib

Apatinib (YN968D1) is a novel, highly potent VEGF-R2 inhibitor with a binding affinity ten times that of sorafenib (Tian et al. 2011). Based on the results of a previous phase I trial showing activity in Chinese patients with metastatic gastric cancer, a phase II randomized, double-blind, placebo-controlled trial was conducted to test this new drug in pretreated gastric cancer patients. The aim of this study was to assess the activity and safety of daily administration of third-line apatinib and to compare the tolerability of a once-daily or a twice-daily regimen. One hundred and forty-four patients were randomly assigned to receive placebo (arm A), apatinib 850 mg once daily (arm B), or apatinib 425 mg twice daily (arm C). The median OS was 2.5 months for arm A (95% CI,1.87–3.70), 4.8 months for arm B (95%

CI, 4.03–5.97), and 4.3 months for arm C (95% CI, 3.83–4.77); the median PFS was 1.4 months (95% CI, 1.20–1.83), 3.7 months (95% CI, 2.17–6.80), and 3.2 months (95% CI, 2.37–4.53), respectively. Both median PFS ($p < 0.001$) and median OS ($p < 0.001$) were statistically longer in the groups exposed to apatinib, and nine patients had a partial response. Toxicities were overall tolerable and easily clinically managed. The most common grade 3–4 adverse events were hand–foot syndrome and hypertension, while severe hematologic toxicities were rare (Li et al. 2013). A recently published randomized, double-blind, placebo-controlled phase III trial of apatinib in patients with chemotherapy-refractory advanced or metastatic gastroesophageal adenocarcinoma assessed the efficacy and safety of apatinib in patients with advanced gastric or gastroesophageal junction adenocarcinoma for whom at least two lines of prior chemotherapy had failed (Li et al. 2016). OS was the primary endpoint. Two hundred and sixty-seven patients were enrolled and were randomly assigned to oral apatinib 850 mg or placebo once daily. Median OS was significantly improved in the apatinib group compared with the placebo group (6.5 months; 95% CI, 4.8–7.6 vs. 4.7 months; 95% CI, 3.6–5.4; $P = 0.0149$; HR, 0.709; 95% CI, 0.537–0.937; $P = 0.0156$). Similarly, apatinib significantly prolonged PFS compared with placebo (2.6 months; 95% CI, 2.0–2.9 vs. 1.8 months; 95% CI, 1.4–1.9; $P < 0.001$; hazard ratio, 0.444; 95% CI, 0.331–0.595; $P < 0.001$). The most common grade 3–4 non-hematologic adverse events were hand–foot syndrome, proteinuria, and hypertension.

Future Directions

While the benefit of systemic chemotherapy in locally advanced and metastatic gastroesophageal cancer has reached a plateau, the possibility of treating gastroesophageal cancer patients with novel drugs has recently emerged. Anti-angiogenic compounds are among the first effective novel drugs in this disease. Recently published studies from *The Cancer Genome Atlas* (TCGA) (Cancer Genome Atlas Research 2014) and the *Asian Cancer Research Group* (ACRG) (Cristescu et al. 2015) proposed a novel gastric cancer classification based on different molecular features. These may serve as a roadmap for future drug development and exploration of novel drug targets. The role of new anti-angiogenic drugs may be of particular relevance in specific subtypes, in which angiogenic pathways are upregulated. Nonetheless, disease anatomy and classical biology parameters remain important and should be integrated with novel molecular classifications. Understanding and targeting the mechanisms of resistance to anti-angiogenic drugs is a key point in order to improve the outcome for patients with advanced gastroesophageal cancer. Mechanisms of resistance can be VEGF axis dependent, stromal dependent, or associated with non-VEGF modulators (Jayson et al. 2016). Overcoming such mechanisms will ensure a better use and results of most novel anti-angiogenic drugs. Beyond that, a crucial issue in further development of anti-angiogenic drugs is the search for predictive biomarker tests that predict which patients will, and will not, benefit before initiation of therapy. Development of biomarkers is important because of the need to balance efficacy, toxicity, and cost. Novel combinations of these drugs with other anti-angiogenics or other classes of drugs are being developed, and the appreciation that these drugs have immunomodulatory and other modes of action will eventually lead to combination regimens that are based on these newly understood mechanisms.

Cross-References

- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Anti-angiogenics in Pancreatic Cancer Therapy

Thilo Hackert, Laura Wüsten, and Markus W. Büchler

Contents

Introduction 416

Pathogenesis 417

Experimental and Clinical Diagnostic Modalities for Angiogenesis in PDAC and pNET 418

Tumorigenesis and Angiogenesis in Pancreatic Adenocarcinoma 421
Pathophysiology 421
Experimental Studies 422

Tumorigenesis and Angiogenesis in pNET 423
Pathophysiology 423
Experimental Studies 424

Clinical Studies 424

PDAC 425
Vaccination Studies 425
Interferon Alpha 426
Antibodies and Targeted Proteins 426
Kinase Inhibitors 427

PNET 428
Kinase Inhibitors 428

Future Directions 428

Cross-References 429

References 429

Abstract

Pancreatic cancer is a highly lethal disease. Up to date, the only curative approach is surgical resection, which is only possible in a limited number of patients by the time of diagnosis. Thus, the development of new therapeutic options besides chemotherapy is extremely important for patients

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who do not qualify for surgery due to local irresectability or systemic tumor spread. During development and progression of pancreatic tumors, angiogenesis is an important mechanism to supply blood, oxygen, and nutrients for the growing tumor mass. Several angiogenic factors play a critical role during this process, including vascular endothelial growth factor (VEGF), as well as multiple factors involved in tyrosine kinase pathways, all of which are potential targets for systemic treatment approaches.

Pancreatic ductal adenocarcinoma represents the biggest proportion among all pancreatic tumor entities. It is histopathologically characterized by a hypovascular appearance and pronounced peritumoral desmoplastic tissue as well as extracellular matrix. In numerous experimental and clinical studies, anti-angiogenic therapy has been evaluated for pancreatic ductal adenocarcinoma with early promising results. However, in clinical phase III studies, only limited effects were achieved with targeted anti-angiogenic approaches.

In contrast, pancreatic neuroendocrine tumors, which are typically hypervascularized, are much more sensitive to anti-angiogenic substances. After a successful phase III study, sunitinib – a multi-targeted kinase inhibitor – has been approved for the treatment of this entity and is incorporated in current international guidelines as a second-line therapy recommendation.

The pathogenesis, diagnostic measures, as well as current experimental and clinical studies regarding angiogenesis and anti-angiogenic therapy of both pancreatic ductal adenocarcinoma and neuroendocrine tumors are summarized described in this chapter.

Keywords

Pancreatic adenocarcinoma · Neuroendocrine tumor · Angiogenesis · Anti-angiogenic therapy

Introduction

Pancreatic cancer (pancreatic ductal adenocarcinoma, PDAC) is one of the most aggressive solid tumor entities and a highly lethal malignancy. The only curative approach is surgical resection; radiotherapy, chemotherapy, or a combination of both can only act as palliative treatment or neo-/adjuvant therapy. Even with advances in surgery and conservative therapy, it remains fourth leading cause for cancer-associated mortality in Western countries with – in contrast to other malignancies – still increasing rates of incidence (Siegel et al. 2015). Symptoms including pain, jaundice, and weight loss show a late onset, and in only 15–20% of all patients, surgery is possible at the time of diagnosis. Thus, offering the chance of long-term survival only to a limited number of patients. When combined with adjuvant chemotherapy, 5-year survival rates of 20–25% can be achieved (Hackert and Büchler 2013). The importance of postoperative adjuvant chemotherapy has been demonstrated in large randomized studies during the last two decades (Valle et al. 2014; Oettle et al. 2013; Ryan et al. 2014) and represents the standard of care for all patients that are considered to be resectable by the time of diagnosis. In contrast, in a situation of systemic spread, especially peritoneal carcinomatosis or liver metastases, only palliative treatment is possible (Tempero et al. 2014; Seufferlein et al. 2012). For this purpose, standard chemotherapies include gemcitabine which can be supplemented by nab-paclitaxel as another cytotoxic substance or erlotinib, a tyrosine kinase inhibitor (see below) (Moore et al. 2007). An alternative treatment in PDAC stage 4 is the application of Folfirinox – a combination of 5-fluorouracil, folinic acid, irinotecan, and oxaliplatin – which seems to be the most effective substance today (Conroy et al. 2011). With the introduction of this regimen, median survival times in the palliative setting have increased from 6.8 months (gemcitabine) to 11.1 months (Conroy et al. 2011). As these outcomes are still unsatisfactory and a high proportion of patients suffer from early progressive disease under a palliative chemotherapy, there is urgent need for other approaches to improve the prognosis. During the last 20 years, the concept of anti-angiogenic therapy as a supplement or alternative to

classical chemotherapy has been examined in a large number of tumor entities and numerous experimental as well as clinical studies have been conducted for PDAC as well as for pancreatic neuroendocrine tumors (pNETs).

Angiogenesis describes the formation of new blood vessels from preexisting vessels and – besides its physiological function, i.e., during wound healing or normal tissue growth – has been recognized as a pathophysiological phenomenon in many solid tumor entities (Craven et al. 2016).

Consequently, the pathophysiology and the factors involved in the de novo generation of vessels by tumors have been examined in many experimental models and translational studies with the aim to target these events and develop selective and potentially effective therapeutic approaches [see below]. Especially vascular endothelial growth factor (VEGF) pathway inhibitors represent the currently most promising substances. However, the clinical results with regard to oncological outcome in terms of progression-free and overall survival strongly depend on the nature of the tumor itself, as some tumors show sensitivity to these approaches (i.e., renal cell or ovarian cancer), whereas other entities are mostly resistant (i.e., prostate cancer or malignant melanoma). Regarding pancreatic tumors, very heterogeneous effects of anti-angiogenic therapy are observed, especially with regard to the differentiation between PDAC and pNET.

Pathogenesis

Tumor angiogenesis is a crucial mechanism for the supply of oxygen, glucose, and other nutrients to sustain the growth of tumor cells after they have initially survived via the mechanism of diffusion or the physiological blood supply at their original location in the parenchyma of an organ. Consequently, two distinct phases can be described during the process of tumor angiogenesis of pancreatic tumors (Bergers 1999).

The first phase, also referred to as pre-vascular phase, is characterized by an increase in tumor cell proliferation with an adequate apoptotic counterbalance resulting in a plateau phase of tumor cell growth. This plateau can exist for years as a premalignant condition and leads to the histopathological appearance of an increasing grade of dysplasia and finally the transformation to carcinoma in situ.

The transition into the next phase is the so-called angiogenic switch and caused by the misbalance between a higher nutrient and oxygen demand due to the increasing cell number and volume with the consequence that the tumor cells cannot cover the needed supply by the existing mechanisms without additional blood vessels from their environment. This stage initiates the subsequent "vascular phase," during which pancreatic tumor cells grow exponentially lacking the counterbalancing apoptotic factors of the normal cell cycle. At the same time an overexpression of pro-angiogenic factors and a loss in physiological angiogenesis inhibitors create an imbalance leading to a de novo chaotic vessel formation with high vascular leakage (Hanahan and Folkman 1996). These mechanisms result in an aggressive tumor expansion, invasion, and possibly distant spread of tumor cells.

As a large variety of cells and signaling molecules are involved in tumor angiogenesis, this complex mechanism has to be regarded as an ambivalent and two-sided event as on one hand it is not completely understood today but on the other hand offers the opportunity to interfere in this process very specifically at many points with the aim to block angiogenesis and develop a targeted therapeutic approach which may be specific and differential for each individual tumor entity.

These specific characteristics of pancreatic tumorigenesis of each histopathological type give an explanation for the ambivalent response to anti-angiogenic therapies. PDAC arises from the ductal epithelium and makes up to 85% of pancreatic cancers; its typical histological appearance consist of a very avascular and fibrotic microenvironment making it difficult for drug delivery (Feig et al. 2012; Erkan et al. 2012). Therefore, PDAC is a mostly hypovascular entity (Fig. 1) and indolent to anti-angiogenics. In contrast, pNETs are characterized by a hypervascularized structure (Fig. 2) and show promising responses, to therapeutic approaches aiming at anti-angiogenic mechanisms.

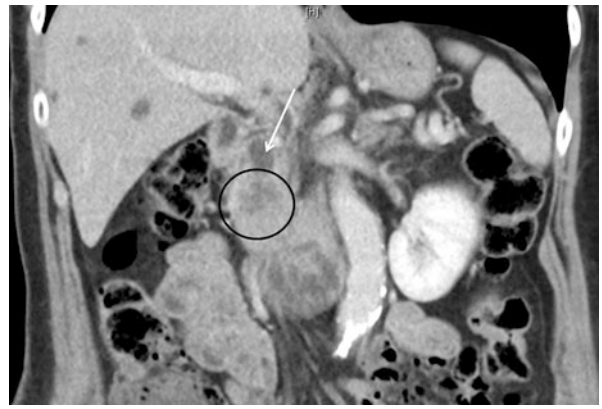

Fig. 1 Contrast-enhanced CT scan (portovenous phase, coronal reformatting) showing the perfusion characteristics of a pancreatic adenocarcinoma in the uncinate process. Tumor depicted as a hypoperfused mass (*black circle*) with a consecutive obstruction and dilation of the bile duct (*white arrow*)

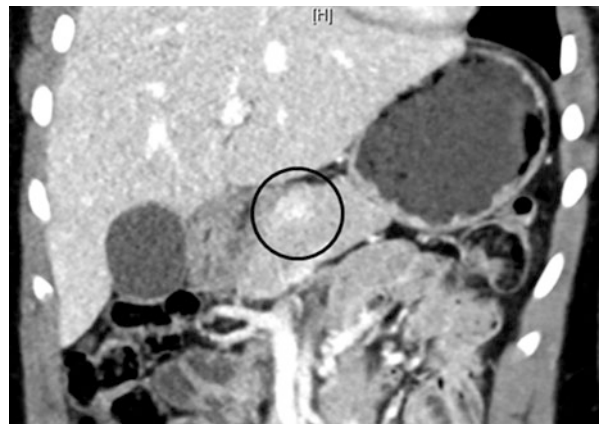

Fig. 2 Contrast-enhanced CT scan (arterial phase, coronal reformatting) showing the perfusion characteristics of a pancreatic neuroendocrine tumor in the body of the pancreas. Tumor depicted as a hyperperfused mass accumulating contrast medium (*black circle*)

Experimental and Clinical Diagnostic Modalities for Angiogenesis in PDAC and pNET

The visualization and quantification of tumor vascularization can be performed by direct or indirect examination. In the experimental setting, there have been several approaches for direct examination of vessel density and morphology including intravital microscopy and ultrasound flow measurement. The latter has also been introduced in the clinical setting but has – comparable to functional cross-sectional imaging (computed tomography (CT) and magnetic resonance tomography (MRI)) or tumor angiography – not reached the level of a routine examination in practice today.

Intravital microscopy (IVM) offers the possibility to directly visualize tumor vessels and blood cell flow by in vivo examination (Tsuzuki et al. 2001a). This experimental method is mostly based on fluorescence imaging of red blood cells, leukocytes, or staining of endothelial cells by respective fluorescence markers (Schmidt et al. 2000). In animal models of PDAC, the generation of abnormal vessels with an increased diameter, reflecting an expansion of endothelial cell surface, has been demonstrated by this method (Fig. 3, (Ryschich et al. 2004)). Furthermore, a pathological leukocyte-endothelium interaction on these altered cell surfaces could be shown, and the fact that the vessel density as investigated by IVM methods during PDAC development was not increased (Schmidt et al. 2000) supports the hypothesis that not the number of vessels itself increases but their abnormal architecture is the key finding during tumorigenesis of PDAC. Due to the need for toxic staining substances for blood cells and endothelium as well as the invasive

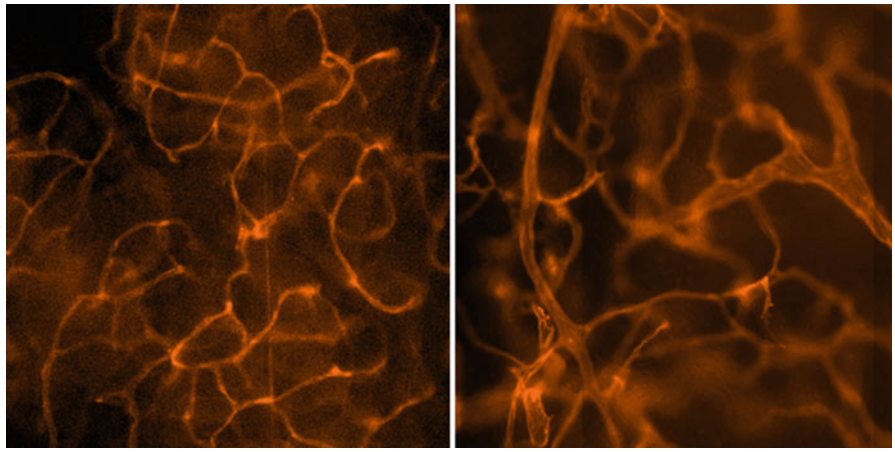

Fig. 3 Intravital fluorescence microscopy in an orthotopic mouse model of PDAC. Normal pancreas (*left side*) and PDAC with vessel irregularities in morphology and diameter (*right side*). Endothelial staining by intravenous injection of RPE-conjugated monoclonal anti-CD146 antibodies

character of this method, IVM has only been applied in the experimental setting and is not a suitable method for clinical use.

Ultrasound examination of tumor vasculature and perfusion is based on contrast-enhanced approaches that allow Doppler flow measurement. In a PDAC mouse model, microbubble-enhanced contrast ultrasound was utilized to evaluate perfusion intensity in small PDAC lesions (Pysz et al. 2015; Foygel et al. 2013; Deshpande et al. 2011). By targeting contrast bubbles against thymocyte differentiation antigen, integrin, endoglin, or VEGF receptor 2 as specific binding markers for PDAC, an increased signal intensity was demonstrated in particular small tumor lesions and correlated with the respective histological finding of a higher vessel density in these areas. This underlines the potential of these biomarkers to facilitate early detection of tumorous lesions based on the perfusion characteristics. Although promising, these approaches bear the disadvantage of the need for specific targeted bubbles, and their accuracy is based on an invasive ultrasound examination that has to be performed on the tumor pancreatic surface directly or with a very close contact of the ultrasound probe to the tumor (i.e., in a subcutaneous model), which is consequently not applicable in a clinical setting but represents the basis for further development of patients-directed diagnostics. Ultrasound Doppler flow measurement during transabdominal or endoscopic ultrasound has been established during the last 15 years, mainly based on the development of the air-based contrast agent Levovist which has been introduced in the clinical practice. This method of ultrasound Doppler flow measurement has become applicable in a number of studies (Nishida et al. 2009; Chen et al. 2004; Scialpi et al. 2005; Hocke and Dietrich 2012; Dyrla et al. 2016; Kobayashi et al. 2014; Gincul et al. 2014; Iordache et al. 2012; Figueiredo et al. 2012).

A 27-patient study on unresectable PDAC evaluated contrast-enhanced ultrasound examination in correlation with dynamic CT scan and VEGF as well as CD34 staining (Fig. 4) to intratumoral vessel density and diameter before and after chemoradiotherapy (Nishida et al. 2009). Ultrasound examination revealed a good correlation with vessel density, VEGF expression, and histological grading. Furthermore, it was also useful to determine therapy effects in terms of partial response or stable disease.

In another clinical study, contrast-enhanced power Doppler sonography was utilized to evaluate the enhancement characteristics of PDAC in correlation with the tumor vascularity observed on digital subtraction angiography (DSA) in 20 patients (Chen et al. 2004). Interestingly, this study showed a large heterogeneity in terms of hypo- (85% of the patients) and hypervascularity (15% of the patients) of the tumors but confirmed

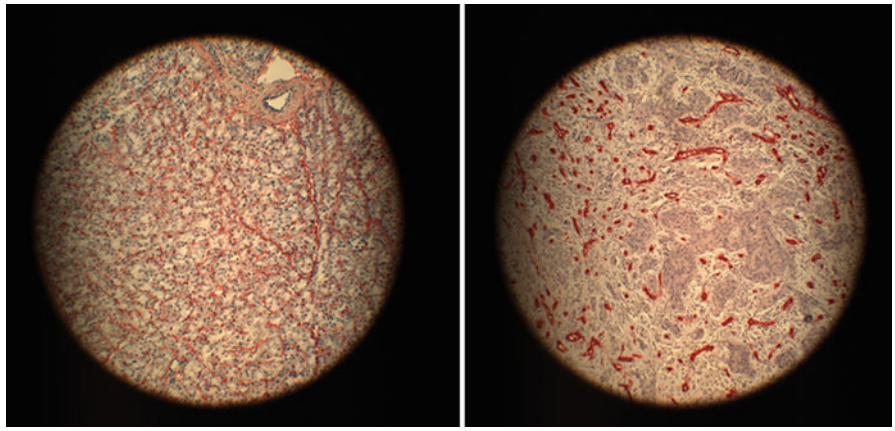

Fig. 4 Immunohistochemistry (magnification 20×) for endothelial staining (anti-CD 34). Regularly structured vessels in healthy pancreas (*left*) and highly irregular vascular diameter and architecture in pancreatic cancer (*right side*)

the high accuracy of ultrasound measurement compared with DSA as the gold standard. Besides the characterization of PDAC alone, contrast-enhanced power Doppler ultrasonography (US) has also been used to differentiate between PDAC and focal chronic pancreatitis as the most important clinical differential diagnosis (Scialpi et al. 2005). A high proportion of PDAC showed an increased number of vessels and irregular vessel characteristics whereas focal chronic pancreatitis presented mostly as avascular masses which confirmed the potential usefulness of this modality to further classify unclear pancreatic masses into malignant or benign lesions.

Regarding other perfusion-directed diagnostic clinical modalities for PDAC detection, functional cross-sectional imaging is currently a field of high interest. This includes MRI and CT imaging (Lemke et al. 2009; Fritz et al. 2016; Klauß et al. 2016; Klauss et al. 2012, 2013; Bangard et al. 2005) as noninvasive methods which can easily be applied as an addition to standard imaging, PDAC patients receive during their diagnostic workup. Both of these radiological modalities are especially helpful to differentiate PDAC versus chronic pancreatitis which can also be found to mimic cancerous lesions in conventional cross-sectional imaging (Klauß et al. 2016). Functional MRI has been established in experimental models of PDAC and angiogenetics by measurement of the endothelial transfer coefficient and fractional plasma volumes by dynamic contrast-enhanced MR imaging (Bangard et al. 2005). More recent clinical imaging studies have focused on functional MRI imaging using the perfusion fraction, and the intravoxel incoherent motion approach showed specific characteristics of PDAC patients, patients with any form of chronic pancreatitis, and healthy control participants (Klauss et al. 2012, 2013). A reduction of these parameters as a surrogate marker for reduced perfusion is observed in PDAC compared to healthy pancreatic tissue and can be used with a high sensitivity and specificity (96% and 100%, respectively) to differentiate between these two types of tissue.

Functional CT scan offers another comparable modality to determine vessel and perfusion characteristics of PDAC (Klauss et al. 2012). Tumor perfusion, blood volume, and permeability show specific changes in PDAC with a significant reduction of these parameters and can be used to differentiate tumor lesions from healthy tissue, especially when these cannot be sufficiently diagnosed in conventional cross-sectional imaging modalities.

As the mentioned findings of a reduced tumor perfusion contradict the proposed impact of tumor neo-angiogenesis, it has to be taken into account that PDAC are – especially in advanced stages – accompanied by a massive peritumoral desmoplastic reaction, which explains the macro-imaging observations of PDAC as rather

low-perfused lesions as the reactive peritumoral fibrosis is known to be a bradytrophic tissue with low vessel density.

To summarize the current state of the abovementioned perfusion-based diagnostic modalities for PDAC detection or differentiation, Doppler sonography is useful in experienced hands as it is always depending on the expertise of the examiner and can only be reproduced individually. Functional cross-sectional imaging is an objective method which is gaining importance in clinical practice. However, it is not regarded as a standard yet, as its availability is still limited in centers around the world and the available study results are still based on small patient numbers. Future studies will help to gain more evidence and increase the routine application of functional MRI and CT in clinical practice.

Tumorigenesis and Angiogenesis in Pancreatic Adenocarcinoma

Pathophysiology

The underlying genetic mutations of pancreatic tumor development can be sporadic but also part of a familial gene mutation. In more than 90% of tumors, an activating mutation in the *KRAS* gene which is part of the mitogenic signaling is found, additional common mutations include the p53 pathway as well as the TGF-ß pathway (Bardeesy et al. 2002; Ahmed et al. 2002; Jonson et al. 2001). Some of these pathways and corresponding factors also contribute to angiogenesis (Table 1). Similar to the tumor sequence model in colorectal cancer, a related adenocarcinoma sequence can be observed in PDAC. The corresponding precursor lesions are classified by their increasing malignant characteristics and defined as "pancreatic intraepithelial neoplasias" (PanIN). The higher the PanIN grade becomes (1–3), the higher the number of mutations that are observed. These sets of mutations correlate with the severity of dysplastic features found in the histological morphology (Brosens et al. 2015). Once the threshold of invasive growth has been reached at the end of the malignant transformation process, the cells display an interaction with the surrounding tissue. This is crucially important for the understanding of the features this tumor entity shows in terms of angiogenesis induction considering the fact that PDAC is – despite this vascularization process – still found to be a hypovascular lesion compared to healthy pancreatic exocrine tissue (Fig. 1).

Table 1 Overview of pancreatic neuroendocrine tumors and the corresponding symptoms, depending on the differentiation

pNET type	Typical symptoms
Gastrinoma	Peptic ulcers (epigastrical pain)
Insulinoma	Hypoglycemia
VIPoma	Diarrhea, achlorhydria, hypokalemia
Somatostatinoma	Hyperglycemia, cholelithiasis, steatorrhea, achlorhydria
Glucagonoma	Glucose intolerance, hypoaminoacidemia, necrolytic migratory erythema, stomatosis

PDAC cells release growth factors such as VEGF that bind to nearby endothelial cells and induce a response that stimulates endothelial cells to divide and form new blood vessels. These signaling pathways consequently play a crucial role in the possibility of PDAC to create a de novo vasculature which is required for growth and nutritional support of the growing tumor mass. VEGF has two functions in these processes. First, it shows a paracrine angiogenic activity and secondly a mitogenic autocrine activity observed. This is important for the understanding of its central function in PDAC growth promotion. The quantity of angiogenesis can be measured by microvascular density (MVD). Based on these considerations, it is not surprising that both high VEGF expression and high MVD count as a surrogate parameter for VEGF release and are clinically correlated with advanced tumor stages, a higher incidence of lymph node or distant metastases, and a high risk of early tumor recurrence, all of which are factors for poor prognosis and impaired survival in PDAC patients (Seo 2000; Itakura et al. 1997; Fujimoto et al. 1998; Khan et al. 2002; Linder et al. 2001; Karademir 2000; Niedergethmann et al. 2002).

As a part of the complex of contributing factors to the development and maintenance of pancreatic tumors, the PDAC microenvironment also plays a

central role. The hypovascular structure observed in computed tomography (Megeibow 1992), and the results of intratumoral oxygen tension measurements (Koong et al. 2000) confirm the presence of hypoxia. One factor contributing to this constant hypoxic environment is the response of cancer-associated fibroblasts originating from pancreatic stellate cells and inflammatory cells (Nielsen et al. 2016; Masamune et al. 2008). These cells form a surrounding desmoplastic tissue which leads to gradually decreasing nourishment and oxygen saturation (Vasseur et al. 2010). Consequently, these changes serve as a stimulus for angiogenesis to overcome the shortage in blood and oxygen supply. Through transcription factors like the hypoxia-inducible factors, the hypoxic stimulus is transferred to the level of gene expression. In PDAC, upregulation of HIF-1α mRNA expression is found and is positively correlated to VEGF mRNA (Buchler et al. 2003). Furthermore, the fibrous stroma seems to cause not only a constant intratumoral hypoxia but also a high interstitial pressure interfering with drug delivery, thus resulting in a two-front effect regarding tumor progression and treatment (Provenzano et al. 2012).

Experimental Studies

Vascular endothelial growth factor (VEGF) represents the most important and most intensely described molecule involved in tumor angiogenesis of PDAC (Itakura et al. 1997). Its essential function in tumor growth has been recognized more than 20 years ago (Folkman 1995; McCulloch et al. 1995). In primary treatment-naive PDAC samples derived from surgical patients, Ikeda et al. (1999) could show a proportion of 68% of patients who exhibited VEGF expression and an even higher proportion of 75% for PD-ECGF expression. VEGF expression correlated with an increase in microvessel density, and both of these parameters were significant prognostic factors and showed an association with poorer survival (Karayiannakis et al. 2003).

The effect of VEGF is promoted via vascular endothelial growth factor receptor-2 (VEGFR2) expressed by PDAC cells. In an experimental setting, inhibition of mRNA for this receptor results in a downregulation of receptor expression and can be targeted by small mRNAs interfering with transcription factors including Sp1, Sp3, and Sp4 that are essential for the transactivation of mRNA that express VEGFR2 (Higgins et al. 2006).

The abovementioned second important function of VEGF as a mitogenic promoter has been demonstrated in human PDAC cell lines by an overexpression not only of VEGF and VEGFR but also of their corresponding kinase mediators KDR, MAPK, and flt-1 (Itakura et al. 1997; von Marschall et al. 2000; Korc 2003). These effects were found in both tumor cells as well as endothelial cells and induced an uncontrolled growth stimulation explained by this so-called autocrine/paracrine mitogenic loop, which shows the features of a positive feedback regulation induced by VEGF.

Another promoter of VEGF effects is NRP-1, originally described as a ligand for neuronal guiding. The expression of this cofactor is highly pronounced in PDAC. NRP-1 enhances the effects of VEGF on its corresponding kinases (i.e., MAPK) (Parikh et al. 2003). The upregulation of NPR-1 is mediated by EGF and can therefore be targeted by blocking EGF receptors which results in a decrease in VEGF-induced kinase activation. NPR-1 seems to be expressed in PDAC tumor cells alone and not by endothelial cells. It shows a positive feedback with VEGF, also mainly produced by tumor cells, whereas the corresponding VEGFR expression does not seem to be located in the tumor cells, but is focused in endothelial cells, which shows the close interaction between these molecules (Li et al. 2004).

The cell adhesion molecule CD146 (MUC-18, MCAM, Fig. 3) is a most recently identified target in PDAC potentially interfering with tumor angiogenesis. Especially the soluble form of CD146 has been demonstrated to have strong angiogenic effects by boosting endothelial progenitor cells (Stalin et al. 2016a). This effect has been transferred to experimental studies on PDAC and the increased tumor cell expression of CD146

correlated well with elevated soluble CD146. Via the binding protein angiomotin, there have been observed angiogenic as well mitogenic effects, suggesting a dual effect, comparable to that described for VEGF (Stalin et al. 2016b). Moreover, targeting the soluble form by monoclonal antibodies showed a high efficacy in terms of decreased vascularization as well as tumor growth.

Another novel mechanism of angiogenesis in PDAC is the identification of miRNA involved in tube formation and endothelial migration which are major prerequisites for de novo vascularization (Li et al. 2015). Specific miRNA involved in this process have been identified. These specific miRNAs can be inhibited by selective miRNA inhibitors, which might be utilized for therapeutic purposes in the future.

Besides the tumor cells – as described before – the tumor microenvironment is an essential factor in the understanding of PDAC growth, expansion, and dissemination. In an experimental mouse study for further examination, Tsuzuki et al. (2001a) showed that the expression of VEGF seems to be promoted by an orthotopic pancreatic microenvironment. VEGF-neutralizing antibodies seem to have the capacity to inhibit these interactions between the tumor and the microenvironment. Regarding the extracellular matrix, the impact of matrix metalloproteinase 9 (MMP-9) produced by inflammatory cells (stromal granulocytes (PMN)) in the tumor environment seems to play an important role (Bausch et al. 2011). MMP-9 has a direct angiogenic effect and furthermore shows the potential to enhance VEGF-mediated vessel growth. It has consequently been identified to be a possible therapeutic target in an experimental setting. A combined blockade of both, VEGF and MMP-9, in a rat model of PDAC showed a synergistic effect of these factors and underlines the interaction of tumor and surrounding tissue (Hotz et al. 2003). Though a monotherapy with either of these agents resulted in an antitumor effect, this effect was markedly enhanced when both substance were administered simultaneously. This approach shows that interfering with one single substance in the cascade of tumor angiogenesis may not be effective and only targeting a combination of angiogenesis promoters may be successful.

Tumorigenesis and Angiogenesis in pNET

Pathophysiology

PNET are comparatively rare with an overall percentage of 1–2% of all pancreatic tumor entities. The neoplasms arise from pancreatic endocrine tissue, which consists of various types of cells and subsequent endocrine function. In 55% of all cases, the tumors are hormonally active (Fischer et al. 2014) and thus classified by their hormonal function, i.e., gastrinoma, insulinoma, glucagonoma, VIPoma, and somatostatinoma, each of which have their very own specific clinical presentation (Table 1). In pNETs, an expression of hormones that are usually not produced by normal islet cells can be present. Some of these hormones are gastrin, vasoactive intestinal peptide (VIP), serotonin, growth hormone (GH), growth hormone-releasing hormone (GHRH), adrenocorticotropin (ACTH), corticotropin-releasing hormone (CRH), parathyroid hormone-related peptide (PTHrp), parathyroid hormone (PTH), calcitonin, ghrelin, human choriogonadotropin (hCG), or renin (Ro 2013). Consequently 45% of all pNETs present as non-functioning tumors. Symptoms in those cases depend solely on location and local tumor mass-related complications (i.e., jaundice in case of compression of the bile duct). More than half of all pNETs, with the exception of insulinomas, are malignant, many of which show a very aggressive presentation (Metz and Jensen 2008). PNETs can develop sporadically or – in approximately 10% of cases – can be part of a familial genetic disease. Syndromal disorders associated with pNET include MEN1, Von Hippel-Lindau syndrome, neurofibromatosis, and tuberculous sclerosis.

Experimental Studies

The most frequent genetic alterations in sporadic pNETs are found in the MEN1 gene, followed by mutations in the alpha thalassemia/mental retardation syndrome, X-linked (ATRX), and death domain-associated protein (DAXX) genes. The nuclear MENIN1 protein, which is encoded by the MEN1 gene, functions as a tumor suppressor in most settings by coordinating chromatin remodeling through transcription regulation. Among other functions, it regulates the expression of cell cycle progression inhibitors, interacts with DNA repair mechanisms, prevents the RAS-promoted activation of the MAPK pathway, might be linked to the Hedgehog pathway (Gurung et al. 2013), and in cases of pregnancy and obesity interestingly promotes proliferation of pancreatic endocrine cells (Karnik et al. 2005; Balogh et al. 2006).

Due to progress in DNA sequencing, recently an increasing amount of mutations in pNETs have been discovered. Right after MEN1, the most common mutations to be found were the alpha thalassemia/mental retardation syndrome, X-linked gene/ATRX), and the death domain-associated protein (DAXX) gene (De Wilde et al. 2012). Physiologically, these proteins are involved in determining histone deposition. Protein expression of these genes is less in pNET with this mutation which seems to play a role in tumor cells gaining "immortality" through the alternative lengthening of telomeres pathway (Heaphy et al. 2011; Capurso et al. 2015).

A higher expression and activity of the mammalian target of rapamycin (mTOR) which is part of a complex pathway called PI3K/Akt/mTOR pathway have been observed. About 14% of sporadic pNETs show mutations in PTEN, TSC2, and PI3K which act upstream of this pathway (Jiao et al. 2011). These mutations in the PI3K/Akt/mTOR pathway have been found to play a role in angiogenesis of pNETs and mTOR functions as a transduction factor that regulates protein translation being associated with cell metabolism, survival, proliferation, and motility (Missiaglia et al. 2010). A correlation of a higher expression of mTOR and p-mTOR with an increased mitotic count, tumor size, staging, vascular invasion, and metastasis has been established.

As endocrine glands need a well-established vascular network for hormone secretion, well-differentiated PNETs are highly vascularized tumors. Thus, they can be distinguished from PDAC by their vascularized appearance in radiological imaging. Interestingly, during tumor progression, a loss of vessel density is observed – a phenomenon called the "neuroendocrine paradox."

With regard to tumor grading, these pNETs are mostly classified as "well- or moderately differentiated (G1-G2)," which explains the good clinical response to a treatment with angiogenesis inhibitors. Therefore, a high microvascular density is – in contrast to findings in many other cancers – associated with a favorable prognosis (Couvelard et al. 2005; Takahashi et al. 2007). In contrast, the low vascular density in poorly differentiated pNETs promotes tumor hypoxia and consequently an angiogenic switch characterized by upregulation of proangiogenic factors and increased endothelial cell proliferation. This results in abnormal vascular architecture similar to PDAC findings and therefore in a poor response to anti-angiogenics (Couvelard et al. 2008). To a degree comparable to PDAC tumorigenesis, factors involved in pNET angiogenesis are vascular endothelial growth factor (VEGF) and its receptor (VEGFR) as well as the platelet-derived endothelial growth factor (PDGF) and fibroblast growth factor (FGF)(Corbo et al. 2012). VEGF-A as one of the most potent factors for angiogenesis along with other VEGF family members plays a role not only in the highly vascularized character of pNET but also in the development of normal pancreatic endocrine cells. This factor is thought to be also part of the switch from normal pancreatic tissues to pNET and reversely can be blocked by anti-angiogenic agents (Bergers 1999; Hanahan et al. 1996).

Clinical Studies

Clinical studies on angiogenesis inhibition have been performed in recent years for PDAC (Table 2) as well as pNET (Table 3) with various observations regarding the efficacy of this

Table 2 Clinical studies targeting angiogenesis in pancreatic ductal adenocarcinoma

Study	Phase	n	Substance	Target	Results
Miyazawa et al. (2010)	I	18	Vaccine	VEGFR2	67% temporary disease control, 8.7 m median survival
Schmitz-Winnenthal et al. (2015)	I	45	Vaccine	VEGFR2	Reduced tumor perfusion
Yamaue et al. (2015)	II/III	153	Elpamotide	VEGFR2	No significant effect
Nukui et al. (2000)		33	IFN-α	VEGF gene, endothelial cells	84% 2-year survival, compared to 54% in controls
Knaebel et al. (2005)	III	110	IFN-α-2b	VEGF gene, endothelial cells	No significant effect
Kindler et al. (2005)	II	52	Bevacizumab	VEGF	67% partial response/stable disease, median survival 8.8 m
Kindler et al. (2010)	III	535	Bevacizumab	VEGF	No significant effect
Van Cutsem et al. (2009)	III	607	Bevacizumab/ erlotinib	VEGF/tyrosine kinase	PFS longer
Rougier et al. (2013)	III	546	Aflibercept	VEGF	No significant survival
Spano et al. (2008)	II	103	Axitinib	VEGFR	No significant effect
Kindler et al. (2011)	III	632	Axitinib	VEGFR	No significant effect
Moore et al. (2007)	III	569	Erlotinib	Tyrosine kinase	OS 6.24 versus 5.9 m, PFS significantly longer
Siu et al. (2006)	I	42	Sorafenib	VEGFR2,3/PDGF/ RAF	57% stable disease
Gonçalves et al. (2012)	III	104	Sorafenib	VEGFR2,3/PDGF/ RAF	No significant effect
Reni et al. (2013)		55	Sunitinib	VEGFR/PDGFR/ kitFlt-3 receptors	PFS 22,% versus 3.6%, stable disease 51.9% versus 21.4%

ORR Overall objective response rate, *SD* stable disease, *PFS* progression free survival

approach in terms of tumor response and patient survival for both entities. In most of the studies for PDAC, these approaches have been combined with a standard chemotherapy regimen (i.e., gemcitabine) whereas in pNET, mainly single-drug approaches have been chosen as no established chemotherapy exists to date.

PDAC

Vaccination Studies

The mechanism of vaccination as an immunotherapy using an epitope peptide for VEGRF2 as an essential factor for tumor angiogenesis has been investigated in a phase 1 study combined with gemcitabine in a palliative patient collective (Miyazawa et al. 2010). Eighteenth patients were exposed to the vaccination peptide (subcutaneously) in a three-level dose-escalation protocol ($n = 6$ patients per group) to assess safety and immunological effects of this treatment. In 67% of the patients, a temporary disease control was observed resulting in 8.7 months of median overall survival. Under medium-level adverse events, all dosages were tolerated and a successful induction of specific cytotoxic T-lymphocytes was achieved in 61% of the study population. A similar phase I approach investigated an oral vaccine for VEGRF2 using a salmonella bacteria-based expression plasmid encoding VEGFR2 in advanced PDAC in combination with gemcitabine (Schmitz-Winnenthal et al. 2015). Although only 3-month observation data are available from this study, the high-dose

Table 3 Clinical studies targeting angiogenesis in pancreatic neuroendocrine tumors

Study	Phase	n	Substance	Target	Results
Faivre et al. (2006)	I	28	Sunitinib	Tyrosine kinase	Intratumoral necrosis
Kulke et al. (2008)	II	66	Sunitinib	Tyrosine kinase	ORR16.7%, SD68%, 1 year–survival 81.1%
Raymond et al. (2011)	III	340	Sunitinib	Tyrosine kinase	PFS 11.4 m versus 5.5 m, ORR 9.3% versus 0%

ORR Overall objective response rate, *SD* stable disease, *PFS* progression free survival

vaccination resulted in a specific immune response on T effector cells and a consequent reduction of tumor perfusion as well an increase of the anti-angiogenic markers VEGF-A and collagen IV. No relevant adverse effects occurred which underlines the safety of this potentially effective vaccination approach.

The VEGFR2-derived epitope peptide elpamotide represented another approach to target tumor angiogenesis by vaccination. It was tested for advanced PDAC on the hypothesis that induction of cytotoxic T-lymphocytes directed against endothelial cells producing VEGFR leads to a reduction of angiogenesis (Wada et al. 2005). A phase I study combining elpamotide with gemcitabine showed a prolonged survival time of 8.7 months when compared to a gemcitabine monotherapy with 5.7 months (Miyazawa et al. 2010). This effect, however, could not be reproduced in a following RCT, where survival times remained unchanged (8.5. and 8.4 months, respectively) regardless of the addition of elpamotide (Yamaue et al. 2015).

Interferon Alpha

IFN-α as an immunomodulatory substance has been shown to have anti-angiogenic effects and a direct impairment of endothelial cell proliferation and migration (Zhu et al. 2008; Indraccolo 2010). The effects of IFN-α on the vasculature have been mainly attributed to inhibition of VEGF gene expression and downregulation of tumor-cell-derived fibroblast growth factor production as well as downregulation of IL-8. The gene expression profile induced by IFN-α in EC has recently been defined, and it was found that several genes encoding negative regulators of angiogenesis are upmodulated thus providing a potential amplification mechanism for this biological activity.

IFN-α has been clinically tested in an adjuvant setting after potentially curative resection of PDAC in several studies. An initial study on 17 resected PDAC patients who received a combination therapy of chemoradiation and IFN-α showed a striking 84% 2-year survival compared to 54% in a control group of 16 patients who received the protocol without IFN-α (Nukui et al. 2000). In the follow-up observation, a 5-year survival of 55% and an actual 10-year survival of 20.1% were observed (Picozzi et al. 2003; Rocha et al. 2016). Despite these promising observational data, the results of the protocol were not confirmed in a phase III RCT which showed similar survival for patients treated with or without IFN-α in an adjuvant setting (Knaebel et al. 2005; Märten et al. 2010).

Antibodies and Targeted Proteins

Bevacizumab is a monoclonal antibody-binding VEGF and approved for therapy in various solid tumors including colorectal, lung, breast, and renal cancer. In advanced PDAC, bevacizumab combined with gemcitabine was tested in an early phase II trial with 52 patients (Kindler et al. 2005). Based on the results of this study with a partial response or stable disease in 67% of the patients and a median survival of 8.8 months, further phase III studies were conducted. In a large RCT comparing bevacizumab and gemcitabine versus gemcitabine alone, 535 patients were included to confirm the results of the phase I trial (Kindler et al. 2010). Despite the encouraging results observed in the pilot setting, this

RCT failed to confirm the efficacy of bevacizumab, and the addition of this antibody resulted in 5.8 months median survival compared to 5.9 months in the control arm and an increase rate of adverse events. In addition, bevacizumab was not beneficial when added to a combination therapy of gemcitabine and the tyrosine kinase inhibitor erlotinib in another RCT including 301 and 306 patients, respectively (Van Cutsem et al. 2009). Consequently, PADC therapy with bevacizumab has been omitted in recent years.

Aflibercept represents an anti-angiogenic fusion protein with antibody properties targeting and inactivating VEGF. Adopted from ocular vascular proliferative diseases, this protein was congruently tested in PDAC in a RCT with the inclusion of 546 patients (Rougier et al. 2013) as an addition to gemcitabine but failed to increase progression-free or overall survival and was in addition burdened by an increased rate of adverse events which finally led to a premature study termination and the omission of this approach.

Axitinib as an oral, potent, and selective VEGFR inhibitor (Hu-Lowe et al. 2008) had shown promising results in a randomized phase II study of 103 patients with locally advanced and metastatic PDAC with an improvement in median overall survival and a greater 1-year survival when combined with gemcitabine versus gemcitabine alone (Spano et al. 2008). Based on these results, it was tested in a larger phase III study on 632 patients in a RCT setting (Kindler et al. 2011). This study failed to confirm the benefit and showed similar survival for both patient groups and increased adverse events.

Kinase Inhibitors

The EGF receptor selective tyrosine kinase inhibitor erlotinib as another approach aiming at angiogenesis inhibition in PDAC was introduced in the palliative setting after a phase II RCT including 569 patients (Moore et al. 2007). However, though statistically significant, the combination of erlotinib with gemcitabine added 0.3 months to the median survival when compared to gemcitabine alone. Although the overall effect of erlotinib was disappointing, the clinical observation showed that a subgroup of patients had a much more pronounced survival benefit when a significant cutaneous rash occurred. To further elucidate this unexpected observation, an analysis of the KRAS and EGFR gene mutation status was performed under the hypothesis of specific genetic variants determining the response to erlotinib (da Cunha et al. 2010). No specific prognostically significant mutation could be identified in this study. A retrospective tissue analysis from another study showed that KRAS wild-type patients had the best prognosis when treated with erlotinib (Boeck et al. 2013). A valuable pre-therapeutic marker to define this subgroup has not yet been established, and the impact of erlotinib has rapidly decreased in the clinical setting today.

Sorafenib is a multi-targeted protein kinase inhibitor directed at VEGFR2 and 3 as well as PDGF and RAF kinase and shows anti-angiogenic properties in addition to antiproliferative effects (Wilhelm et al. 2004). Based on experimental data, a phase I study was conducted which showed stable disease in 57% of PDAC patients when combined with gemcitabine (Siu et al. 2006). A consecutive phase III study could not confirm these observations in a 104-patient collective (Gonçalves et al. 2012). In this RCT, neither response rates nor progression-free or overall survival (9.2 vs. 8 months, respectively) showed a superiority of sorafenib in comparison to gemcitabine monotherapy.

Sunitinib is another multi-targeted kinase inhibitor aiming at VEGFR, PDGFR, KIT, and Flt-3 receptors that are overexpressed in PDAC and therefore represent a therapeutic aim. An RCT investigated its effect as a maintenance therapy in 55 PDAC patients after 6 months of an initial chemotherapy followed by 3 months of sunitinib application (Reni et al. 2013). The anti-angiogenic therapy resulted in an improvement of progression-free survival (22.2% vs. 3.6%) and more patients with stable disease (51.9% vs. 21.4%). Although not statistically significant, overall 2-year survival showed promising outcomes as well (22.9% vs. 7.1%) which might qualify this approach of maintenance therapy for further clinical application in the future.

An important clinical aspect with regard to both, sorafenib and sunitinib, is the observation of drug-related mortality. A current meta-analysis on this adverse effect including more than 14,000 patients from 41 studies on tyrosine kinase inhibitors for various solid tumors could show a 1.9% risk of treatment-related death (Hong et al. 2014). Especially when combined with chemotherapy, the risk for cardiovascular failure or thromboembolic events may be increased by tyrosine kinase inhibitors which this must be carefully weighed against the benefit of these drugs.

Overall, the pathway of angiogenesis inhibition in clinical PDAC therapy has led to mainly disappointing results with regard to approaches using antibodies or targeted proteins except for tyrosine kinase inhibitors that seem to be useful for selected subgroups of patients. The approach of active vaccination may be promising but needs to be evaluated in further phase II and III studies.

PNET

Kinase Inhibitors

In contrast to PDAC, in pNET therapy, sunitinib therapy has gained a much more important significance during the last decade. In an initial phase I study including 28 patients with various malignancies, a potent antitumor activity under sunitinib therapy was shown, characterized by radiological response and especially the development of intratumoral necrosis which underlines the anti-angiogenic effect with a consecutive decrease of vascularization which could be confirmed by imaging modalities in this study (Faivre et al. 2006).

Based on the clinical benefit observed in this study, a consecutive phase II trial on pNET was conducted (Kulke et al. 2008). In 66 patients with advanced pNET, the objective response rate was 16.7% with 56.1% of patients showing stable disease for more than 6 months with a 1-year survival of 81.1% without relevant clinical side effects. To evaluate these observations in a phase III, a large international double-blind RCT compared sunitinib to placebo in patients with well-differentiated PNET who had radiological evidence of tumor progression (Raymond et al. 2011). The trial was designed to show a 50% improvement of progression-free survival, and 340 patients were intended for inclusion on this statistical basis. As a higher occurrence of deaths and serious adverse events in patients receiving placebo was observed, the trail was stopped earlier. At that point of time, progression-free survival in patients receiving sunitinib was more than double the progression-free survival in the placebo group. In addition, an improved overall survival as a secondary end point provided additional evidence of the efficacy of sunitinib in pNET therapy. Similar to the radiological observations in the phase 2 study, patients showed a high proportion of changes toward hypodense lesions in CT scans of the primary tumor as well as liver metastases which can be regarded as tumor necrosis and underlines the mechanisms of antitumor activity on the basis of anti-angiogenesis.

On the basis of these data, FDA and EMA approvals have been obtained for sunitinib in advanced pNET, and the current ENETS guidelines have included the recommendation for this therapy as a second- or third-line therapy and also allow the consideration of this approach in the first-line setting when an alternative treatment with somatostatin analogues, chemotherapy, and/or locoregional therapies are not feasible or promising (Falconi et al. 2016).

The efficacy of sunitinib in advanced pNET appears to be similar regardless of preceding chemotherapy or somatostatin analogue treatment which underlines the impact of anti-angiogenesis as a specific and successful therapeutic approach in the distinct tumor entity.

Future Directions

To improve the outcome of patients suffering from PDAC as a highly lethal disease, an interdisciplinary approach is necessary to improve screening tools and develop potentially new diagnostic methods for early detection as well as innovative systemic therapies, including approaches of targeted and personalized oncological therapy.

Anti-angiogenic therapies as targeted approaches currently show a diverse range of results in PDAC depending on individual patient factors and supposed subtypes of cancer, which are not completely defined to date. Though promising experimental and phase I studies in PDAC suggest a potential role of anti-angiogenics, phase II/III study has failed to show a significant impact on disease control or survival for all patient, and only subcollectives of patients may benefit from such approaches. Thus, new directions, including vaccination, immunomodulation, or specific anti-angiogenic antibodies have to be adopted to meet individual patient characteristics. This adoption could be based on specific genetic profiling (i.e., as shown for the kinase inhibitor erlotinib (da Cunha et al. 2010) or for the efficacy of adjuvant chemotherapy in the ESPAC trials (Greenhalf et al. 2014)). However, these specific approaches are not introduced into clinical routine, and more research needs to be conducted to determine respective subgroups and thereby improve efficacy of individual screening and personalized cancer therapy in which future anti-angiogenic approaches could play an important role.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Anti-angiogenics in Hepatocellular Cancer Therapy

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Contents

Introduction 436

Inhibition of Angiogenesis in HCC 436

Inhibition of Angiogenesis with *Transarterial Chemoembolization* 437

Inhibition of Angiogenesis with Systemic Therapy 438
Sorafenib 438
Sunitinib 439
Brivanib 440
Linifanib 440
Cabozantinib and Tivantinib 440
Regorafenib 441

Bevacizumab 441
Ramucirumab 442

Combination of Local and Systemic Therapy 442
TACE in Combination with Systemic Therapy 442

Conclusion 443

References 444

Abstract

Hepatocellular carcinoma (HCC) is one of the most common and deadly malignancies worldwide. HCC is a highly vascularized malignant tumor providing a rationale to consider angiogenesis as a therapeutic target. Anti-angiogenic strategies include locoregional and systemic treatments in HCC. Depending on the stage of the disease, different anti-angiogenic approaches are currently being employed in the treatment of HCC.

For patients at intermediate stage disease, transarterial chemoembolization (TACE) has been widely accepted as the standard of care and is the most common therapy for this patient group. TACE is a locoregional intervention, and its main mechanism of action is the embolization of the tumor-feeding arteries. For patients with advanced HCC, the multiprotein

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kinase inhibitors sorafenib and regorafenib provide systemic treatment options. Their efficacy in terms of survival prolongation has been shown in the palliative setting. Both drugs target among others the receptor of the vascular endothelial growth factor (VEGF), and their antitumor efficacy is believed to partly depend on the anti-angiogenic properties.

Keywords
Hepatocellular carcinoma · VEGF · VEGFR · PDGFR · PLGF · FGFc-MET · TACE · HAP · STATE · Regorafenib · Tivantinib · Lenvatinib · Brivanib · Sorafenib · Bevacizumab · Ramucirumab

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in men worldwide and represents the third most frequent cause of cancer death (El-Serag 2011; Bosetti et al. 2014). The prognosis of patients with HCC is dismal and the mortality rates are almost the same as the incidence rates. In the year 2008, 748,300 new HCC and 695,900 deaths have been registered (Jemal et al. 2011). In 70–80% of the cases, HCC is diagnosed in patients with liver cirrhosis and a compromised hepatic function. For these patients, the cumulative 5-year risk to develop a HCC is 5–30% (El-Serag 2011). Major risk factors to develop liver cirrhosis and subsequently HCC are chronic infections with hepatitis B and C and alcohol abuse. Additionally, nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as an additional risk factor for the development of liver tumors.

In clinical practice, several therapeutic options are available for patients with HCC depending on the stage of the disease, which depends on tumor burden and liver function. Potentially curative treatments for patients at early stage disease are liver transplantation, resection, and radiofrequency or microwave ablation (Bruix et al. 2011). Early stage disease is characterized by a low tumor burden with small tumor lesions, no more than three lesions, lack of vascular invasion and extrahepatic spread, and a preserved hepatic function. However, HCC is most often diagnosed at intermediate or advanced stage, where therapeutic options are mostly restricted to palliation due to high tumor burden and/or impaired liver function (Llovet et al. 2003; Park et al. 2015). HCC is a highly vascularized malignant tumor providing the rationale for anti-angiogenic strategies through locoregional and systemic treatments. Intermediate stage patients have liver-limited disease, with multiple and large HCC lesions, but no vascular invasion or extrahepatic spread, making them appropriate candidates for local treatment. Transarterial chemoembolization (TACE) has been widely accepted as the standard of care for patients at intermediate stage disease and is the most common therapy in clinical practice for this patient group (Park et al. 2015; Llovet et al. 2002; Lo et al. 2002; Malek et al. 2014; Kirstein et al. 2016). TACE combines the administration of cytotoxic drugs, with or without lipiodol, and embolizing agents by means of a catheter directly to the hepatic artery.

For patients with advanced disease, high hepatic tumor burden, and/or evidence of vascular invasion or extrahepatic tumor manifestations, the multi-tyrosine kinase inhibitor sorafenib is so far the only approved systemic drug (Llovet et al. 2008). Sorafenib targets the receptor of the vascular endothelial growth factor (VEGF) among others suggesting that inhibition of angiogenesis is one of its anti-tumoral mechanisms of action. More recently, evidence has been provided for the efficacy of the multi-tyrosine kinase inhibitor regorafenib in patients with progressive disease upon sorafenib. In the following we will summarize the anti-angiogenetic approaches for patients with HCC at intermediate and advanced stage disease.

Inhibition of Angiogenesis in HCC

HCC is a highly vascular tumor, and angiogenesis, mediated mainly through VEGF, is thought to play a major role in development, progression, and prognosis of this cancer. Inhibition of angiogenesis is achieved through local and systemic

therapies in HCC. TACE is a local, embolizing procedure most commonly performed in combination with the administration of local chemotherapy. On the other hand, sorafenib is the established therapy for patients at advanced stages with higher hepatic tumor burden and/or vascular invasion and extrahepatic metastases. Up to date, sorafenib is the only approved agent for HCC. After the approval of sorafenib, multiple molecular agents with anti-angiogenic properties have been investigated to improve overall survival in patients with advanced HCC. Most of these drugs failed in phase II or III clinical trials until very recently, when the positive phase III trial with the multi-kinase inhibitor regorafenib has been reported. In the following, we will describe and discuss the different approaches that have been undertaken to target angiogenesis in HCC.

Inhibition of Angiogenesis with *Transarterial Chemoembolization*

TACE is the most common first-line treatment for patients with HCC. Two early randomized trials have confirmed significantly improved survival rates of patients with intermediate stage disease treated with TACE so that TACE has become the standard treatment in these patients. In the first trial reported by Llovet et al., more than 900 Caucasian patients were screened during a period of 4 years. Out of these 903 patients, 113 were included in the trial, and a survival benefit was reported for 35 patients treated with TACE as compared to 38 patients treated with best supportive care (BSC); only survival in the BSC arm was very long with 17.8 months indicating that a highly selected patients population was included in the trial with a good liver function and low tumor burden. Survival in the TACE arm was significantly improved to 28.7 months. In contrast to the survival the pivotal trial, outcome of patients treated with TACE in clinical practice is still very poor with median overall survival rates of 20 months or less (Sieghart et al. 2013; Kadalayil et al. 2013; Hucke et al. 2014).

TACE is frequently part of a multimodal treatment strategy at any stage. A high variability of second-line treatments after TACE has been reported in real-life cohorts, where TACE was most often followed by other local therapies rather than by systemic therapies (Park et al. 2015; Kirstein et al. 2016). In order to select the best patients for TACE, several prognostic scores such as the *hepatoma arterial-embolization prognostic* score (HAP), the modified HAP-II, *Selection for TrAnsarterial chemoembolization TrEatment* (STATE), as well as an individual prognostic calculator have been established (Kadalayil et al. 2013; Park et al. 2016; Hucke et al. 2014; Cappelli et al. 2016). In addition, predictive scores have been proposed in order to select the most appropriate patients for continuation with TACE (Sieghart et al. 2013; Adhoute et al. 2015). It still remains unclear though, at which time patients should be switched from TACE to systemic therapy according to the currently available scores. Specifically, it has never been shown that patients with a poor prognosis would benefit from a switch to systemic therapy and to which extent frequent TACE sessions may compromise post-TACE survival due to impairing liver function.

The most popular TACE technique has been established by the administration of lipiodol followed by embolic agents. Lipiodol is used as a vehicle to carry the chemotherapeutic agent inside the tumor and as a microembolic agent for tiny tumor vessels. A recent systematic review of 101 mostly single-arm and/or non-randomized studies including 10,108 subjects revealed that the most commonly used chemotherapeutic agents, either as single agents or in combination regimens, are doxorubicin, epirubicin, cisplatin, and mitomycin (Lencioni et al. 2016a). Median overall survival (OS) in these studies was 19.4 months, which is consistent with the data reported in previous meta-analyses. The survival rates were 81.0% at 6 months post-TACE, 70.3% at 1 year, 51.8% at 2 years, 40.4% at 3 years, and remarkably 32.4% at 5 years indicating that TACE can be very efficacious in in specific subgroups. Median PFS was only evaluated in a few studies and ranged between 3 and 9 months, and the objective response rate, defined as sum of complete and partial response, was approximately 50%.

Using TACE with lipiodol, local embolization (conventional TACE) of the vessels that supply HCC induces inflammation and necrosis of the lesions (Shim et al. 2008). Several attempts have been made to improve the efficacy of TACE in intermediate stage HCC. One approach, which is increasingly used and standard of care in several prospective trials, is the use of drug-eluting beads. TACE with embolic doxorubicin-eluting beads (DC Bead; Biocompatibles UK Ltd.; DEB-TACE) was developed to simplify the procedure, reduce peak concentrations and total systemic exposure to doxorubicin, and ensure high concentrations in the tumor and adequate arterial occlusion. One randomized phase II trial found that DEB-TACE reduced the rates of systemic adverse events and liver toxicity compared with conventional TACE with lipiodol and doxorubicin. The drug-eluting bead group showed numerical higher rates of complete response, objective response, and disease control compared with the conventional TACE group (27% vs. 22%, 52% vs. 44%, and 63% vs. 52%, respectively) in the intention-to-treat (ITT) population of 212 patients, which however did not reach the level of significance (Lammer et al. 2010). Similarly, in one randomized study, comparing conventional TACE and DEB-TACE, there was no significant difference in median OS with 28 and 29 months, respectively, suggesting equal antitumor efficacy for lipiodol TACE compared to beads TACE (Golfieri et al. 2014). One very recent study readdressed the question, whether the addition of doxorubicin has any additional effect on response and outcome after embolization with beads (Brown et al. 2016). In this single-center study, 92 patients with comparable characteristics underwent 129 embolizations to complete their initial treatment, with a total of 209 embolizations during the entire study. Median progression-free survival (PFS) was 6.2 versus 2.8 months (HR 1.36, $p = 0.11$) and median PFS 19.6 versus 20.8 months (hazard ration [HR], 1.11, $p = 0.64$) for TACE without and with doxorubicin, respectively. Moreover, there was no significant difference in the response rate measured by Response Evaluation Criterial In Solid Tumor (RECIST) 1.1 and modified RECIST (mRECIST). This finding supports a previous study in which patients were randomized to TAE with polyvinyl alcohol (PVA) particles alone or sequential TACE with cisplatin 50 mg administered intra-arterially 4–6 h before PVA embolization (Meyer et al. 2013). In this study, median OS and median PFS were 17.3 versus 16.3 ($p = 0.74$) months and 7.2 versus 7.5 ($p = 0.59$), respectively, indicating that the efficacy of TACE primarily depends on the mechanical anti-angiogenic effect than on the antitumor effect of the chemotherapy.

Inhibition of Angiogenesis with Systemic Therapy

Sorafenib

Sorafenib is an oral multi-kinase inhibitor, which inhibits proliferation of tumor cells and induces apoptosis. Target structures are the serine-threonine kinases Raf-1 und B-Raf, the platelet-derived growth factor receptor-b (PDGFR-b), and also the receptor tyrosine kinases of the vascular endothelial growth factor (VEGF). The VEGF family consists of five ligands VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PIGF) and the three receptor tyrosine kinases VEGFR1, VEGFR2 and VEGFR3. Of the VEGF receptors, VEGFR2 expression is restricted to the vasculature and appears to play a key role in angiogenesis.

The safety and efficacy of sorafenib have been shown with the multicentric, randomized, placebo-controlled SHARP trial (Sorafenib HCC Assessment Randomized Protocol) (Llovet et al. 2008). Six hundred two patients with advanced disease were included from 21 countries. The study was preliminary stopped after the second interims analysis, which revealed a significant survival benefit for sorafenib. The median OS was 10.7 months in the sorafenib arm and 7.9 months in the placebo group (HR 0.69; $p < 0.001$). Time to radiological progression was also significantly prolonged from 2.8 to 5.5 months with sorafenib (HR 0.58; $p < 0.00001$). The results of the SHARP trial led

to the approval by the FDA and EMA in 2007 for advanced HCC, not suitable for local therapy.

Later on, the survival benefit for sorafenib treatment was confirmed in Asian patients also within another large phase III trial, the Asia-Pacific Trial (HR 0.68, $p < 0.05$) (Cheng et al. 2009). Moreover, for differentiated conclusions of HCC-therapy in real-life clinical practice, 2,770 patients were selected from 37 countries for a systemic treatment with sorafenib to participate within the GIDEON trial (Global Investigation of Therapeutic Decisions in Hepatocellular Carcinoma and of its Treatment with Sorafenib) (Lencioni et al. 2010). The survival advantage and the delay in progression were particularly confirmed for patients with a well-preserved hepatic function (Child-Pugh A). Accordingly, sorafenib is recommended for patients with Child-Pugh A within the current German and international guidelines (Bruix et al. 2011; Malek et al. 2014). The efficacy of sorafenib has formally not been shown in patients with advanced disease and more impaired hepatic function (CP B) and is accordingly not recommended by the guidelines. A more profound prolongation of OS may also be conceivable in patients with a less advanced tumor stage. However, an "earlier" administration of sorafenib at intermediate stage disease has so far not been sufficiently investigated, and there are no head-to-head trials comparing sorafenib with TACE.

In order to better understand the mechanism of action of sorafenib and to identify patients that respond to the drug alone or in combination with other systemic drugs, several biomarkers have been investigated in previous studies. One prospective study found that lower baseline plasma levels of insulin-like growth factor-1 and higher plasma VEGF levels correlate with advanced clinical pathologic parameters and poor OS in 288 patients with HCC suggesting that high VEGF levels are of prognostic relevance in HCC (Kaseb et al. 2011). Subsequently, an analysis of the 602 patients in the SHARP trial also observed that baseline plasma concentrations of angiopoietin 2 and VEGFA were independent prognostic predictors of patient survival in the entire patient population (Llovet et al. 2012). These data were further supported by a recent analysis aimed to identify biomarkers predicting prognosis or response to sorafenib with or without erlotinib in HCC patients from the phase III SEARCH trial. Treatment arm-independent analyses showed that elevated hepatocyte growth factor (HGF; HR,1.687 (high vs. low expression) and elevated plasma VEGFA (HR, 1.386) were significantly associated with poor overall survival (OS) in multivariate analyses (Zhu et al. 2016a). Furthermore, a multi-marker signature of HGF, VEGFA, KIT, EPGN, and VEGFC correlated with improved median OS in the multivariate analysis. These biomarkers were also tested in predictive analyses in both trials to determine whether their baseline concentrations correlated with treatment benefit. However, none of them – either alone or in combination – significantly predicted benefit from sorafenib.

Sunitinib

Sunitinib is an oral, multi-targeting tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, and VEGFR-3 and other receptor tyrosine kinases such as platelet-derived growth factor receptors (PDGFRs), c-Kit, Flt-3, and RET receptors. The drug has shown promising antitumor activity in three phase II studies of patients with advanced HCC (Raymond et al. 2007; Faivre et al. 2009; Zhu et al. 2009). Each study evaluated a different dosing regimen: 37.5 mg/d on a 4-week-on-2-week-off schedule (schedule 4/2), 50 mg/d on schedule 4/2, and 37.5 mg via continuous daily dosing. The 50 mg/d schedule 4/2 regimen was associated with pronounced toxicities, and the 37.5 mg CDD schedule was selected for further study in the large phase III SUN1170-HCC study in order to compare the efficacy of sunitinib against sorafenib (Faivre et al. 2009; Cheng et al. 2013). The primary objective was to demonstrate at least non-inferiority of sunitinib as compared to sorafenib in terms of OS. As a result of a planned safety review by the independent data monitoring committee, carried out after the first interims analysis, the trial was terminated and enrolment was stopped for futility and safety

reasons. Moreover, interims analysis revealed that 18% of the death in the sunitinib arm were related to the drug. Despite similarities in PFS and TTP, the lack of OS benefit emphasizes the limitations of surrogate endpoints in HCC. In the final analysis, an OS of 7.9 months in the sunitinib arm and 10.2 months in the sorafenib arm was reported (HR 1.30, $p < 0.01$) and confirmed therefore the approval data for sorafenib from the SHARP trial. Interestingly, the survival difference between sunitinib and sorafenib was specifically seen in patients from non-Asian regions, many of whom were HCV positive. In contrast, median OS was similar in HBV-infected patients. These data are in agreement with an exploratory subgroup analysis from the SHARP study, in which survival with sorafenib was longer in HCV-infected patients compared to patients with alcohol- or HBV-related HCC suggesting that HBV-related and HCV-related HCC may respond differently to targeted therapies (Bruix et al. 2012).

Brivanib

Another anti-angiogenic agent, which has been extensively investigated in HCC, is brivanib. Brivanib is an oral, selective dual inhibitor of the VEGFR and the FGFR, which exhibit both antiproliferative and anti-angiogenic activity (Bhide et al. 2010). Preclinical evidence suggested that both pathways play a role in the pathogenesis of HCC and that the FGFR family at least partly mediate resistance to VEGF-driven angiogenesis. Based on promising preclinical and phase II data that indicated that brivanib could have a comparable activity as sorafenib, three phase III trials were initiated in the first-line (BRISK-FL) and second-line (BRISK-SL) setting and in combination with chemoembolization (BRISK-TA). The BRISK-PS study investigated the efficacy of brivanib compared to best supportive care after failure of sorafenib or intolerance to sorafenib (Llovet et al. 2013). The primary endpoint for the study was OS, which was however not reached. While the study was mostly well stratified, there was an imbalance in the number of patients that had vascular invasion, which was masked by the fact that the majority of patients had extrahepatic spread. In contrast to OS, TTP, objective response rate, and disease control rate were significantly improved with brivanib suggesting that these imbalances in the patient population could have contributed to missed OS benefit.

Similarly, the BRISK-FL study did not meet its primary endpoint of non-inferiority compared to sorafenib (Johnson et al. 2013). This study enrolled over 1,100 patients randomized 1:1 to brivanib or sorafenib and stratified similarly to the SHARP study. mOS did not differ significantly from the sorafenib survival of 9.9 months but exceeded the upper-limit HR 95% CI of 1.08 to prove non-inferiority. Results of the BRISK-TA study are reported below. After these discouraging results, all investigations on brivanib in HCC were stopped.

Linifanib

Linifanib is an ATP-competitive inhibitor of all VEGF and PDGF receptor tyrosine kinases. In an open-label, phase II trial, linifanib demonstrated clinical activity as monotherapy in mainly Asian patients with advanced HCC with an TTP of 5.5 months and a mOS of 9.7 months. Based in these promising data, the efficacy and tolerability of linifanib versus sorafenib were tested in patients with advanced HCC who had not received prior systemic therapy. Patients receiving linifanib had a longer TTP, PFS, and ORR than patients receiving sorafenib. These improvements however did not translate to an improvement in OS, which was not significantly different between the two treatments all in prespecified subgroups. None of the predefined superiority and non-inferiority OS borders for linifanib were met within the trial (Cainap et al. 2015).

Cabozantinib and Tivantinib

Another class of drugs of increasing interest in HCC is the inhibitors of the receptor tyrosine kinase c-MET with its ligand the hepatocyte

growth factor (HGF), either alone or in combination with VEGF inhibition. C-MET has been implicated in tumorigenesis, and overexpression or activation of c-MET has been shown within several retrospective trials in HCC (Kaposi-Novak et al. 2006; Ke et al. 2009). Cabozantinib is a dual MET/VEGFR2 inhibitor. Cabozantinib has been tested against placebo in a phase II discontinuation trial with 41 patients with progressive HCC (Verslype et al. 2012). With a PFS of 4.4 months and a promising OS of 15.1 months, a relevant antitumor activity was assumed, and a phase III trial has been initiated testing cabozantinib against placebo in sorafenib-pretreated patients (Abou-Alfa et al. 2015). The trial is designed to enroll 760 patients with advanced HCC who received prior sorafenib. Patients are randomized 2:1 to receive 60 mg of cabozantinib daily or placebo. Following an interims analysis in 2016, which was scheduled to take place when 50% of the events for the primary endpoint of OS had occurred, the trial's Independent Data Monitoring Committee (IDMC) determined that the study should continue without modifications of the study protocol.

However, challenging the assumption that VEGF inhibition is the key mediator of the antitumor activity of cabozantinib, tivantinib, a selective, non-ATP-competitive inhibitor of the MET-tyrosine kinase, also showed promising results. Tivantinib has been investigated in a multicenter, placebo-controlled phase II trial (Santoro et al. 2013), in which 107 patients after failure of sorafenib or intolerance to sorafenib were included. Primary endpoint was time to progression. In the total population, there was no improvement, but in patients with an immunohistochemically high c-MET expression, median OS was improved from 3.8 to 7.2 months as compared to placebo. Moreover, a negative prognostic value of c-MET has been reported as c-MET high-expressing patients had a significant shorter OS compared to patients with a low c-met expression. Based on these data, a phase III trial with tivantinib in the second-line setting in c-MET-overexpressing patients has been conducted and the results are awaited for 2017 (NCT01755767).

Regorafenib

Regorafenib is a multi-tyrosine kinase inhibitor that is structurally almost identical to sorafenib with the addition of only one fluorine atom in the center phenyl ring (Ravi and Singal 2014). In addition to VEGFR-1, VEGFR-2, and VEGFR-3, additional angiogenic targets of regorafenib are PDGFR and FGFR-1 and FGFR-2 and, to a lesser degree, the tyrosine kinase of the immunoglobulin and epidermal growth factor homology domain 2 (TIE-2) receptor, another promoter of angiogenesis. Within a phase II trial, regorafenib revealed an acceptable safety profile and a relevant efficacy with an OS of 13.8 months and a TTP of 4.3 months in patients after sorafenib failure (Bruix et al. 2013). Based on these results, the phase III RESORCE trial (REgorafenib after SORafenib in patients with hepatoCEllular carcinoma) has been conducted, and for the first time following the SEARCH study, a significant improvement of OS could be demonstrated within a phase III trial. Importantly, a significant benefit of regorafenib versus placebo could be demonstrated in all efficacy endpoints: mOS was prolonged from 7.8 to 10.6 months (HR 0.62, $p < 0.001$); mPFS was 3.1 months for regorafenib versus 1.5 months for placebo (HR = 0.46; $p < 0.001$). Accordingly, median TTP was 3.2 months for regorafenib versus 1.5 months for placebo (HR 0.44; $p < 0.001$). ORR was 10.6% versus 4.1% ($p = 0.005$). The most frequent adverse events (≥grade 3) were hypertonia (15.2% vs. 4.7%), hand and foot reaction (12.6% vs. 0.5%), fatigue (9.1% vs. 4.7%), and diarrhea (3.2% vs. 0%). Based on these results, regorafenib is expected to be approved as second-line treatment option for patients with HCC following progression on sorafenib.

Bevacizumab

Bevacizumab is a monoclonal antibody targeting VEGFA and is the classical inhibitor of angiogenesis approved for various cancer types. By targeting VEGFA, bevacizumab impacts on VEGFR1 and VEGFR2 and the non-catalytic

co-receptors neuropilin-1 and neuropilin-2. VEGFA is a central regulator of endothelial cell proliferation and survival, tumor angiogenesis, and vascular permeability. Although the precise mechanism of action is incompletely understood, bevacizumab is thought to decrease tumor vascularity and growth by directly binding to VEGF. Bevacizumab may also help to normalize tumor vasculature, improving oxygenation and the delivery of cytotoxic drugs. Bevacizumab has been evaluated in several small trials in HCC. A recently published meta-analysis summarized the results of eight trials with more than 300 patients (Fang et al. 2012). In six trials, bevacizumab was given as first- or second-line treatment and in seven trials in combination therapy with erlotinib, capecitabine, and/or oxaliplatin. The response rate in most trials was approximately 20% with a disease control rate of 20–79%. Median PFS was between 1.5 and 6.9 months, and median OS was between 5.9 and 15.7 months. In all trials, though accompanied with manageable, drug-typical toxicities, bevacizumab was generally well tolerated. Overall, these published data suggest that bevacizumab could be an effective treatment for advanced HCC, but to our knowledge, further investigations regarding bevacizumab have been largely stopped.

Ramucirumab

Ramucirumab is a monoclonal antibody against VEGFR-2, where it binds to the extracellular VEGF-binding domain with high degree of specificity and affinity, thereby preventing the binding of the VEGF ligand to the VEGFR2 receptor. In a small phase II and biomarker study, patients with advanced HCC and no prior systemic treatment received ramucirumab 8 mg/kg every 2 weeks until disease progression or limiting toxicity (Zhu et al. 2013). In this study, median PFS was 4.0 months, ORR 9.5%, and median OS 12.0 months suggesting that the drug may confer anticancer activity in advanced HCC. The exploratory biomarker studies revealed that there was an increase in serum VEGF and placental growth factor (PlGF) and a transient decrease in soluble VEGFR-2 following treatment with ramucirumab.

Based on these data, the global, randomized, double-blind REACH trial was initiated comparing ramucirumab to placebo as a second-line treatment in patients with HCC after being treated with sorafenib in the first-line setting. Median OS was 9.2 months on the ramucirumab arm compared to 7.6 months on the placebo arm (HR 0.866; 95% CI: 0.717–1.046; $p = 0.1391$). While the median OS was not statistically significant, a prespecified subgroup of patients with an elevated baseline of alpha-fetoprotein (AFP) $\geq$400 ng/mL showed a greater survival improvement with ramucirumab treatment regardless of Child-Pugh score (Zhu et al. 2016b). Median OS in this subgroup of patients was 7.8 months in the ramucirumab arm compared to 4.2 months in the placebo arm (HR 0.674; 95% CI 0.508–0.895; $p = 0.0059$) supporting the use of baseline AFP as a method to identify those patients most likely to benefit from ramucirumab. Serum AFP has long been recognized as a diagnostic and prognostic marker, and the analyses from this study thus confirmed elevated AFP levels as a marker of poor prognosis in HCC. The association between efficacy and baseline AFP could be because of the unique selective inhibition of only VEGFR-2 by ramucirumab, which might be relevant in this poor prognosis group. Further investigation of the efficacy and safety of ramucirumab in patients with HCC and elevated baseline AFP are ongoing in the phase III REACH-2 trial (NCT02435433).

Combination of Local and Systemic Therapy

TACE in Combination with Systemic Therapy

As both TACE and systemic therapy can target angiogenesis and the existing tumor-feeding arteries and do not have overlapping toxicities, a combination of both has been thought to increase clinical outcome in patients with intermediate stage HCC. Moreover, TACE has been shown to lead to a spike in the intratumoral concentration of

VEGF and FGF, which have been shown to be associated with increased risk of tumor growth, recurrence, metastasis, and poor survival providing a rationale to combine both treatments. The addition of systemic therapy to TACE may therefore shrink or stabilize tumors remaining after TACE, prevent tumors from spreading outside of the liver, and may also suppress growth of microscopic lesions not treatable by TACE. Several single-arm phase I and II trials have explored the combination of sorafenib plus conventional TACE or DEB-TACE indicating that these combinations are feasible in patients with intermediate stage HCC. Two reviews and meta-analysis including four and, respectively, two randomized trials concluded that the combination of TACE and sorafenib does not improve OS or overall response rates but improves time to progression (Zeng et al. 2016; Wang et al. 2016). However, recent data from four well-performed randomized, placebo-controlled trials have shown discouraging results for the combination of TACE with sorafenib, brivanib, and orantinib.

Three hundred and seven patients were randomized in the phase II SPACE-trial (Lencioni et al. 2016b). Patients were 1:1 randomized to receive either sorafenib or placebo. Systemic treatment started on day 1, and the first TACE was performed on days 3–7 using drug-eluting beads (DEB-TACE). The primary outcome was time to progression. Sorafenib plus DEB-TACE improved TTP according to the predefined statistical threshold for this exploratory study, but the median TTP was the same (169 vs. 166 days, respectively; HR 0.8; $p = 0.072$), and the combination did not improve TTP in a clinically meaningful manner compared with DEB-TACE alone. The overall response rates (ORRs) for patients in the sorafenib and placebo groups were 55.9% and 41.3%, respectively, and not significantly different. Similarly, the HR of OS in the sorafenib plus DEB-TACE versus the placebo plus DEB-TACE group was 0.898 ($p = 0.295$), with the median OS not reached in either group after a median follow-up of approximately 270 days.

The results of the randomized-controlled phase III TACE-2 trial have recently been presented (ASCO 2016 annual meeting, abstract #4018)). Patients with intermediate stage HCC were randomized 1:1 to continuous sorafenib (400 mg BD) or placebo. After randomization patients were treated with the study drug. DEB-TACE was performed at 2–5 weeks. The primary outcome progression-free survival (PFS) was not met (7.8 for sorafenib vs. 7.7 months for placebo; HR 1.03; $p = 0.85$). Moreover, there were no differences between both arms in the secondary measures OS (18.8 vs. 19.6 months; HR 1.03; $p = 0.87$) and overall response (34.7% vs. 31.3%).

The efficacy of brivanib in combination with TACE was evaluated in large prospective trials with more than 500 patients. The final analysis did not reveal an improvement in the primary endpoint of mOS with a mOS of 26.4 months in the brivanib group and 26.1 months in the placebo group (HR: 0.9). There was also no improvement in the composite endpoint of time to disease progression (TTDP) (defined as the time from the first TACE to the development of extrahepatic spread or vascular invasion, deterioration of liver function or ECOG-PS, or death) with brivanib versus placebo (median 12.0 vs. 10.9 months) and in respect to ORR (48% in the brivanib group and 42% in the placebo group). In contrast, TTES/IV (time to extrahepatic spread or vascular invasion) (median not reached vs. 24.9 months; HR, 0.64; $p = 0.0096$) and TTP (median, 8.4 vs. 4.9 months; HR, 0.61; $p < 0.0001$) were longer in the brivanib group than in the placebo group, suggesting that brivanib may have some antitumor efficacy in this setting, which was however not sufficient to improve OS.

The combination of TACE has also been tested with another multi-kinase inhibitor, orantinib in a smaller phase II study. The results of the study have so far been only reported in abstract form. Similarly, data of a combination of TACE with sunitinib are awaited for full publication.

Conclusion

Impairing arterial perfusion and vascularization of HCC by means of TACE is effective and is recommended as the standard of care for intermediate stage patients and as bridging therapy at

earlier stage disease. Systemic therapy has been shown to be effective in the first-line setting with sorafenib and in the second-line setting with regorafenib. So far, sorafenib is the only approved drug for patients with advanced HCC. Regorafenib however is the first drug, following the SHARP study, to show a significant survival benefit in patients with failure of sorafenib and provides now a treatment option in second line. For both drugs, however, the respective impact on anti-angiogenesis for their antitumor efficacy has never been proven and likely involves additional impact on other signal cascades within the tumor cells.

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Part XI

Anti-angiogenics in GIST

Inhibition of Tumor Angiogenesis in GIST Therapy

Charlotte Benson and Michela Libertini

Contents

Introduction 450
Angiogenesis 450
GIST 451

Anti-angiogenic Therapy of GIST 452
Imatinib 453
Sunitinib 454
Regorafenib 454
Other Anti-angiogenic Inhibitors 455

Tumor Response Evaluation in the TKI Era 456

The Other Side of the Coin: Mechanism of Acquired Resistance and Toxicity 457

Conclusion 459

Cross-References 459

References 459

Abstract

Gastrointestinal stromal tumors (GISTs) are rare tumors accounting 10–15 new cases/year per million individuals and only represent 2–3% to all gastrointestinal malignancies. They usually arise from the stomach, less frequently from the small bowel, rectum, and esophagus. The hallmark of GISTs is the presence of activating mutations in *KIT* or platelet-derived growth factor-alpha (*PDGFRA*) genes, which are considered key drivers in the molecular pathogenesis and that represent important predictive factors. Before the angiogenic inhibition era, GIST was found to be resistant to cytotoxic chemotherapeutic agents. The introduction of small molecules able to inhibit angiogenesis and tumor growth has utterly changed the clinical history of this rare tumor. GISTs have represented for years a model for anti-angiogenic treatment in solid cancer. Tyrosine kinase inhibitors against pro-angiogenic targets, such as imatinib, sunitinib, and regorafenib, are currently available in GIST treatment. One of the main concerns with this molecular therapy is acquired resistance due to a huge variety of factors, including activation of parallel angiogenic pathways. A

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second important aspect in learning curve of tyrosine kinase inhibitor mechanism of action is the different toxicity profile compared to cytotoxic chemotherapy.

Keywords
GIST · KIT mutation · PDGFRA mutation · Anti-angiogenic therapy · TKI · Imatinib · Sunitinib · Regorafenib · Resistance · Toxicity

Introduction

Over 40 years ago, Folkman and colleagues developed the concept that tumor growth and metastatic spread were angiogenesis dependent. They observed that the tumor growth was severely reduced in the absence of new vessel development. Furthermore, the observation that tumors implanted in an avascular area induced growth of new capillaries indicated that tumors could release pro-angiogenic factors (Folkman et al. 1971). Since then, the attention of scientific community has been focused on the inhibition of tumor ability to create new vessels as potential target treatment of cancer. Gastrointestinal stromal tumors (GISTs) represented for years a model for anti-angiogenic treatment in solid cancer, thanks to the discovery, in 2001, of a tyrosine kinase inhibitor called imatinib. Joensuu et al. in 2001 described the first patient diagnosed with metastatic GIST treated with imatinib. This case report, also known as "the case zero," revolutionized the history of GIST treatment.

Angiogenesis

The process of forming new blood vessels from existing vasculature is defined as angiogenesis. In adult, angiogenesis occurs in physiological conditions such as the female reproductive cycle and wound healing. Normally, angiogenesis is regulated by a perfect balance between pro- and anti-angiogenic mediators. The alteration of this balance is known as the "angiogenic switch." Angiogenesis-related tumor growth traditionally consists in two phases: the first, avascular phase, in which occult tumors not bigger than 1–2 mm are in a dormant status and the second phase that is characterized by a vasculo-mediated growth (Ribatti et al. 1999). The angiogenic switch represents one of the most important key roles in tumorigenesis determining the passages from phase one to two. It is caused by several pathological factors such as oncogenic mutation, hypoxia, nutrition deprivation, and mechanical stress. Endothelial cells are stimulated to migrate to the extracellular matrix, proliferate, and organize into new capillaries, through a complex process that is still not fully understood. The tumor vessels lose their physiological quiescence and enable an uninterrupted growth resulting in abnormal architecture of new vessels which are tortuous, dilated, lack in pericyte coverage and rich in arteriovenous shunts. This aberrant pattern leads to a highly hypoxic microenvironment and consequently to a hyper-expression of numerous genes encoding pro-angiogenic factors.

The vascular endothelial growth factor (VEGF) family is likely the most ubiquitous one and is a momentous player in angiogenesis. The VEGF family includes VEGFA, VEGFB, VEGFC and VEGFD, and placental growth factor (PIGF) and their associated transmembrane tyrosine kinase receptors VEGFR1, VEGFR2, and VEGFR3. The VEGF family stimulates proliferation and migration of endothelial cells. VEGF is also implicated in lymph-angiogenesis that may also contribute in tumor growth and metastatic spread.

The second most important growth factor is platelet-derived growth factor (PDGF) which consists in four members (from -A to -D) and its cognate receptor tyrosine kinases, PDGFRα and PDGFRβ. PDGF induces angiogenesis by upregulating VEGF production and modulating the proliferation and recruitment of perivascular cells (Raica and Cimpean 2010).

Besides VEGF and PDGF, other growth factors have been showed to have a significant pro-angiogenic effect, such as fibroblast growth factor (FGF), endogen, angiopoietin and endothelin, and many others.

Tyrosine kinase receptors (TKRs) are essential for the transduction of extracellular signals into the cells. TKRs consist of an extracellular domain, a transmembrane domain, a juxta-membrane

domain, and a tyrosine kinase (TK) domain that splits into two domains, TK I and TK II, by a kinase insert. Ligands, such as growth factors, bind to the extracellular domain of the receptors and promote receptor activation (dimerization and autophosphorylation). The activated receptor catalyzes the transfer of a phosphate group from ATP to a hydroxyl group of serine or threonine (phosphorylation) and triggers downstream signaling pathways, including MEK/MAPK kinase and PI3K/Atk pathways that regulate multiple cell types involved in angiogenesis.

Activation of MEK by protein kinase C (PKC) stimulates the MAPK pathway and activates various transcription factors and cell proliferation.

PI3K and its downstream-activated serine/threonine kinases Akt/protein kinase B (PKB) are involved in translating several important processes of angiogenesis, including endothelial cell migration, proliferation, and survival (see Fig. 1).

GIST

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal neoplasm of the gastrointestinal tract. Nevertheless, GISTs are a rare cancer with an estimated incidence of approximately 10–15 new cases per million individuals and only represent 2–3% to all gastrointestinal malignancies. Commonly, GISTs originate in the stomach (60%) and small intestine, including the duodenum (20–30%), but can occur in other sites including the rectum and rarely esophagus and colon. The main sites of metastatic spread are the liver and peritoneum. Less frequently occurring are extra-abdominal sites of metastases including the bone, lung, and lymph nodes.

GISTs can be histologically identified as highly cellular spindle cell or epithelioid mesenchymal tumors. The expression of Kit (CD117 antigen), which is a diagnostic criterion, is common to all histological subtype of GIST. Anoctamin-1 (DOG1) is another equally specific marker that often stain in CD117-negative GIST.

However, the hallmark of GIST is the presence of activating mutations in KIT and/or PDGFRA genes, which are considered to be key drivers in the pathogenesis of GIST. Both *KIT* and *PDGFRA* genes map to chromosome 4q12 and encode for third-type (or III type) tyrosine kinase receptor (TKR).

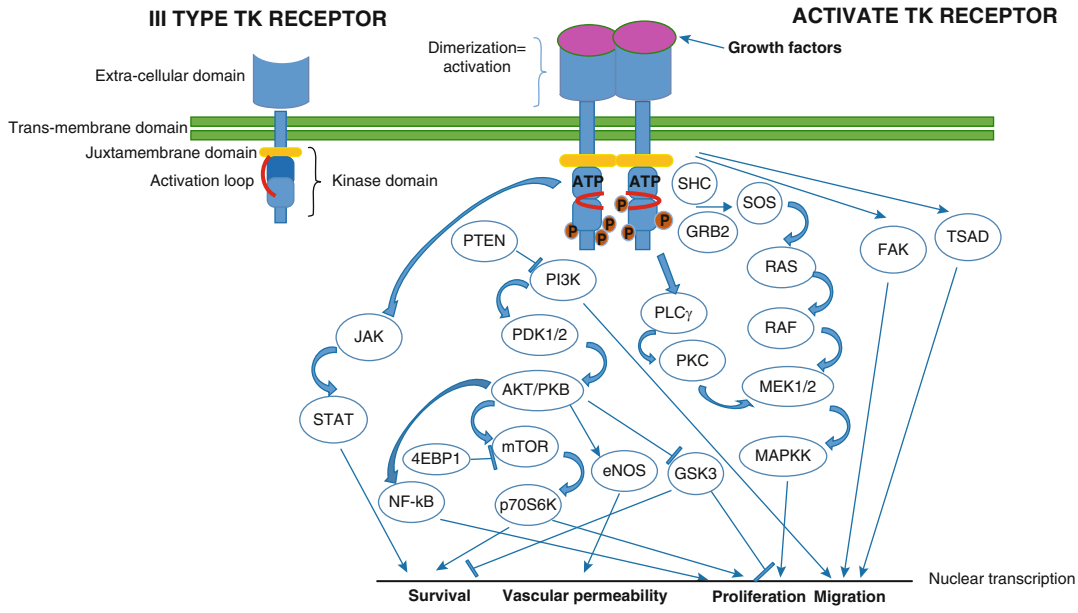

Fig. 1 Tyrosine kinase receptor and activated signaling pathway

Growth factor binding activates receptor tyrosine kinases by inducing receptor dimerization.

KIT gene mutation is the most frequent gain-of-function mutation seen in GIST. It occurs in 65% of all GISTs and usually involves exon 11, which encodes for the juxta-membrane domain. Rarely, mutations can also affect extracellular domain (exons 9) or TK I and TK II domains (exons 13 and 17) (Hirota et al. 1998). Approximately 30–35% of GISTs are wild-type KIT gene; around 20% of these cases have PDGFRA mutations.

PDGRFA plays an essential role in the regulation of embryonic development, cell survival, and chemotaxis. The most common mutations in PDGFRA gene are substitutions (or point mutations) of exon 12 and exon 18 (Hirota et al. 2003).

Mutations of KIT and PDGFRA activate receptor tyrosine kinases by rendering them constitutive for phosphorylation. In this way, they activate the mitogen-activated protein kinase (MAPK), PI3K/Akt, and JAK/STAT signaling pathways, which promote cell cycle activation, cell proliferation, and inhibition of apoptosis.

Ten to fifteen percent of GISTs have neither KIT nor PDGFRA mutations, and they are designated as "wild type"; this group forms a heterogeneous group classified in succinate-dehydrogenase (SDH)-deficient GIST, pediatric GIST, neurofibromatosis type 1 (NF1)-associated GIST, and GIST driven by mutations downstream the TK pathway, such as BRAF or NF1.

SDH complex is a component of Krebs cycle present in mitochondria that metabolize succinate to fumarate. SDH-deficient tumors principally accumulate succinate. The loss of SDH increases succinate and causes the activation of hypoxia inducible factor 1, which upregulates in the transcription of vascular endothelial growth factor (VEGF) and insulin- like growth factor-1 (IGF1) and further stimulates the cell growth (Wada et al. 2016).

GIST mutations can be divided in primary mutations related to GIST pathogenesis and secondary mutations, which occur during tyrosine kinase inhibitor (TKI) treatment, which causes treatment resistance. KIT and PDGFRA are believed to be mutually exclusive (the presence of KIT mutation excludes the presence of PDGFRA mutation and vice versa) both in primary and in metastatic sites.

Understanding the molecular pathology of GIST has allowed the development of specific agents against the targets that promote tumor growth.

GIST mutations have, also, a prognostic and predictive significance. To date, knowing the molecular characterization of a GIST has revealed important biological information which helps to predict response to TKI treatment and the natural history of the tumor (Lasota and Miettinen 2008).

KIT exon 11 mutations are the most sensitive to imatinib treatment and are found most frequently in gastric GISTs. In clinical trials of advanced GIST, those patients with exon 9 mutation seemed to have a better outcome if treated with a higher dose of imatinib (800 mg instead of 400 mg). Furthermore, according to a long-term analysis of the adjuvant study of imatinib 400 mg, GISTs harboring exon 9 mutations of KIT have a lower relapse-free survival (RFS) compared with exon 11 mutation albeit in a very small group of patients. PDGFRA mutations are sensitive to imatinib, with the exception of the rare D842V PDGFRA mutation, which shows primary resistance to imatinib. PDGFRA mutations have been diagnosed more frequently in adjuvant trials than in metastatic series, suggesting that these tumors have a lower recurrence rate. Wild-type GIST tends to develop in younger patients and may have a more indolent behavior; they are also less sensitive to imatinib.

Anti-angiogenic Therapy of GIST

Before the TKI era, GIST was found to be resistant to cytotoxic chemotherapeutic agents. Historically the median duration of survival for patients with metastatic GIST was 19 months and 9 months for those patients with metastatic disease and local recurrence (Dematteo et al. 2000). The introduction of tyrosine kinase inhibitors has utterly changed the history of GIST treatment.

The first approach to localized GIST remains surgery, which can play a role also in selected

cases with metastatic disease, although this is not yet clearly defined. The role of radiotherapy is debated both in localized and metastatic disease. Historical series show poor efficacy of radiotherapy treatment in GIST, even though there is some clinical evidence that, on the contrary, radiation therapy can be employed in GIST treatment particularly where hemostasis is required or for palliation.

Imatinib

Imatinib mesylate is a small molecule, tyrosine kinase inhibitor, which acts against c-ABL and Bcr-Abl, but is also able to target KIT and the platelet-derived growth factor receptors (PDGFR). Imatinib was first used for chronic myeloid leukemia (CML) and other chromosome Philadelphia-related leukemias. The target in CML, the Bcr-Abl gene product, presents in 95% of CML, a protein tyrosine kinase constitutively activated and believed to be at the basis of CML pathogenesis (Druker et al. 2001).

As already mentioned, GISTs express the cell-surface transmembrane receptor KIT that is a protein tyrosine kinase, constitutively activated. Imatinib mesylate acts by inhibiting the tyrosine kinase activity of KIT, resulting in a proapoptotic and antiproliferative action.

Given the multi-target inhibition spectrum, it was proposed that imatinib could play a role in GIST treatment. This hypothesis was confirmed in preclinical studies demonstrating decreased proliferation and apoptotic cell death in GIST cell lines (Tuveson et al. 2001). The first evidence from bench-to-bedside was in 2002. Demetri et al. (2002) published the results of an open-label phase II trial on the efficacy and safety of imatinib mesylate in metastatic GIST. Patients were randomized to receive either 400 mg or 600 mg of imatinib daily. The authors found 53.7% of partial response, 27.9% of stable disease, and 13% of progression disease. No patient had a complete response. Long-term result of this trial published in 2008 showed an overall survival of 50% after 5 years (Blanke et al. 2008), certainly longer than in historical series treated with chemotherapy. A subsequent meta-analysis demonstrated no difference in overall survival (OS) and progression-free survival (PFS) between high dose (800 mg per day) and standard dose (400 mg per day) of imatinib, with the exception of GISTs with exon 9 mutation. Within patients with exon 9 mutation, PFS was longer for patient treated with high doses of imatinib. No difference was observed in OS (MetaGIST 2010).

More recently, it has been also suggested that imatinib inhibits also VEGF-independent angiogenesis by targeting neuropilin 1-dependent ABL1 activation in endothelial cells. It is known that neuropilin 1 (NRP1) is expressed by endothelial cells (EC) to enable new blood vessel growth. NRP1 function is essential for angiogenesis. Raimondi et al. in 2014 discovered that NRP1 regulates angiogenesis by forming a complex with ABL1 that promotes EC motility in corneal cells in vitro and in vivo (Raimondi et al. 2014).

However, most patients develop resistance to imatinib treatment after a median of 2 years.

Interestingly, it is believed that the time of secondary resistance is correlated with tumor burden at diagnosis; thus, in 2009, the scope of imatinib treatment was extended to the adjuvant setting within a randomized phase III clinical trial (Dematteo et al. 2009). Patients with resected GIST and tumor dimension >3 cm were randomly assigned to receive adjuvant imatinib versus placebo for 1 year. The primary end point was recurrence-free survival (RFS). The imatinib arm showed 8% of patients with tumor recurrence or death compared with 20% in placebo arm at median follow-up of 19.7 months. The trial shows a significant improvement of RFS in imatinib versus placebo arm (98% vs. 89%, respectively, $p < 0.0001$). These results led to the introduction of adjuvant imatinib in clinical practice. In a subsequent study, a total of 400 enrolled patients with localized high-risk GISTs were randomly assigned to receive imatinib 400 mg for 1 year versus 3 years. Authors observed a 17% difference in 5-year relapse-free survival in favor of the 3-year treatment arm. A further study is ongoing, comparing 3 years versus 5 years of adjuvant imatinib treatment.

However, imatinib is thought to have a "cytostatic" rather than curative action on GIST, and it is able to delay the progression of the disease. Indeed, a drop in relapse-free survival curves 2–3 years later at the end of adjuvant imatinib treatment has been observed.

Sunitinib

Sunitinib malate is an oral multi-target tyrosine kinase inhibitor with a potent anti-angiogenic activity. Sunitinib inhibits VEGFR1, VEGFR2, VGFR3, PDGFRβ, KIT, fms-related tyrosine kinase 3 (FLT3), RET, and CSF1 receptor (CSF1R). It clearly appears that sunitinib plays a crucial role in the inhibition of cancer cell proliferation, angiogenesis, and lymph-angiogenesis.

Sunitinib use has been approved for imatinib-resistant or intolerant advanced GISTs in 2007 after a multicenter phase III trial, comparing placebo versus sunitinib in second-line treatment (Demetri et al. 2006). The primary end point of the study was median time to progression (TTP). TTP was significantly higher in the sunitinib arm as compared to placebo (27.3 weeks vs. 6.4 weeks). PFS results were similar to TTP in both sunitinib and placebo arms, with 24.2 weeks compared with 6 weeks, respectively. A long-term analysis showed a median OS of 72.7 weeks. OS for placebo arm was quite similar (64.9 weeks) probably because patients on placebo arm were crossed over on sunitinib treatment resulting in a converging OS curve (Demetri et al. 2012). In this study, authors examined also plasma level of pro-angiogenic factors including VEGF-A, sVEGFR-2, and sVEGFR-3 and sunitinib efficacy in a subset of patients. As known, plasma level of these factors changes from baseline according with sunitinib treatment schedule. Unfortunately, there was not found any predictive value of angiogenic factor plasma levels and sunitinib efficacy.

Since sunitinib is a smaller molecule than imatinib, structural studies demonstrated that its activity in imatinib-resistant patients is due to the ability to avoid acquired stereotactic block entrance to the KIT protein. In particular, it has been demonstrated that secondary point mutation associated with imatinib treatment resistance is located in the drug/adenosine triphosphate (ATP) binding pocket of the receptor (encoded by exons 13 and 14) or in the activation loop (encoded by exon 17). Sunitinib shows activity against KIT exon 13 or exon 14 resistance acquired mutations but not exon 17 mutant kinases (Prenen et al. 2006).

However, as for imatinib, the failure of the treatment is due to resistance mechanisms. Sunitinib resistance occurs usually within 1 year of treatment, and it is characterized by a larger number of mutations (from one to five) than in imatinib-resistant GIST (usually two).

Regorafenib

Regorafenib is the latest tyrosine kinase inhibitor approved in 2013 for advanced GIST, imatinib and sunitinib resistant, in third-line of treatment. Regorafenib is a potent oral TKI that inhibits multiple protein kinases involved in oncogenesis, angiogenesis, and the tumor microenvironment, including KIT; PDGFRA; RET; RAF1; BRA; VEGFR1, VEGFR2, and VEGFR3; TIE2, and fibroblast growth factor receptor (FGFR). The randomized double-blind study (GRID study) enrolled 199 patients with metastatic GIST after failure of both consecutive imatinib and sunitinib treatments (Demetri et al. 2013). The patients were randomly assigned with 2:1 ratio to receive either regorafenib or placebo. The median PFS was 4.8 months on regorafenib and 0.9 months on placebo (HR 0.27, $p < 0.0001$). Six (4.5%), and one (1.5%) of the patients assigned to regorafenib and placebo had partial response (PR), respectively, and 71.4 and 33.3% had stable disease (SD). No complete responses (CR) were observed.

There is increasing evidence that regorafenib has activity against KIT exon 17 mutations which are known to be more prominent following exposure to multiple TKIs. In the long-term follow-up results of phase II trial of regorafenib in advanced pretreated GIST, interestingly the median PFS for patients with tumor that harbored a secondary

mutation in exon 17 was 22 months (95% CI 6–NR), suggesting a more sensitive inhibition of this common KIT secondary mutation by regorafenib (Ben-Ami et al. 2016).

Furthermore, regorafenib is believed to be effective in wild-type GISTs, which usually are relatively insensitive to other tyrosine kinase inhibitor treatment.

As already mentioned, succinate dehydrogenase is a member of a complex chain of enzymatic reactions responsible for the oxidation of succinate to fumarate. This complex chain is an important regulator of hypoxia-inducible factor-1α (HIF-1α), a subunit of HIF1. In SDH-deficient GIST, succinate dehydrogenase inactivation leads to accumulation of HIF-1α and to high expression of pro-angiogenic genes (including VEGF) (Pollard et al. 2005). In the Ben-Ami et al. study (Ben-Ami et al. 2016), authors report that all six patients with SDH-deficient GIST (KIT/PDGFRA wild-type GIST) experienced clinical benefit from regorafenib with tumor response or stabilization for 16 weeks or more. Other potent VEGF inhibitors used in this subtype, such as pazopanib and sorafenib, achieved clinical benefit and tumor response, suggesting the important role of VEGF factor and its inhibition in this population.

Other Anti-angiogenic Inhibitors

Based on mechanism of angiogenic inhibition, several other drugs have been developing since imatinib approval.

Nilotinib is a very similar molecule to imatinib. Nilotinib potently inhibits receptor tyrosine kinases KIT, PDGFRα, and PDGFRβ as well as ABL1 and BCR-ABL. A phase III randomized trial compared nilotinib versus best supportive care with or without TKI (Reichardt et al. 2012). This study failed in demonstrating superiority in PFS (according to the central radiology review), but nilotinib was associated with a non-statistically significant improvement in median OS in the ITT population. A following phase III study (ENESTg1) compared first-line imatinib versus nilotinib in patients with metastatic or unresectable GIST (Bernard Lyon et al. 2015). Two-year progression-free survival was higher in the imatinib group (59.2%) than in the nilotinib group (51.6%). Based on the results of these trials, nilotinib is not in use in the treatment of GIST patients.

Pazopanib is an oral tyrosine kinase inhibitor, which inhibits KIT, VEGFR1, VEGRF2, VEGFR3, PDGFRα, and PDGFRβ. This drug has been recently approved for the treatment of patients with previously treated metastatic soft-tissue sarcomas, excluding liposarcoma, osteosarcoma, and GIST. A randomized phase II trial evaluated the efficacy of pazopanib after first line in metastatic GIST patients (Ganjoo et al. 2014). Patients were randomly assigned to receive either pazopanib 800 mg daily or best supportive care (BSC). The study enrolled 27 patients but evaluated 25 patients (two patients did not receive pazopanib because of proteinuria). The median PFS of the total cohort enrolled was 1.9 months, and the median OS was 10.5 months. Two patients in the cohort had SDH-deficient wild-type GIST; one interrupted the treatment after one cycle due to toxicity; the second one, interestingly, experienced a long stabilization of disease (more than 17 cycles administered). This suggests a possible activity of pazopanib in this subtype. Although the mechanism of action is unknown, the potent anti-angiogenic activity of pazopanib could probably play a role. Indeed, as already mentioned, SDH deficiency leads to high level of VEGF that might represent the key role in pazopanib efficacy.

Vatalanib is an oral protein tyrosine kinase inhibitor of KIT, PDGFRα, as well as VEGFR1, VEGFR12, and VEGFR13. Since vatalanib inhibits protein kinases more selectively, it seems to be better tolerated than other TKIs such as sorafenib or sunitinib. A phase II trial investigated the efficacy of vatalanib in second- and third-line treatment, after either imatinib or imantinib and sunitinib treatment failure (Joensuu et al. 2011). Although the PR rate was low (4.4%), many patients (35.6%) had SD lasting for 6 months, suggesting some vatalanib activity in GIST.

Sorafenib was initially developed as a specific inhibitor of protein serine-threonine kinase RAF.

Actually, sorafenib is a multi-kinase inhibitor, and its action is also against receptor protein tyrosine kinases such as KIT, VEGFR2, VEGFR3, and PDGFR. In in vitro experiments, sorafenib has showed activity in imatinib- and sorafenib-resistant cell lines and in GIST xenograft models. Montemurro et al. (2013) published a retrospective series of patients treated with sorafenib after imatinib, sunitinib, and nilotinib failure. The analysis showed a median PFS of 6.4 months and of median OS of 13.5 months with no statistical differences between patients who received sorafenib as third- or fourth-line treatment. Furthermore, no difference in terms of response was seen among different mutations.

Masitinib is approved for the treatment of mastocytosis in dogs. Masitinib is a TKI that inhibits wild-type c-Kit; its constitutively activated mutated form, PDGFRα; and PDGFRβ. Masitinib, at 12 mg/kg dosage, showed activity in a phase I trial with 19 GIST patients. Among them one of two imatinib-intolerant patients had PR, and 29% of imatinib-resistant patients had SD as best response (Soria et al. 2009). Given the good early activity in the phase I study, a more selective profile of action and thus minor "off target" toxicity was expected, as well as the possibility of individualize treatment by the adjustment of dose; masitinib activity was subsequently explored in a phase II trial. Thirty imatinib-naïve patients with locally advanced or metastatic GIST were enrolled. Best responses were a complete response in 1/30 patient (3.3%), partial response in 15/30 patients (50%), stable disease in 13/30 patients (43.3%), and progressive disease in 1/30 patient (3.3%). Estimated median PFR was 41.3 month, and OS at 2 and 3 years was 89.9% (Le Cesne et al. 2010). In a small randomized open-label study, masitinib versus sunitinib were randomly assigned to imatinib-resistant patients. In the overall population, OS was significantly increased in the masitinib treatment arm with a median estimated OS >21.2 months compared with 15.2 months in the sunitinib arm. These results have to be confirmed in ongoing phase III trials (NCT00812240 and NCT01694277) (Adenis et al. 2014).

Tumor Response Evaluation in the TKI Era

Since the imatinib introduction on GIST treatment, radiological changes of tumor appearance have been observed. As already explained, targeted therapies induce changes in lesion structure, including decrease in tumor density, enhancement of intra-tumoral nodules, and decrease in tumor vessels, that are consistent with tumor response even without a change in tumor size. As Response Evaluation Criteria in Solid Tumors (RECIST) take into consideration as parameter of tumor response the anatomic information only (tumor size), they could underestimate the tumor response to imatinib in patients with metastatic GIST. Due to this evidence, the concern about the use of traditional tumor response criteria has increased. Positron emission tomography (PET) using [18F]fluorodeoxy-glucose (FDG) has subsequently been proposed as an early, sensitive marker to detect early tumor response to anti-angiogenic agents by following the decrease in glucose metabolism. FDGPET could be also useful in predicting long-term response to imatinib in patients with metastatic GIST. Choi et al. (2007) proposed a study in which they evaluated whether the changes on CT scan of patients on imatinib treatment correlated with the changes in glucose metabolism on FDG-PET (measured as SUV_{max}). The author suggested new contrast-enhanced CT criteria (see Table 1) in which they took into consideration many tumor characteristics, such as tumor density (determined by measuring CT attenuation coefficient in Hounsfield unit [HU]), enhancing tumor nodules and tumor vessels in addition to tumor size. In the study, they found that response evaluated by Choi criteria, a 10% decrease in size or a 15% decrease in density on contrast-enhanced CT, was more predictive of time to tumor progression (TTP) than response by RECIST. Due to this study, Choi criteria have become a possible new paradigm for the evaluation of tumor response in GIST.

Since then, other radiologic techniques have been explored to better evaluate tumor response such as dual-energy CT (DECT). By using two

Table 1 Choi criteria definition

Complete response (CR)	Disappearance of all lesions. No new lesions
Partial response (PR)	A decrease in size of ≥10% or a decrease in tumor density (HU) ≥15% on CT. No new lesions
Stable disease (SD)	Does not meet the criteria for CR, PR, or PD. No symptomatic deterioration attributed to tumor progression
Progression of disease (PD)	An increase in tumor size of ≥10% and does not meet criteria of PR by tumor density (HU) on CT. New lesions. New intratumoral nodules or increase in the size of the existing intratumoral nodules

different energy and iodine contrast, DECT allows to improve lesion-to-background contrast and the quality of vascular imaging (Agrawal et al. 2014). Some studies have shown that DECT could represent a better predictor of therapeutic benefit in advanced GIST patients treated with tyrosine kinase inhibitors than other response criteria.

The Other Side of the Coin: Mechanism of Acquired Resistance and Toxicity

Even though anti-angiogenic therapy has changed the approach to GIST treatment, two subsequent important concerns have been come up: resistance to treatment and TKI-related toxicity.

The mechanisms of resistance are heterogeneous and still not completely understood. They are linked to the presence of secondary mutations on KIT or PDGFRA genes, genomic amplification of KIT, histological changes, or decrease of imatinib bioavailability. Furthermore, acquired resistance to anti-angiogenic therapy in cancer may also be explained with the redundancy and the diversity of angiogenesis mechanisms, including activation of parallel angiogenic pathways and production of alternative angiogenic factors.

The most common and prevalent cause of resistance to TKI are point mutations within the kinase domain, which lessens the affinity of the TKIs to binding domains.

In GIST, the most common point mutations harboring in the KIT gene involve exons 13, 14, and 17. Exons 13 and 14 encode for drug/adenosine triphosphate (ATP)/binding pocket, and, when mutated, they affect the imatinib binding without affecting the whole kinase receptor. The point mutation of exon 17 encodes for the activation loop that stabilizes the active conformation of the KIT kinase and prevents imatinib binding, which occurs only in the inactive conformation. Interestingly, secondary mutation resulting in KIT activation is not linked to the histology or response to imatinib (Antonescu et al. 2005). As already mentioned, exon 13 and exon 14 mutations are sensitive to sunitinib treatment, and exon 17 seems to be more sensitive to regorafenib.

Furthermore, VEGF pathway could be activated by others means, such as PI3K/Akt signaling that could be promoted by angiopoietin-Tie. PI3K/Akt regulates cell proliferation, survival, and angiogenesis, and it is associated with the expression of mammalian target for rapamycin (mTOR). Deregulation of upstream pathway effectors can lead to hyperactivation of the protein kinase mTOR. Everolimus, an oral mTOR inhibitor, has shown in preclinical studies synergic, antiproliferative effects when combined with imatinib in imatinib-resistant GIST cell lines (Bauer et al. 2007). A phase I–II study combined imatinib with mTOR inhibitor, everolimus, in patients with imatinib-resistant GIST (Schöffski et al. 2010). The study suggested a potential therapeutic effect of the combination. Ongoing trials are investigating the combination of imatinib plus a MEK inhibitor (MEK162/binimetinib) as first-line treatment (NCT01991379) and imatinib plus a PI3K inhibitor (BYL719) as third-line treatment (NCT01735968).

Interesting preclinical data have been recently published, showing the effects of combined inhibition of Akt, the downstream effector of PI3K, and KIT in a panel of imatinib-sensitive and -resistant GIST cell lines (Zhou et al. 2017). The authors demonstrated in vitro the enhanced activity in combining AKT inhibitor and imatinib, opening the way for a future study in vivo.

Given the high expression of VEGFA in GIST, which correlates with poor prognosis, in 2008, a phase III randomized trial (SWONG trial) investigated the efficacy of the combination of imatinib

and bevacizumab, the first anti-angiogenic treatment licensed against VEGF. Unfortunately, the study was closed due to poor accrual; therefore any conclusions cannot be made about this hypothesis (Blanke et al. 2015).

Furthermore, a preclinical study has shown that the combination of imatinib with PD1-PDL1 blockade can enhance the antitumor activity of imatinib. In particular, in a mouse model of GIST, anti-PD-1 and anti-PD-L1 showed any antitumor effect when used alone but could improve the efficacy in combination with imatinib (Seifert et al. 2017).

Another important concern with TKI therapy is toxicity. A huge variety of side effects has been described during the TKI treatment including nausea, diarrhea, edema, musculoskeletal complaints, fatigue, hemorrhage, hand and foot skin reaction, skin and hair discoloration, mucositis, hypertension, cardiac toxicity, hypothyroidism, hepatic enzymes alteration, and hematological toxicity. These side effects are completely different from those classically related with chemotherapeutic agents both in terms of duration and presentation. In particular, whereas chemotherapy side effects are mainly seen some days after its administration (nadir, short-term side effects) but also many years later (long-term side effects), TKI toxicity is strongly associated with the administration of therapy and usually improves as soon as therapy is discontinued. It could be accounted for as the direct receptor inhibition by anti-angiogenic agents. A famous example of this switch on/off of tyrosine kinase inhibitor side effects has been reported with a hair discoloration resulting in the patient's hair turning white during the weeks on sunitinib and brown during the week after treatment due to c-kit inhibition in melanocytes (Botchkareva et al. 2001). Mechanisms of toxicities, related to anti-angiogenetic kinase inhibitors, might be due to the so called "off-target effects" due to the inhibition of hidden targets of multi-kinase inhibitors. To some extent, "off target effects" may lead to the discovery of unknown signaling pathways. However, selective inhibitors may induce toxicities because of the expression of the kinase targets in endothelial cells (Gotink and Verheul 2010).

One of the most common drug-related toxicity is hypothyroidism. The first evidence of this side effect came in 2006 and showed up in patients with metastatic GIST treated with sunitinib (Desai et al. 2006). The authors found that 60% of patients with basal normal thyroid function developed thyroid dysfunction. Thyroiditis has been suggested as a possible cause of hypothyroidism. Nevertheless, the exact mechanism of thyroid dysfunction cannot be explained. It is commonly believed that there are many factors involved including a direct effect of sunitinib on sodium iodide symporter or TSH receptor (Mannavola et al. 2007) or indirect effect due to inhibition of targets such as VEGFR-2, KIT, BRAF, and RET. Moreover, several studies demonstrated that drug-related hypothyroidism might be correlated with better prognosis (Bilen et al. 2016).

Important side effects connected to TKI treatment could also affect the cardiovascular system. The cardiovascular effects of small molecule TKIs include peripheral edema and congestive heart failure, systemic and pulmonary hypertension, acute coronary syndromes, and cardiac arrest due to QTc prolongation. A recent meta-analysis conducted to reveal the relative risk of congestive heart failure associated with multi-targeted VEGFR tyrosine kinase inhibitors showed an increased risk of arterial thromboembolic and cardiac events with the use of VEGFR TKIs (Ghatalia et al. 2015). VEGFR plays a crucial role in maintaining a well-vascularized myocardium both under normal conditions and after ischemic changes. Among these cardiovascular events, special mention has to be given to hypertension. The physiopathology of hypertension is directly correlated with abnormalities in endothelial function and angiogenesis. Alterations of the microvascular network result in a significant increase in blood pressure during anti-angiogenic treatment, probably due to a direct effect at the level of the microvascular structure. Furthermore, a deficient production of the vasodilator nitric oxide (NO) from endothelial cells or decreased NO levels seem to play a central role. VEGF activates endothelial NO synthase through AKT, and VEGF pathway inhibitors have been shown to inhibit this process leading to a decrease in NO

levels. Inhibition of NO may cause vasoconstriction and hypertension. NO is also implicated in the tubular regulation of sodium excretion, and its inhibition results in renal-mediated hypertension (León-Mateos et al. 2015).

A vast range of skin reactions should be mentioned in TKI-related toxicities, in particular hand and foot skin reaction (HSFR) or palmoplantar erythrodysesthesia syndrome, which most frequently occurs in patients treated with TKI, such as sorafenib and sunitinib. So far, the mechanism of these toxicities cannot yet be explained. It is probably associated with a damage of the endothelium in hands and feet.

Conclusion

The anti-angiogenic approach has dramatically changed cancer treatment in the last decade, especially in GIST, traditionally known to be insensitive to chemotherapy and radiotherapy. Imatinib was the first protein tyrosine kinase inhibitor used in metastatic GIST resulting in a substantial stretch of overall survival. Unfortunately, although three lines of treatment are available, there are no standard options beyond regorafenib.

As extensively discussed, molecular analysis plays a central role in GIST management. Its importance is undeniable in diagnosis and prognosis of GIST. For this reason, circulating tumor DNA collection by bloodstream, also known as liquid biopsy, is an emerging technique, which allows the detection of early secondary mutations and unknown primary resistance. Identical point mutations were found in several studies between surgical tissue and circulating DNA. This technique could lead to monitoring tumor status during treatment with a minimally invasive blood test.

Furthermore, several new target therapies are under evaluation for imatinib-resistant GIST patients, especially against insensitive mutations, including crenolanib, an inhibitor of imatinib-resistant PDGFRA kinases as well as D842V, and ponatinib with a pan–BCR-ABL and KIT inhibitory profile in cellular assays, showing activity in refractory GIST in preclinical study. In addition, ongoing clinical trials are evaluating the efficacy of inhibitors of downstream pathway kinases (PI3K and MAPK) and heat shock protein (HSP), as well as an immune-modulated approach.

The association of different inhibition mechanisms could represent the future direction against imatinib resistance.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Combination of Anti-angiogenics and Other Targeted Therapies
- ▶ Imaging Tumor Angiogenesis
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part XII

Anti-angiogenics in Other Soft Tissue Sarcomas

The Value of Anti-angiogenics in Soft Tissue Sarcoma Therapy

Bernd Kasper and Charlotte Benson

Contents

Introduction 466
Angiogenesis and Sarcoma 466
Preclinical Data 466
Anti-angiogenic Agents 467
Systemic Chemotherapy 470
Future Directions 471
Conclusion 471
Cross-References 471
References 472

Abstract

Soft tissue sarcomas are a rare and diverse group of tumors of mesenchymal origin with a spectrum of differing behaviors. While surgery is the mainstay of treatment in localized disease, a large proportion may metastasize. In the metastatic or advanced disease setting, cytotoxic chemotherapy is of modest benefit and there is a clear need for new treatments. Emerging preclinical data have shown that angiogenesis is a credible target in soft tissue sarcomas. Early clinical trials and retrospective case studies have shown that a number of anti-angiogenic agents have evidence of benefit. One drug, pazopanib, has recently been approved for the treatment of patients with advanced soft tissue sarcomas. However, a lot still has to be gained in understanding of biological markers of response and correct selection of histological subtypes.

Keywords

Monoclonal antibody · Tyrosine-kinase inhibitor · Bevacizumab · Sunitinib · Pazopanib · Sorafenib · Cediranib · Regorafenib · Paclitaxel

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Introduction

Soft tissue sarcomas (STS) are a rare and heterogeneous group of soft tissue tumors of mesenchymal origin. They may arise in any anatomical site and span all age groups making them a diverse and challenging group of tumors to treat. Surgery is the mainstay of treatment in localized disease; however, a significant proportion (around 50%) of patients may later develop metastatic disease or indeed may present with disease at an advanced stage. Conventional cytotoxic chemotherapy is typically used in the metastatic setting where the main goals of treatment are disease palliation. Doxorubicin and ifosfamide remain the principal active cytotoxic agents that are used in the first line setting. Other active drugs include gemcitabine ± docetaxel, trabectedin, and dacarbazine. However, response rates are modest, around 20%, and debate continues whether there is an overall survival benefit associated with systemic treatment (Judson et al. 2014a). Further challenges abound in clinical trial design given both the disease rarity and the varied response rates and sensitivities of differing sarcoma subtypes which are often combined in large phase III trials which may lead to difficulty in interpretation for specific subtypes. Clearly, new treatment strategies are required, preferably those that exploit our increasing knowledge of underlying tumor biology. Anti-angiogenesis is one such area that has been explored leading to development of some promising novel therapies in the field of STS. The aim of this chapter is to review recent clinical trial data of anti-angiogenic therapies and supporting translational research as well as indicating interesting ongoing clinical trials for STS patients.

Angiogenesis and Sarcoma

Angiogenesis is simply the process of new vessel formation. In order for small tumors to grow larger than 2 mm and develop invasive and metastatic behavior, they need to have angiogenic properties, i.e., the ability to develop new blood vessels (Folkman 1995). This change in balance between pro-angiogenic factors and loss of angiogenic inhibitors is known as the angiogenic switch. This may be caused by oncogenic mutations, hypoxia, and stress and allows tumor cells to adapt to hypoxic surroundings. There are many molecules that are positive regulators of angiogenesis including vascular endothelial growth factor (VEGF) and its three associated receptor forms (VEGFR-1, VEGFR-2, and VEGFR-3), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF) among others, in a complex interaction of pro- and anti-angiogenic factors; all have a critical role in tumor angiogenesis.

Anti-angiogenic therapy is a potentially exciting strategy because tumor vascular supply is absolutely fundamental to tumor growth. There are many different possible pathways involved in this highly complex process, many of which are possible for targeting treatment. Potential modes of action of anti-angiogenic therapy include inhibition of action of these factors by monoclonal antibody therapy, receptor protein tyrosine kinase inhibitors, and inhibitors of the signal transduction pathway.

Preclinical Data

There are interesting preclinical data showing varying levels of VEGF expression in different STS subtypes. Those tumors with the highest expression included epithelioid sarcoma and alveolar soft part sarcoma (Kuhnen et al. 2000). Another group showed high serum levels of VEGF expression in about 25% of patients with soft tissue sarcoma. Those subtypes with highest expression included malignant fibrous histiocytoma, dermatofibrosarcoma protuberans, and leiomyosarcoma (Potti et al. 2004). Serum VEGF levels in STS have been correlated with tumor size, grade, and risk of recurrence. Poorly differentiated tumors have been shown to have higher VEGF levels and are perhaps a marker of more aggressive behavior (Yoon et al. 2004; Chao et al. 2001; Graeven et al. 1999; Hayes et al. 2004). Furthermore, in tumor xenograft work, VEGF transfected xenografts formed very vascular tumors with accelerated growth, increased

chemoresistance, and a higher rate of pulmonary metastases (Zhang et al. 2006).

There is also data exploring the role of VEGFR expression. A Japanese group investigated the immunohistochemical expression of vascular endothelial growth factors and their receptors in a series of angiosarcomas (Itakura et al. 2008). VEGFR-1 was expressed in 94%, VEGFR-2 in 65%, and VEGFR-3 in 79% of tumor samples, and those patients that expressed low or no VEGFR-2 showed a significantly poorer prognosis. Subsequent work examined sequencing of the VEGFR-2 gene in a group of patients with angiosarcoma and showed evidence of VEGFR-2 overexpression in a proportion of patients. Further cell line work showed evidence of efficacy of both sunitinib and sorafenib suggesting that VEGFR-2 mutations may favor response to these anti-angiogenic agents (Antonescu et al. 2009).

Clearly, in a heterogeneous group of tumors, the behavior and interaction of the angiogenic pathway may well vary between histological subtypes. Another important factor is hypoxia-inducible factor (HIF-1α), a transcription factor that supports the adaption of human tumor cells to hypoxia, tumor growth, and progression. Expression of HIF-1α has been shown to be a biomarker of outcome in STS (Shintani et al. 2006). A study of 49 tumor specimens showed that those patients with a strong or moderate expression of HIF-1α had a significantly shorter overall survival rate compared to those with negative or week expression.

This important tranche of translational work has provided a good biological rationale for the scientific exploration of anti-angiogenic drugs as a potential therapeutic strategy in STS.

Anti-angiogenic Agents

Anti-angiogenic therapies may be categorized into three main groups: the monoclonal antibodies, the protein tyrosine kinase inhibitors, and systemic chemotherapy.

Bevacizumab is the most widely studied **monoclonal antibody** against VEGF and one of the earliest anti-angiogenic agents to be investigated in STS. It is a humanized monoclonal anti-VEGF antibody and has been studied both as a single agent and in combination with cytotoxic treatment and also in conjunction with radiotherapy.

One of the earliest trials was with bevacizumab in combination with doxorubicin (D'Adamo et al. 2005). This was a phase II trial comprising 17 patients with advanced soft tissue sarcoma. Although two responses were seen, alongside with several stable diseases (n = 11), the incidence of cardiac toxicity was high, despite the use of the cardioprotective agent dexrazoxane for the latter cycles of doxorubicin. Furthermore, there was one patient death due to bilateral pneumothorax. Pneumothoraces, following pulmonary cavitation, are seen not uncommonly as part of disease response in STS, partly because lung metastases are a common phenomenon and possible also due to the underlying disease biology and do represent a difficult therapeutic challenge in a responding patient.

Other combination schedules with bevacizumab include the addition to temozolomide and more recently with gemcitabine and docetaxel, the so-called Axtell regimen. The bevacizumab and temozolomide study was developed to investigate activity in a STS subtype, solitary fibrous tumor, which is known not only for its poor response rate to conventional chemotherapy but also its angiogenic properties (Park et al. 2011). Disease response was evaluated by Choi criteria (Choi et al. 2007) which was initially developed to assess response in gastrointestinal stromal tumor (GIST) and takes into account not only change in tumor dimension but also variations in tumor density. In this study, 11 out of 14 patients were found to have a Choi response and median progression free survival (PFS) was 9.7 months, which is certainly very encouraging.

The gemcitabine and docetaxel regimen is commonly used in soft tissue sarcoma with activity in a number of subtypes including leiomyosarcoma and pleomorphic sarcomas. Several groups have investigated the addition of bevacizumab (Verschraegen et al. 2012) including a recently published randomized double blind

phase III trial of gemcitabine and docetaxel ± bevacizumab in patients with metastatic uterine leiomyosarcoma (Hensley et al. 2015). Disappointingly, accrual was stopped early due to futility as no improvement was seen in PFS, overall survival (OS), or overall response rate (ORR).

Finally, bevacizumab in combination with weekly paclitaxel as treatment for angiosarcoma has been recently reported in a randomized phase II trial (Ray-Coquard et al. 2015). Toxicity was greater in the combination arm with one fatal intestinal occlusion, but there was no increase in response rate.

Bevacizumab has also been investigated as a single agent in a number of different STS subtypes. It has shown hints of activity in HIV-related Kaposi's sarcoma (Uldrick et al. 2012), desmoplastic small round cell tumor (DSRCT) (de Araujo and Araujo 2014), alveolar soft part sarcoma (Azizi et al. 2006), as well as angiosarcomas and epithelioid haemangioendotheliomas (Agulnik et al. 2013). Bevacizumab has also been shown to be safe in the pediatric population (Glade Bender et al. 2008).

Bevacizumab in combination with radiotherapy is also an area of interest and has been reported on in the preoperative setting (Yoon et al. 2011) with impressive rates of pathological necrosis and now is being studied in a prospective clinical trial (NCT00356031).

The challenge remains in identifying the STS subtype most likely to benefit from bevacizumab. Bevacizumab in addition to conventional chemotherapy has not yielded as many benefits as had been hoped and has led to overlapping toxicities and occasional toxic deaths and so, currently, is not part of the standard treatment paradigm in STS.

The tyrosine **kinase inhibitors (TKIs)** comprise a much wider group of agents with a varying specificity and spectrum of targets.

Sunitinib is a multi-targeted TKI with a broad range of activity against targets including VEGFR-1–3, PDGFR, KIT, FLT3, RET, and CSF-1. Its role in STS was initially investigated by George and colleagues in an open label study, utilizing continuous daily dosing of sunitinib, 37.5 mg, in a group of 53 patients (George et al. 2009). Response evaluation included the use of PET scans on a subset of patients, at baseline and after 10–14 days of treatment in order to assess metabolic response. Disease control/stable disease was seen in a proportion of patients (10 patients for greater than 16 weeks) and one patient with the rare sarcoma subtype desmoplastic small round cell tumor (DSRCT), with an aggressive disease biology, had a prolonged partial response, remaining on study for over a year.

The role of sunitinib in three of the more common STS subtypes, leiomyosarcoma, liposarcoma, and malignant fibrous histiocytoma (MFH, now known as undifferentiated pleomorphic sarcoma), was also investigated in a single center phase II study (Mahmood et al. 2011). In this study, the 50 mg dose for 4 weeks every 6 weeks was used and patients were divided into treated and not pretreated groups. Liposarcomas and leiomyosarcomas with no prior systemic treatment fared best with PFS rates at 3 months of 75% and 60%, respectively. This apparent benefit may in part be attributed to the varying behavior of liposarcoma, which can behave in an indolent fashion. MFH or pleomorphic sarcoma has an aggressive disease biology and chaotic karyotype and so it is perhaps unsurprising that these patients did not respond as well.

Patients with uterine leiomyosarcoma who had progressed following prior chemotherapy have also been investigated in a phase II trial (Hensley et al. 2009). Disappointingly, the median number of treatment cycles delivered was 1. Two out of the 23 patients achieved a partial response and only four were progression free at 6 months. Given the known toxicity profile of sunitinib and the lack of clinically meaningful benefit, this is unlikely to be a useful treatment strategy for this particular group of patients.

The Milan group has demonstrated several uses for sunitinib for patients with rare and unusual STS subtypes. Sunitinib has shown to be of benefit in a number of small retrospective studies including extra skeletal myxoid chondrosarcoma (Stacchiotti et al. 2014), solitary fibrous tumor (Stacchiotti et al. 2012), and clear cell sarcoma (Stacchiotti et al. 2010). In some of

these ultra-rare subtypes, the challenge remains to achieve funding for drugs such as sunitinib given that large randomized trials are likely never to be feasible where the patient numbers are so small.

The challenge of drug development in rare disease is amply demonstrated by the treatment of alveolar soft part sarcoma (ASPS), a malignant, highly vascular soft tissue tumor affecting young adults that accounts for only 1–2% of all STS. It is refractory to conventional chemotherapy and generally has an indolent course but a high metastatic potential and a median overall survival of around 40 months. ASPS has a pathognomonic translocation t (X, 17) (p11; q25) leading to the ASPL-TFE3 transcription factor, which in turn leads to upregulation of angiogenesis. Sunitinib has been shown to have activity in this rare disease type, again by the Milan group (Stacchiotti et al. 2011). A randomized phase II trial is in process comparing the activity of sunitinib versus **Cediranib**, another potent anti-angiogenic agent with activity against VEGFR and KIT (NCT01391962) in addition to a randomized phase II clinical trial of cediranib versus placebo (NCT01337401). Signs of activity with cediranib were first seen in early phase I trials and have now been confirmed in two phase II studies. These have shown an objective response rate of 35% and disease control rate of 84% at 24 weeks (Judson et al. 2014b; Kummar et al. 2013). It is hoped that the two ongoing randomized trials will demonstrate sufficiently compelling data to allow for funding of these two drugs that appear to have real benefit in this orphan disease.

Sorafenib is a multi-targeting anti-angiogenic agent with activity against VEGFR-2, VEGFR-3, PDGFR, and KIT among other targets. Its activity was first explored in a phase II trial, which was stratified by histological subtype (Maki et al. 2009). Angiosarcoma was the only subtype to meet the RECIST rate primary endpoint, and the rate of dose reduction was high, 61%, due to mainly skin related adverse events. Following on from this, the French Sarcoma Group designed a trial of sorafenib in patients with either superficial or visceral angiosarcoma (Ray-Coquard et al. 2012); however, the median progression free survival was short (1.8 and 3.8 months, respectively) and response rate was low. Another stratified trial did show hints of activity in vascular sarcomas but again no partial responses (von Mehren et al. 2012).

However, more encouragingly sorafenib has been shown to be active in desmoid tumor/deep fibromatosis with 25% patients achieving partial response and 70% noting clinical benefit, which correlated with T2 signal change on magnetic resonance imaging (Gounder et al. 2011). A prospective randomized placebo-controlled phase III trial is underway in the United States (NCT02066181).

Regorafenib is also a multi-targeting agent which binds to and inhibits VEGFR-1, -2, and -3; RET; KIT; PDGFR; and Raf leading to inhibition of tumor angiogenesis and proliferation. The phase I trial included a response from a patient with soft tissue sarcoma (Mross et al. 2012). Therefore, a multinational randomized placebo-controlled phase II trial is in process (REGOSARC), investigating activity in liposarcoma, leiomyosarcoma, synovial sarcoma, and other subtypes (NCT01900743). Interim data presented at ASCO 2015 showed the median PFS of patients with leiomyosarcoma was 4 months on regorafenib versus 1.9 months with placebo. The median PFS of those patients with other subtypes were 4.6 and 1.0 months with regorafenib and placebo, respectively. The 6-month OS rate of leiomyosarcoma patients was significantly higher in the treatment arm (87.0 versus 75.9%); this difference was not significant in the other sarcoma cohort (79.0 versus 62.0%). These early results demonstrate promising activity in both patient groups and the final results are eagerly awaited.

Pazopanib is perhaps the most widely studied and arguably most promising anti-angiogenic agent in STS (Kasper and Hohenberger 2011). It has activity against VEGFR-1,-2,-3; PDGFR; and KIT and is the first drug in this class to have proven benefit in a randomized phase III trial. Initially, a stratified phase II trial was conducted by the European Organisation for Research and Treatment of Cancer (EORTC) (Sleijfer et al. 2009). The aim of this study was to look for activity in four STS groups: liposarcoma,

leiomyosarcoma, synovial sarcoma, and "'other." All these categories save for liposarcoma met the progression free rate at 12 weeks (44% in leiomyosarcoma, 49% in synovial, and 39% in the 'other' group). This led to the randomized, double blind phase III **PALE**TTE study for all major STS subtypes excluding liposarcoma (Van der Graaf et al. 2012). 369 patients were enrolled in a 2:1 randomization. Median PFS was 4.6 months for pazopanib compared to 1.6 months for placebo and was highly statistically significant ($p < 0.001$). There was no significant difference in overall survival, most likely due to crossover and further treatment on disease progression. The results of this trial led to EMA and FDA approval of pazopanib in advanced non-adipocytic soft tissue sarcoma. Further work on the data from the two EORTC pazopanib trials (phase II and III) has shown that those patients with a good performance status, low/intermediate grade tumors, and normal hemoglobin level at baseline were advantageous for long-term survival (Kasper et al. 2014). In addition, the presence of hypertension was explored as a potential biomarker of efficacy in patients treated within the phase II and III trials but was found not to correlate (Duffaud et al. 2015).

The publication of PALETTE has led to an explosion of interest in the role of pazopanib in specific subtypes and differing treatment settings. Like sunitinib, pazopanib has activity in a number of STS subtypes including solitary fibrous tumor (Maruzzo et al. 2015), DSRCT (Frezza et al. 2014), extra-osseous Ewing sarcoma (Attia et al. 2015), and desmoid fibromatosis (Martin-Liberal et al. 2013). A phase I clinical trial in children with advanced STS showed that this drug was well tolerated and of potential clinical benefit, again in a patient with DSRCT (Glade Bender et al. 2013).

Pazopanib in the preoperative setting for patients with high risk STS is being explored by the German Interdisciplinary Sarcoma Group (GISG) (NCT01543802) investigating metabolic response rate via the proportion of patients with >50% reduction of standardized uptake value on FDG PET/CT scan with associated translation studies (Ronellenfitsch et al. 2016). Data from the Netherlands Cancer Institute has shown that pazopanib at the standard 800 mg dose may be safely delivered in combination with neoadjuvant radiotherapy at a dose of 50 Gy and a phase II PAZART trial is underway (NCT02575066) (Haas et al. 2015).

A phase Ib/II trial of pazopanib in combination with gemcitabine/docetaxel chemotherapy in the neoadjuvant setting has shown that this combination is toxic with a high frequency of grade 3 adverse events. Although pathologic responses were seen, no objective responses occurred (Munhoz et al. 2015). A German phase II trial (PAPAGEMO) comparing pazopanib alone versus the combination of pazopanib with gemcitabine in the metastatic setting finalized recruitment; final data will be presented. Finally, there is an ongoing study, still actively recruiting from the German Interdisciplinary Sarcoma Group (GISG), evaluating the combination of pazopanib and paclitaxel in angiosarcoma patients (NCT02212015).

Currently, challenges remain in the correct sequencing and dosing of anti-angiogenic agents and conventional sarcoma chemotherapy.

Systemic Chemotherapy

The anti-microtubule agent **Paclitaxel** is a form of systemic chemotherapy that also has anti-angiogenic properties (Belotti et al. 1996). Paclitaxel has activity in a very particular sarcoma subtype – angiosarcoma. This was initially noted in patients with angiosarcoma of the scalp and then in a retrospective study by the EORTC with a response rate approaching 75% (Fata et al. 1999; Schlemmer et al. 2008). The French Sarcoma Group published a prospective study of paclitaxel in unresectable or advanced angiosarcomas which showed PFS rates at 2 and 4 months of 74% and 45%, respectively (Penel et al. 2008). Interestingly, in the previously mentioned trial by the same group of paclitaxel ± bevacizumab, the response rate to paclitaxel alone was higher than in the 2008 trial, perhaps reflecting more stringent trial entry criteria (Ray-Coquard et al. 2015). Paclitaxel also has the advantage of being well tolerated in all age groups and without the

cardiotoxicity of anthracycline (Letsa et al. 2014). Furthermore, paclitaxel has activity in the closely related Kaposi's sarcoma (Kim et al. 2011). Future trial approaches aim to harness the potential anti-angiogenic activity of paclitaxel in combination with other novel anti-angiogenic agents.

Future Directions

Clearly the development of anti-angiogenic therapy has been a promising strategy in the treatment of STS in all its differing subtypes. Challenges remain in understanding which specific disease biology will benefit most from treatment, and further work is needed to examine and understand potential predictive and prognostic biomarkers. There are potential biomarkers available such as VEGF-A, VEGF-B to name two as well as circulating endothelial cells but further prospective validation is needed. It is imperative for future trial design that suitable parallel translational studies are in place, not only at a molecular biology level but also incorporating modern imaging techniques such as FDG PET, or dynamic contrast enhanced magnetic resonance imaging to examine the tumor vasculature, in order to gain signals of responding patients. Trial endpoints are also important, especially in the knowledge that in STS tumor stability may be as important as partial response. Classic radiological response criteria such as RECIST may not be so relevant in this tumor group particularly as angiogenesis inhibitors slow or stop tumor growth rather than overt tumor shrinkage. Perhaps tumor density as well as change in dimensions should also be considered as per Choi criteria in gastrointestinal stromal tumor. Understanding mechanisms of resistance also need further work, not only primary resistance but also secondary as tumor responses may be relatively short-lived. An understanding of how to combine anti-angiogenic therapy with other targeting drugs, as well as cytotoxic therapies and radiotherapy may also maximize benefits of this treatment type. So-called window of opportunity or preoperative trials with pre- and post-tumor biopsies may also shed further light on underlying mechanisms of action as well as give indications of pathological response. As always in soft tissue sarcoma, due to the rarity of the disease and in particular differing biology of individual tumor, subtypes means that international collaboration is key and it is necessary to work as part of larger collaborative groups for the benefit of all patients. The success of the phase III PALETTE trial underlines this point well. The challenge of drug development in a competitive market is also significant, particularly in the less common subgroups and orphan disease in often financially insecure times.

Conclusion

There is now a significant body of evidence to underline the potential and promise of anti-angiogenic therapy in the field of STS. Now the challenge remains in selection and identification of suitable patients and careful objective evaluation of their response to treatment, symptomatically, radiologically, and preferably with suitable biomarkers. Careful clinical trial design is also paramount to ensure that potentially active drugs are not discarded prematurely. Of all the drugs studied so far in STS, pazopanib and sunitinib seem to have the most potential, perhaps because of their multi-targeting effects. However, other drugs such as cediranib in the rare ASPS are also very important, and it is critical that this drug continues to be available for this orphan disease type. The challenge of combination of these drugs with cytotoxic chemotherapy remains and is worthy of further exploration.

Cross-References

- ▶ Anti-angiogenics and Radiation Therapy
- ▶ Cytotoxics and Anti-angiogenics: Metronomic Therapies
- ▶ Inhibition of Tumor Angiogenesis in GIST Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part XIII

Anti-angiogenics in Head and Neck Cancer

The Value of Anti-angiogenics in Head and Neck Cancer Therapy

Valérie Cristina and Sandrine Faivre

Contents

Introduction 478

Targeting Angiogenesis in Head and Neck Squamous Cell Carcinoma 479
Monoclonal Antibody Inhibiting VEGF: Bevacizumab 480

Bevacizumab for the Treatment of Locally Advanced HNSCC 480
Combining Bevacizumab with Cisplatin and Radiation Therapy 480
Combining Bevacizumab with Radiation Therapy and Other Chemotherapeutic Regimens 481
Combining Bevacizumab with Radiation Therapy and Both Chemotherapy and Anti-EGFR Therapy 482

Bevacizumab for the Treatment of Recurrent and Metastatic HNSCC 483
Combining Bevacizumab with Anti-EGFR Therapies 483
Combining Bevacizumab with Anti-EGFR Therapies and mTOR Inhibitors 485
Combining Bevacizumab with Chemotherapy 485
Tyrosine Kinase Inhibitors 485
Single-Agent Sorafenib in the Setting of Recurrent and/or Metastatic HNSCC 486
Sorafenib in Combination with Chemotherapy in the Setting of Recurrent and/or Metastatic HNSCC 487

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Sorafenib in Combination with Anti-EGFR Therapies in the Setting of Recurrent and/or Metastatic HNSCC 488
Sorafenib in the Setting of Locally Advanced Disease 488
Sunitinib in the Setting of Recurrent and/or Metastatic HNSCC 489
Sunitinib in the Setting of Locally Advanced HNSCC 490

Novel TKI Under Study 490
Vandetanib 490
Pazopanib 491
Axitinib 491
Mechanisms of Resistance to VEGF-/VEGFR-Targeted Therapy 491

Conclusion 492

Cross-References 492

References 492

Abstract

Squamous cell carcinoma of the head and neck is mostly diagnosed at an advanced stage, and the prognosis remains poor despite advances in the multimodality treatments involving surgery, radiotherapy, chemotherapy, and/or targeted therapy. Improvement in understanding the molecular biology of cancer has led to new promising research strategies, as the development of molecular-targeted therapies like anti-angiogenic therapies. The monoclonal antibody inhibiting VEGF (vascular endothelial growth factor), bevacizumab, has been studied in combination with chemotherapy and/or anti-EGFR (epidermal growth factor receptor) therapy (cetuximab) and radiation therapy in the locally advanced setting and in combination with chemotherapy and/or targeted therapies in the recurrent/metastatic setting. Activity has been shown in preliminary studies, and phase III trials are currently ongoing with bevacizumab and chemotherapy in the recurrent/metastatic setting. Tyrosine kinase inhibitors targeting VEGFR have been reported as not effective when used as monotherapy, warranting combinations with radiation therapy, chemotherapy, or other targeted therapies. A major concern of targeting angiogenesis in squamous cell carcinoma is the risk of increased bleeding, especially in pretreated areas. A careful selection of patients is mandatory to minimize the risk of severe bleeding adverse events in future trials exploring anti-angiogenics that are not recommended in routine practice in head and neck cancers. Validating biomarkers to better select patients who will benefit of anti-angiogenic therapy is also a key point for further development of these therapies in the treatment of head and neck squamous cell carcinoma.

Keywords

Head and neck carcinoma · Squamous cell carcinoma · Anti-angiogenic therapy · Bevacizumab · Sorafenib · Sunitinib · Vandetanib · Pazopanib · Axitinib

Introduction

Head and neck squamous cell carcinoma (HNSCC), including cancer of the oral cavity, oropharynx, hypopharynx, pharynx, and larynx, is an important cause of cancer. It accounts for nearly 600,000 new cases annually worldwide. The risk factors most frequently associated with HNSCC are tobacco use, alcohol consumption, and human papillomavirus (HPV) infection. HPV-positive HNSCC arises typically in the oral cavity in younger men who do not consume tobacco and alcohol. This subtype of HNSCC is characterized by a distinct biology and a better prognosis than HPV-negative HNSCC.

Most patients with HNSCC are presented with advanced disease. Despite advances in the multimodality treatments involving surgery, radiotherapy, chemotherapy, and/or targeted therapy, the disease-free survival, the overall survival, and the functional outcome remain poor. Although those treatments are effective in treating HNSCC, more than 50% of patients with locally advanced disease will relapse locally, and more than 20% will develop metastases. Patients with recurrent or metastatic disease have a worse prognosis with an overall survival of less than 1 year.

Therefore, new therapeutic approaches are needed to increase the cure rate of locally advanced HNSCC and to improve the overall survival. Inducing angiogenesis is one of the hallmarks of tumorigenesis; it plays a central role in growth, invasion, and metastatic spread of the disease. So it is an important target for anticancer treatment. Angiogenesis has been studied in various cancer types including HNSCC. Targeting angiogenesis is currently under investigation in the treatment of HNSCC.

Targeting Angiogenesis in Head and Neck Squamous Cell Carcinoma

Angiogenesis leads to the formation of new vessels. It is a complex process under both positive and negative control by growth factors in the tumor microenvironment. The dominant growth factor inducing angiogenesis is the vascular endothelial growth factor A (VEGF-A), usually referred to as VEGF. It belongs to the platelet-derived growth factor (PDGF) superfamily, which also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). VEGF expression is induced by hypoxia (through the expression of hypoxia-inducible factor), cytokines, and oncogenes. The binding of VEGF to a group of specific cell surface tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) triggers downstream angiogenesis-related signals. While VEGFR-1 is mainly involved in the early inflammation process, the most important receptor in tumor angiogenesis development is VEGFR-2. VEGF-A binds to both VEGFR-1 and VEGFR-2, but it exerts its mitogenic, chemotactic, and vascular permeabilizing effects by interacting with VEGFR-2. Those specific receptors are expressed on the surface of endothelial and tumor cells. Other factors involved in cancer neovascularization are prostaglandins, COX-2, IL-6, and epidermal growth factor (EGF).VEGF signaling increases the vascular permeability and the neovascularization (especially development of tumor blood vessels). It also promotes tumor-cell proliferation, migration, and cancer invasion by activating predominant pathways in tumorigenesis, such as the MAPK and PI3K-AKT pathways (Vassilakopoulou et al. 2015).

Overexpression of VEGF has been found in many HNSCC and is associated with more advanced disease, disease aggressiveness with resistance to cytotoxic agents, and a worse prognosis. In a meta-analysis of 12 studies conducted by Kyzas et al. (2005), VEGF expression was evaluated in more than 1000 patients with HNSCC. VEGF expression was associated with a worse overall survival with a 1.88-fold higher risk of death in 2 years. The adverse prognostic effect of VEGF is thought to be primarily the result of angiogenesis. For this reason VEGF pathway appears as an interesting target for HNSCC treatment. Indeed, targeting angiogenesis can induce tumor regression, reduce the probability of recurrence, and enhance the response to standard chemotherapy and radiotherapy. Molecular-targeted therapies which inhibit tumor angiogenesis have been evaluated within the last years leading to the approval of anti-angiogenic therapies for treatment of several solid tumors such as colorectal cancer and renal cell cancer. However, the use of anti-angiogenics remains currently experimental in the treatment of HNSCC. Two types of anti-angiogenic therapies have been developed: monoclonal antibodies inhibiting VEGF (bevacizumab) and tyrosine kinase inhibitors targeting VEGFR tyrosine kinase (e.g., sorafenib, sunitinib, vandetanib). Here we summarize the main results of preclinical and clinical studies exploring anti-angiogenics in head and neck models and patients.

Monoclonal Antibody Inhibiting VEGF: Bevacizumab

Bevacizumab is a recombinant humanized anti-VEGF monoclonal antibody developed in the late 1990s. It binds VEGF and inhibits its function. Its clinical use is approved in the USA and Europe for the treatment of various solid tumors, either in combination with chemotherapy (colorectal cancer, lung cancer) or as monotherapy (glioblastoma). It is the anti-angiogenic monoclonal antibody and the most extensively anti-angiogenic agent studied in the field of head and neck cancer.

In HNSCC, bevacizumab has been evaluated in two settings: in patients with locally advanced HNSCC in combination with both radiation therapy and systemic therapy including chemotherapy and/or targeted therapy (like anti-EGFR therapies) and in patients with recurrent or metastatic HNSCC in combination with chemotherapy and/or anti-EGFR therapies.

Bevacizumab for the Treatment of Locally Advanced HNSCC

Early-stage HNSCC is treated with surgery or radiotherapy. But in many cases, HNSCC is diagnosed at advanced stage with locally advanced disease. The standard treatment of such cases requires multimodality treatment combining surgery when possible and radiotherapy combined with systemic therapy (platinum-based chemotherapy being the recommended standard). To improve the patient's outcome and tolerance, new therapeutic approaches such as targeted therapy have been studied. In this field of research, cetuximab (a recombinant chimeric anti-EGFR monoclonal antibody) has been approved as monotherapy in combination with radiation for treatment of locally advanced disease for few years, as an alternative to platinum chemotherapy in combination with radiation. Indeed, the expression of EGFR in HNSCC is detected in more than 90% of all the HNSCC. It has been demonstrated that high level of this protein expression is associated with resistance to radiotherapy, locoregional treatment failure, and increased rates of distant metastases (Agulnik 2012). Nevertheless, despite improvement in the multimodality treatment, those patients' outcome remains poor, and approximately half of them will relapse locally or develop metastatic disease. Combining anti-angiogenic therapies and radiation therapy has been studied with the aim of enhancing therapeutic index and improving tumor control. Currently radiation dose escalation is limited by normal tissue toxicities. For this reason, combining targeted therapies to improve radiation impact on tumor cells without increasing toxicities on normal cells appears attractive. Bevacizumab is the most studied anti-angiogenic agent in the treatment of potentially curable HNSCC.

As explained before, hypoxia induces secretion of pro-angiogenic cytokines like VEGF which increases the vascular permeability. In preclinical studies, it has been demonstrated that bevacizumab is a radiation sensitizer that can enhance the antitumor efficacy of the combination of radiation therapy and chemotherapy. Indeed, in cell culture, bevacizumab induces a two- to threefold increase in endothelial cell apoptosis following radiation. In head and neck tumor xenograft models, the concurrent administration of bevacizumab and radiation reduced tumor blood vessel formation and inhibited tumor growth compared with either modality alone (Hoang et al. 2012). Therefore, these data supports the strategy of blocking the VEGF signaling pathway and targeting tumor blood vessels to improve therapeutic index of radiation.

Combining Bevacizumab with Cisplatin and Radiation Therapy

Patients with locally advanced HNSCC, not amenable for surgery, are treated with a combination of radiation therapy and chemotherapy or targeted therapy (cetuximab). Cisplatin is the most used chemotherapeutic agent given concurrently with radiation therapy.

In a phase I trial conducted by Nyflot et al. (2015), 10 patients with locoregionally advanced

squamous cell carcinoma of the oropharynx (stage IV disease) were treated with a combination of bevacizumab (administrated 3 weeks before chemoradiation and then 3 times every 3 weeks during chemoradiation), cisplatin (administrated 7 times with weekly doses), and radiation therapy (with a curative intent to 70 Gy in 33 fractions). Although HPV status was not incorporated into trial eligibility, all patients were p16 positive, a surrogate test for HPV positivity. This trial demonstrated that the incorporation of bevacizumab into a regimen of curative-intent chemo-/radiation therapy for HNSCC is safe and tolerable. All patients successfully complete radiation therapy, and there was no occurrence of dose-limiting toxicity, although there was some increase in the acute toxicity profile associated with concurrent weekly cisplatin. Experimental imaging demonstrated that the majority of patients had a strong reduction in tumor proliferation (evaluated by fluorothymidine PET/CT), clear changes in tumor hypoxia (evaluated by Cu-ATSM-PET/CT), and perfusion (evaluated by dynamic contrast-enhanced CT) in HNSCC tumors after bevacizumab monotherapy then also after 1 or 2 weeks of chemo-/radiation therapy.

In a nonrandomized phase II study conducted by Fury et al. (2012), 42 patients with locally advanced HNSCC mainly of the oropharynx (stage III/IV disease) were treated with bevacizumab and cisplatin administrated every 3 weeks and concurrent radiation therapy. The 2-year PFS rate was the primary endpoint and reached 76%. In this study, extensive exclusion criteria were used to minimize risk of bleeding complications. The initial version of the protocol included an additional 6 months of maintenance bevacizumab after chemo-/radiation therapy, but the first patient presented a grade 4 pulmonary hemorrhage that occurred during the maintenance bevacizumab treatment after the completion of chemo-/radiation therapy. For this reason, the authors amended the protocol and canceled the maintenance bevacizumab treatment. With the exception of this complication, no bleeding was observed. Therefore the addition of bevacizumab to standard dose of cisplatin plus radiotherapy did not appear to increase toxicity to an unacceptable level, and the efficacy results were considered as encouraging. Two nonrandomized phase II studies have been completed recently and the results are pending. The first one aims to determine the effectiveness of treatment with bevacizumab, cisplatin, cetuximab, and intensity-modulated radiation therapy (NCT00968435). The second one evaluates the combination of bevacizumab, cisplatin, and intensity-modulated radiation therapy (NCT00423930).

Combining Bevacizumab with Radiation Therapy and Other Chemotherapeutic Regimens

Bevacizumab was also studied in combination with radiation therapy and a concomitant chemotherapy with docetaxel for the treatment of locally advanced HNSCC. Some patients in need of definitive concurrent chemo-/radiation therapy are actually not candidates for cisplatin-based treatment. Docetaxel was selected based upon its documented potent radiosensitizing effects and its favorable toxicity profile when compared to cisplatin. In a nonrandomized phase II clinical trial conducted by Yao et al. (2015), 30 patients with locally advanced HNSCC (stage III/IV disease) were included and treated with a novel combination of concurrent bevacizumab (administrated every 2 weeks), docetaxel (administrated once per week), and radiation therapy. Despite a careful selection of patient regarding bleeding risk, two patients developed grade 4 hemorrhage (the first one in the radiation field and the second one in the abdomen). Life-threatening bleedings were already reported in patients with locally advanced non-small cell lung cancer treated in a study using bevacizumab in combination with a chemotherapy of carboplatin and paclitaxel. In the phase II study conducted by Yao et al. (2015) and mentioned above, bleeding was associated with squamous cell histology, tumor necrosis, and disease location close to major blood vessels. It emphasizes the importance of carefully selecting patients with HNSCC candidates for anti-angiogenic therapy. In addition, a favorable locoregional control was obtained in this phase II trial contrasting with

a high distant metastasis rate with a 3-year distant metastasis-free survival of only 80.5%. As a result, the primary end point did not meet the expected efficacy with a 3-year PFS of 75%.

A randomized phase II study conducted by Salama et al. (2011) was early terminated because of an unexpected high locoregional recurrence in the bevacizumab arm. In this study, 26 patients with intermediate stage and selected T4 N0-1 HNSCC were randomized for 5-fluorouracil, hydroxyurea, and twice-daily radiation therapy, with or without bevacizumab. Seventeen patients were treated with bevacizumab and nine patients without bevacizumab. The reported 2-year locoregional control was 67% in the bevacizumab arm and 100% in the arm without bevacizumab. This difference could be explained by the fact that it has been necessary to reduce the doses of 5-fluorouracil and hydroxyurea in the arm with bevacizumab, compared to the standard doses, because of elevation of hepatic enzymes. This could have led to a reduction of the radiosensitizing effect of this regimen.

Combining Bevacizumab with Radiation Therapy and Both Chemotherapy and Anti-EGFR Therapy

There is a cross talk between EGFR and VEGFR pathways. It has been demonstrated that EGFR expression upregulates VEGF signaling (Tabernero 2007). Moreover VEGF-mediated angiogenesis has been linked to resistance to anti-EGFR agents (Viloria-Petit and Kerbel 2004). For this reason, the combination of anti-VEGF and anti-EGFR therapies has been studied in the treatment of HNSCC and could overcome resistance to EGFR inhibition. Furthermore, poor outcome of patients with HNSCC and resistance with single-agent molecular therapy have yielded to the development of strategies that target synchronously multiple signaling pathways, especially the dual inhibition of VEGFR and EGFR pathways.

In a preclinical study on a head and neck cancer orthotopic model conducted by Bozec et al. (2008), it was demonstrated that concomitant administration of bevacizumab, erlotinib (an EGFR tyrosine kinase inhibitor), and radiation therapy produces a marked and significant supra-additive decrease in tumor volume. In this study, they also observed that the strong increase in tumor angiogenesis known to be induced by radiotherapy was no longer observed when erlotinib and bevacizumab were combined.

The combination of chemotherapy, radiation therapy, and bevacizumab and anti-EGFR therapy was studied in three nonrandomized phase II clinical trials in patients with locally advanced HNSCC. The first study conducted by Hainsworth et al. (2011) was designed to evaluate the feasibility and the efficacy of adding bevacizumab to an induction chemotherapy followed by the combination of targeted therapies and paclitaxel to a chemo-/radiation therapy as first-line treatment of patients with locally advanced HNSCC (stage III/IV disease). An induction chemotherapy with 6 weeks of paclitaxel, carboplatin, infusional 5-fluorouracil, and bevacizumab was used, followed by a regimen of weekly paclitaxel, bevacizumab, and erlotinib performed during radiation therapy. Sixty previously untreated patients were included in this study. The most frequent severe toxicity was mucosal toxicity, responsible of more frequent treatment interruptions than previously described with other chemo-/radiation regimens, so targeted therapies may have increased this toxicity. Based on this study, the authors concluded that adding an angiogenesis inhibitor and an EGFR inhibitor into combined modality treatment was feasible and safe. The estimated 3-year progression-free survival and overall survival were, respectively, of 71% and of 82% after a median follow-up of 32 months. This study was not randomized, but these results suggest an improvement in efficacy because the authors reported in a previous study a 3-year overall survival rate of 51% using the same chemo-/radiation regimen without bevacizumab or erlotinib.

The second phase II study was conducted by Yoo et al. (2012) and also aimed to examine the safety and the efficacy of combining bevacizumab, erlotinib, chemotherapy, and radiation therapy. A curative-intent cisplatin-based

chemotherapy and a twice-daily hyperfractionated radiotherapeutic regimen were used in 28 patients with locally advanced HNSCC (stage III/IV disease). The treatment was feasible, and there was no bleeding adverse event, but a significant risk of osteoradionecrosis and soft tissue necrosis was noted: 18% of the patient presented grade 3 osteonecrosis or soft tissue necrosis, and 21% of the patients developed ulceration, tissue necrosis, or fistulas. These adverse events may be due to the use of bevacizumab. It is unknown whether if the rate of these adverse events would have been lower with a conventional one-daily radiotherapy and/or with another concurrent chemotherapy. It is interesting to note that in this study, changes in the baseline dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and in the follow-up DCE-MRI were correlated with treatment efficacy.

The third study recently published by and conducted by Fury et al. (2016) included 30 patients with locally/regionally advanced HNSCC who received two cycles of cisplatin and bevacizumab every 3 weeks with weekly cetuximab, administered concurrently with radiation therapy. The results of this study were also encouraging with a 2-year progression-free survival of 88.5% and a 2-year overall survival of 92.8%. This regimen was well tolerated: all the patients completed the full planned dose of radiation therapy, and 93% of the study subjects received both planned cycles of bevacizumab. In this study, mucositis was the most frequent severe toxicity.

The major limitation of the above studies is the lack of a randomized design to assess the proper effect of bevacizumab, which addition to radiation and chemotherapy seems to be well tolerated for treatment of locoregionally advanced HNSCC. Further investigation of bevacizumab in a phase II randomized trial, recently published by Argiris et al. (2016), compared a non-platinum-containing regimen with pemetrexed and cetuximab with or without bevacizumab in combination with radiotherapy for the treatment of patients with locally advanced HNSCC. In the bevacizumab arm, patients received a maintenance with this drug for 6 months. Seventy-eight patients were randomized (mostly oropharynx cancer including 42 HPV-positive tumor). Two-year PFS was 79% without bevacizumab and 75% with bevacizumab. It was concluded that the combination of radiotherapy with a non-platinum regimen of cetuximab and pemetrexed was feasible. However, the adjunction of bevacizumab increased toxicity without clear benefit in terms of efficacy. Finally, a recently published phase I trial investigated the combination of cisplatin, docetaxel, 5-FU, and erlotinib as induction therapy followed by a combination of cisplatin, bevacizumab, and erlotinib with concurrent radiotherapy for advanced head and neck cancer (Ahn et al. 2016). It is important to note that the goal of this study was the maximal tolerated dose of erlotinib, not of bevacizumab. Thirteen patients were enrolled in this study. Gastrointestinal toxicities (bleeding and perforation) were a cause for the high rate of study withdrawal. As a conclusion this combination was active but toxic, and a phase II study was not recommended.

It remains unknown if targeting angiogenesis with bevacizumab could be appropriate for deintensification trials for patients with HPV-related oropharyngeal cancer. Ongoing trials with bevacizumab in the treatment of locally advanced HNSCC are summarized in Table 1. At this time to our knowledge, there is no planned phase III trials with bevacizumab in the setting of locally advanced HNSCC.

Bevacizumab for the Treatment of Recurrent and Metastatic HNSCC

In the setting of recurrent and metastatic HNSCC, bevacizumab has been studied in combination with chemotherapy or with anti-EGFR therapies.

Combining Bevacizumab with Anti-EGFR Therapies

EGFR is a validated target for the treatment of HNSCC. In a phase III trial conducted by Vermorken et al. (2008), it was demonstrated that compared to the chemotherapy (platinum

Table 1 Ongoing trials with anti-angiogenic therapies in the treatment of locally advanced HNSCC

Anti-angiogenic agent	Disease setting	Phase	Regimen	Sample size (target)	Primary endpoint	NCT
Bevacizumab	Stage III/IV HNSCC	Phase II	Bevacizumab + cisplatin + cetuximab + IMRT	40	2-year PFS	NCT00968435
Bevacizumab	Stage III/IV HNSCC	Phase II	Bevacizumab + cisplatin + IMRT	42	2-year PFS	NCT00423930
Bevacizumab	Stage III/IV HNSCC	Phase II	Induction therapy with bevacizumab + cetuximab + cisplatin + docetaxel, followed by concurrent radiotherapy + cisplatin + cetuximab + bevacizumab	33	Response rate	NCT01588431

plus 5-fluorouracil) alone, the addition of cetuximab, an anti-EGFR antibody, to chemotherapy in patients with recurrent or metastatic HNSCC improved the response rate (36% vs 20%) and the overall survival (10.1 months vs 7.4 months). Anti-EGFR therapies have only a modest activity as single agent in the treatment of recurrent and metastatic HNSCC. In a phase II study, cetuximab was given as a single agent to patients with recurrent and metastatic HNSCC resistant to platinum (Vermorken et al. 2007). The overall response rate was 13%, disease control rate of 46%, and median overall survival of 5.9 months.

As reported before, VEGF-mediated angiogenesis is implicated in the resistance to anti-EGFR therapy. That is why dual inhibition of VEGFR and EGFR pathways has been studied with the combination of an anti-EGFR therapy with bevacizumab. To improve cetuximab efficacy without enhancing toxic effects, the combination of cetuximab and bevacizumab was studied. Preclinical studies were conducted in vitro in human endothelial cells and in vivo in head and neck and lung cancer xenograft models (Argiris et al. 2013). It was shown that combining cetuximab and bevacizumab substantially enhances the inhibition of human umbilical vein endothelial cell growth in cell culture. In head and neck and lung tumor xenograft models, antitumor activity is increased with cetuximab + bevacizumab combination as compared with bevacizumab alone. These results support the strategy of targeting both EGFR and VEGF signaling pathways in the treatment of HNSCC.

In a phase I/II conducted by Cohen et al. (2009), bevacizumab and erlotinib were combined in patients with recurrent or metastatic HNSCC already treated with no more than one previous regimen of chemotherapy. Forty-eight patients were included in the phase II section of this study. Erlotinib 150 mg daily and bevacizumab 15 mg/kg every 3 weeks were used. The response rate was only of 15%, but four patients showed complete response, warranting further investigation to identify the subset of patients who could benefit from this combination. The median overall survival was 7.1 months and the median progression-free survival was 4.1 months. Even with selection of patients and excluding those at risk for bleeding, three patients presented serious bleeding events of grade 3 or higher (one fatal). But it is necessary to underline that patients with recurrent or metastatic HNSCC are particularly at risk for bleeding even without any treatment. Overall the combination of bevacizumab and erlotinib was well tolerated, the more common adverse events being rash, diarrhea, fatigue, stomatitis, and anorexia. Biomarker analysis suggested that patients with high ratios of tumor-cell phosphorylated VEGF receptor-2 (pVEGFR2) over total VEGFR2 and high endothelial cell pEGFR over total EGFR in pretreatment biopsies could have a better clinical benefit.

In another phase II multicenter trial conducted by Argiris et al., 48 patients with previously treated HNSCC received a combination of cetuximab and bevacizumab (Argiris et al. 2013). The results were similar to the previous phase II study. The median overall survival of 7.5 months appeared promising, but there was no major improvement in response rate (16% in this study), the primary objective of this study. The disease control rate was 73%. Grade 3–4 adverse events were those expected and occurred in less than 10% of patients.

Combining Bevacizumab with Anti-EGFR Therapies and mTOR Inhibitors

The combination of bevacizumab with either cetuximab or with the mTOR inhibitor temsirolimus in advanced solid tumors was studied in a phase I study recently published (Liu et al. 2016). Twenty-one patients were enrolled in this study. Nine of them had HNSCC. For those patients, there was an evidence of clinical activity even if it was not the goal of the study. The overall response rate was 25% and the disease control rate was 38%. The maximum tolerated dose was determined to be bevacizumab 10 mg/kg biweekly, temsirolimus 5 mg weekly, and cetuximab 100/75 mg/m2 weekly. Several toxicities were reported, hyperglycemia and hypophosphatemia showing the highest prevalence.

Combining Bevacizumab with Chemotherapy

In a phase II trial conducted by Argiris et al. (2011), bevacizumab was combined with pemetrexed in previously untreated patients with recurrent or metastatic HNSCC. Forty patients were included in this study. The objective response rate was 30% including a complete response rate of 5%. The median time to progression was 5 months, and the median overall survival was 11.3 months, being comparable with the outcome achieved with the combination of platinum, 5-FU, and cetuximab (even if a real comparison is not possible because the studies were not randomized). A significant rate of serious bleeding was also reported in six patients (15%) with grade 3–5 bleeding adverse events (two fatal).

A phase III study is currently ongoing to compare a platinum-based chemotherapy (cisplatin or carboplatin plus either 5-FU or docetaxel) with or without bevacizumab in recurrent or metastatic squamous cell carcinoma of the head and neck (NCT00588770). The primary endpoint is overall survival. An interim analysis of this study was presented at the ASCO 2015 (Argiris et al. 2015). A total of 403 patients were included. It was shown that the addition of bevacizumab significantly increased treatment-related grade 3–5 bleeding adverse events with a rate of 7.3%. Patients of older age (>65 years old), females, and those with performance status of 1 had a higher risk of bleeding on treatment with bevacizumab. Final toxicity and efficacy data are pending.

Bleeding adverse events are a potential concern for the safety of bevacizumab in HNSCC. It is not uncommon that patients with HNSCC present hemorrhagic complications even without any drug. But it is very important to select carefully patients to reduce the incidence of those adverse events. Controlled randomized clinical trials will be necessary to evaluate any increased risk for bleeding and other complications related to bevacizumab added to chemotherapy in the setting of recurrent or metastatic HNSCC (Vassilakopoulou et al. 2015). Ongoing trials with bevacizumab in the treatment of metastatic/recurrent HNSCC are summarized in Table 2.

Tyrosine Kinase Inhibitors

Sorafenib

Sorafenib, a bisaryl urea, is an oral multiple tyrosine kinase inhibitor targeting VEGFR-2, VEGFR-3, platelet-derived growth factor (PDGFR), and c-kit, among others, and a serine/threonine kinase inhibitor targeting C-Raf and B-Raf. As a result, sorafenib targets two major mechanisms of carcinogenesis: the EGFR-Ras-

Table 2 Ongoing trials with anti-angiogenic therapies in the treatment of recurrent/metastatic HNSCC

Anti-angiogenic agent	Disease setting	Phase	Regimen	Sample size (target)	Primary endpoint	NCT
Bevacizumab	Recurrent or metastatic HNSCC	Phase III	Cisplatin-based chemotherapy ± bevacizumab	400	Overall survival	NCT00588770
Pazopanib	Recurrent or metastatic HNSCC refractory to platinum-based chemotherapy	Phase II	Pazopanib monotherapy	45	Objective response rate	NCT01377298
Sorafenib	Recurrent or metastatic HNSCC	Phase II	Carboplatin + paclitaxel + sorafenib	40	Progression-free survival	NCT00494182
Sorafenib	Recurrent or metastatic HNSCC	Phase I-II	Cisplatin + docetaxel + sorafenib	41	Progression-free survival	NCT02035527
Pazopanib	Recurrent or metastatic HNSCC	Phase I	Pazopanib + cetuximab		Maximum tolerated dose	NCT01716416

Raf-MEK-ERK signaling pathway, a key regulator of cell growth, apoptosis, differentiation, and cellular transformation, and the VEGFR pathway which promotes tumor growth and metastasis by inducing angiogenesis. Sorafenib is approved in the USA and Europe as monotherapy for the treatment of renal cell carcinoma, hepatocellular carcinoma, and thyroid cancer. It is currently under investigation in various solid tumors as a single agent and in combination with chemotherapy, radiation therapy, or other targeted therapies. It has also been studied for treatment of head and neck cancer, especially in the setting of recurrent or metastatic HNSCC.

Single-Agent Sorafenib in the Setting of Recurrent and/or Metastatic HNSCC

The use of sorafenib as a single agent in the treatment of HNSCC yielded disappointing results. In a phase II study conducted by Elser et al. (2007), sorafenib was used as a single agent in patients with recurrent and metastatic HNSCC but also nasopharyngeal carcinoma to evaluate both efficacy and toxicity. Twenty-seven patients previously treated with none or one line of chemotherapy were included in this study, 26% presenting a nasopharyngeal carcinoma. Only one patient achieved partial response, but disease stabilization was obtained at a rate of 37%. The median time to progression was 1.8 month, and the median overall survival time was 4.2 months. However, the treatment was well tolerated. The most common grade 3 toxicities were lymphopenia (17%) and fatigue (7%).

Sorafenib as a single agent was also evaluated in another phase II study in chemotherapy-naïve patients with metastatic, persistent, or recurrent HNSCC. In this study conducted by Williamson et al. (2010), 41 patients received this treatment orally at 400 mg twice daily on a continuous basis. Sorafenib was well tolerated with a toxicity profile similar to that observed in other trials with this agent. The most common grade 2–3 adverse events were fatigue, anorexia, stomatitis, hand-foot syndrome, weight loss, and hypertension. Only one confirmed partial response was reported, yielding a partial response rate of 2%. Although the response rate was poor, the disease control rate was 51%, median progression-free survival being 4 months, and median overall survival being 9 months. Of note, it is important to underline that in hepatocellular carcinoma, sorafenib

provides a statistically significant PFS and OS benefit, despite being associated with a very low response rate of 2% (Llovet et al. 2008). Furthermore, it is also interesting to note that the survival results of sorafenib in this study with chemotherapy-naïve patients seem comparable to those achieved with more toxic chemotherapy regimens. A study, based on tissue sample of different patients enrolled in this phase II trial, has evaluated the prognostic value of the expression of different proteins by immunohistochemical analysis (Mehta et al. 2013). Several angiogenesis markers like SMA, Raf-1, VEGF, and VEGFR were studied, but none of them was significantly associated with response to sorafenib. Negative HER-2 status was associated with a longer progression-free survival. It is interesting to note that angiogenesis markers were almost always overexpressed in these samples, further supporting the evaluation of anti-angiogenic therapies in HNSCC.

Another phase II trial evaluating the efficacy and the safety of sorafenib as single agent was conducted and recently published by Lalami et al. (2016). As in other trials, sorafenib was administered orally at 400 mg twice daily on a continuous basis. Twenty-three patients with recurrent or metastatic HNSCC were included. They may have received previous platinum-based palliative systemic therapy. Sorafenib showed a modest antitumor activity with one patient who had a partial response (rate of 5%) and a rate of 55% for the patients having stable disease. The median progression-free survival was of 3.4 months, and the median overall survival was of 8 months. Toxicities were similar as the other trials except that two patients presented a grade 3–4 bleeding adverse event (tumor bleeding) considered as related to sorafenib. In this study, an original evaluation of the early metabolic response with 18FDG PET-CT was performed during the first cycle. It was found that the early metabolic response (response rate of 38%) was higher than the late RECIST-defined tumor responses (response rate of 5%). These data support the hypothesis that sorafenib has a cytostatic effect inducing early metabolic response but not always leading to a significant tumor shrinkage. It is unknown if these early metabolic changes do really indicate treatment efficacy. In this study half of the patients with clinical benefit under sorafenib therapy presented an early metabolic response at 18FDG PET-CT. Furthermore, the median progression-free survival was significantly correlated with the metabolic response during sorafenib treatment: 2.2 months in early metabolic nonresponders and 7.4 months in the eight patients with early metabolic response ($p = 0.006$).

These trials demonstrate that sorafenib has a modest anticancer activity when used as a single agent in HNSCC; thus additional evaluation of sorafenib as a single agent was not considered appropriate. However, development of biomarkers or genomic analysis to better select responding patients or combining sorafenib with other therapeutic modality should be explored because it is a well-tolerated treatment, and the disease stabilization rate in the later study was promising.

Sorafenib in Combination with Chemotherapy in the Setting of Recurrent and/or Metastatic HNSCC

The results of a phase II combining sorafenib and a chemotherapy of carboplatin and paclitaxel were presented at the ASCO 2012 but remain unpublished at this time (Blumenschein et al. 2012). Forty-eight patients with recurrent or metastatic HNSCC were included and received paclitaxel 20 mg/m2 and carboplatin AUC 6 on day 1 followed by sorafenib 400 mg orally twice daily (days 2–19) in every 3-week cycle. Response rate was 55% and disease control was 84%. The median progression-free survival was 8.5 months and the median overall survival was 22.6 months. Grade 3 toxicities were hand and foot syndrome, fatigue, neutropenia, anemia, elevated lipase, hypertension, and neuropathy. This combination was rather well tolerated and had encouraging activity in this setting. But final outcomes and toxicity data are not yet reported.

Two phase II trials are currently ongoing combining with chemotherapy in patients with

recurrent and/or metastatic HNSCC. The first one evaluates the combination of sorafenib with a doublet of cisplatin and docetaxel (NCT 02035527). The second one combines sorafenib with a doublet of carboplatin and paclitaxel (NCT00494182).

Sorafenib in Combination with Anti-EGFR Therapies in the Setting of Recurrent and/or Metastatic HNSCC

Sorafenib and cetuximab both have a modest antitumor activity when use as single agents. Furthermore, as explained before, there is rationale to target both EGFR and VEGFR pathways since activation of EGFR upregulates expression of VEGFR. For these reasons it was hypothesized that a combination of EGFR and VEGFR-2 inhibitors could have a synergistic effect.

Herein, a randomized phase II trial was conducted by Gilbert et al. (2015) to compare a treatment of cetuximab with or without sorafenib in patients with recurrent, refractory, or metastatic HNSCC. Fifty-two patients, who may have received up to one prior palliative chemotherapy regimen, were randomized to receive cetuximab plus sorafenib or cetuximab plus placebo, and 43 patients were evaluable for response. The primary objective was progression-free survival. The overall response rate was of 8% in both arms. The median progression-free survival was of 3.2 months with sorafenib and of 3 months without sorafenib. The median overall survival was of 5.7 months with sorafenib and of 9 months without sorafenib. The combination of cetuximab and sorafenib was well tolerated but more toxic than cetuximab monotherapy, especially regarding fatigue, diarrhea, and oral mucositis. Unfortunately, this study failed to demonstrate a clinical benefit of cetuximab/sorafenib combination as compared with cetuximab alone, and the combination added toxicities. The study was early terminated based on a planned interim analysis.

Ongoing trials with sorafenib in the treatment of metastatic and/or recurrent HNSCC are summarized in Table 2.

Sorafenib in the Setting of Locally Advanced Disease

Until now there has been no clinical trial published with sorafenib in the setting of locally advanced disease. Sorafenib has been evaluated in preclinical studies to increase the radiosensitivity of HNSCC cells. The objective is to target pathways involved in the cellular radiation response leading to specific radiosensitization of tumor cells that could enhance tumor response to radiotherapy and reduce the total radiation dose needed. Radiosensitization has already been achieved with cetuximab which targets EGFR. The rational underlying the strategy of using sorafenib to radiosensitize HNSCC cell is that VEGF could induce chemoresistance and radioresistance in endothelial cells by upregulating Bcl-2 protein that protects endothelial and tumor cells against radiation-induced apoptosis by upregulating the Raf-MAPK-ERK (extracellular signal-regulated kinase) pathway (Kumar et al. 2004, 2007).

In a preclinical study, sorafenib was used with the aim of investigating its potential to radiosensitize HPV-negative HNSCC cells using six cell lines (Laban et al. 2013). It was demonstrated that sorafenib enhanced radiosensitivity and inhibits DNA double-strand break repair and proliferation. Additionally, sorafenib had a strong cytotoxic effect independent of the radiosensitizing effects, thus could be explored with irradiation to radiosensitize HNSCC cell lines. Sorafenib may also be efficient in combination with a platinum-based chemoradiotherapy for HNSCC cell lines. In a preclinical study, both in vitro and in vivo models were used to evaluate the efficacy of sorafenib either as a single agent or in combination with chemoradiation (Yadav et al. 2011). It was demonstrated in vitro that sorafenib induces a dose-dependent inhibition of tumor and endothelial cell proliferation. These effects were more pronounced when the combination of low doses of sorafenib, cisplatin, and radiation therapy were used. In vivo this combination significantly inhibited tumor growth and tumor angiogenesis in a severe immunodeficient mouse xenograft model. Sorafenib increased tumor-cell sensitization to chemo-/radiation therapy by

downregulating ERCC-1 and XRCC-1, which are DNA repair proteins often overexpressed by tumor cells developing chemoresistance and radioresistance and regulated by MAPK pathway. In a third preclinical study, the radiosensitivity and chemosensitivity with or without sorafenib were measured in four HNSCC cell lines and normal fibroblasts (Möckelmann et al. 2016). The antiproliferative effect of cisplatin was enhanced by sorafenib in HNSCC without affecting apoptosis induction. Added prior to irradiation, sorafenib increased radiosensitivity of three cell lines.

In conclusion, the combination of sorafenib and radiation therapy seems to be promising in preclinical studies to improve antitumor activity against HNSCC models. Sorafenib in combination with radiation therapy could be explored in clinical trials to improve the therapeutic efficacy of HNSCC treatment without increasing toxicities on normal tissue. To our knowledge, there is currently no clinical trial ongoing evaluating the combination of radiation therapy and sorafenib in HNSCC.

Sunitinib

Sunitinib is an orally bioavailable multiple tyrosine kinase receptor inhibitor. It targets VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, RET, and the stem-cell factor receptor (KIT) among others. Inhibition of these receptors can inhibit cell growth, metastasis, and angiogenesis and it induces apoptosis. Sunitinib is approved in the USA and Europe as monotherapy for the treatment of metastatic renal cell carcinoma, pancreatic neuroendocrine tumors, and imatinib-resistant gastrointestinal stroma tumor (GIST).

Sunitinib in the Setting of Recurrent and/or Metastatic HNSCC

Sunitinib has been studied in several phase II studies as a single agent in patients with recurrent and/or metastatic HNSCC, unsuitable for chemotherapy or with progression after platinum-based therapy.

In the first phase II study conducted by Choong et al. (2010), the tolerability and the efficacy of sunitinib were evaluated in patients with recurrent and/or metastatic HNSCC with no more than two prior chemotherapy regimens. Sunitinib was administered orally in 6-week cycles at the dose of 50 mg daily during 4 weeks followed by 2 weeks off. Twenty-two patients were included in this study, which was closed prior to completing the planned accrual because efficacy parameters were not met at the interim analysis. The response rate was low: only one patient experienced partial response, and stable disease as best response occurred only in 25% of the patients. It has to be noted that one patient did not respond to sunitinib based on standard RECIST criteria but developed central necrosis of the tumor. Tumor cavitation is a classic effect of anti-angiogenic agents and has little impact on response assessment. This suggests that despite a low response rate in this study, sunitinib could have some antitumor activity. During this study, eight patients experienced hemorrhagic events. As for tumor hemorrhage, it is unclear if the bleeding events relate either to disease response to antitumoral therapy or to disease progression or natural evolution of the disease.

The second phase II clinical trial conducted by Fountzilas et al. (2010) and designed to evaluate the activity and safety of sunitinib in chemotherapy-naïve patients with recurrent and/or metastatic HNSCC was performed with sunitinib 50 mg per day administered during a 4-week period followed by a rest period of 2 weeks as first-line treatment. Seventeen patients were included and the median of administration of sunitinib was two cycles. Efficacy results were disappointing without any objective response, and only three patients had stabilization disease so the study was terminated prematurely due to lack of efficacy, despite that sunitinib was well tolerated in this trial.

The third phase II clinical trial to assess the efficacy and safety of sunitinib monotherapy in patients with recurrent and/or metastatic HNSCC, unsuitable for chemotherapy or who had experienced disease progression after platinum-based chemotherapy, was conducted by Machiels et al. (2010) with sunitinib 37.5 mg daily continuously. The primary endpoint was the disease control rate defined as stable disease or partial response.

Thirty-eight patients were included in this study. Among them one patient presented a partial response according to RECIST criteria, and 18 patients had stable disease resulting in a disease rate control of 50%. However, the median progression-free survival of 2 months and the median overall survival of 3.4 months are rather low compared with other trials in similar patients. The most frequent grade 3–4 toxicity in this study was fatigue (32% of patient). The rate of bleeding adverse events of grade 3–5 was as high as 16% of patients. Five of the patients who presented these severe hemorrhage complications had tumors located less than 5 mm from the carotid artery. Another patient developed a large ulcer in a previously irradiated area with subsequent carotid rupture. Furthermore, local complications including occurrence or worsening of tumor skin ulceration or tumor fistula were observed in 15 patients. The severity of those complications shows that it is very important to select carefully patient for future studies investigating use of anti-angiogenic agents in HNSCC, especially sunitinib.

According to those studies, sunitinib has not demonstrated sufficient antitumor activity in HNSCC, and no further development of this drug as monotherapy in this indication is encouraged. There is no current clinical trial ongoing with sunitinib in the treatment of recurrent and/or metastatic HNSCC.

Sunitinib in the Setting of Locally Advanced HNSCC

As for sorafenib, until now there is no clinical trial with sunitinib reported in the setting of locally advanced disease. In a preclinical study, the combination of sunitinib, cetuximab, and radiation therapy has been evaluated in vivo on orthotopic xenografts of head and neck cancer in nude mice (Bozec et al. 2009). In this study, the combination of sunitinib and cetuximab produced a significant supra-additive decrease in tumor growth. The addition of radiotherapy completely abolished the tumor growth.

Novel TKI Under Study

Vandetanib

Vandetanib is a multi-kinase inhibitor that targets VEGFR-2, RET, and EGFR protein tyrosine kinases. Its use is currently approved in the USA and in Europe for the treatment of medullary thyroid carcinoma. A preclinical in vivo study showed that the combination of vandetanib and radiation therapy is effective in both EGFR-positive and EGFR-negative tumor xenografts (Gustafson et al. 2008). In another preclinical study, vandetanib increased tumor cell apoptosis and tumor-associated endothelial cell apoptosis and decrease microvessel density. Therefore, vandetanib could reduce tumor metastasis and restore sensitivity to cisplatin and to radiation therapy (Sano et al. 2011).

Based on these preclinical data, a phase I study was recently published using vandetanib with radiation therapy with or without cisplatin in locally advanced head and neck squamous cell cancer (Papadimitrakopoulou et al. 2016). In this study, 33 patients with previously untreated locally advanced HNSCC received vandetanib daily for 14 days and then vandetanib in combination with radiotherapy alone or with radiotherapy and cisplatin for 7 weeks. The vandetanib plus radiotherapy-alone arm was stopped because of poor recruitment. Five patients discontinued vandetanib because of adverse events. The most common grade 3–4 adverse events were dysphagia, stomatitis, and mucosal inflammation. Overall, vandetanib with chemoradiotherapy was considered as feasible.

In recurrent and/or metastatic HNSCC setting, a randomized multicenter phase II was conducted by Limaye et al. (2013) to evaluate docetaxel with or without vandetanib in patients who had previously been treated with platinum in primary or recurrent/metastatic setting. Twenty-nine patients were included. The most frequent toxicities in the combined arm were diarrhea, constipation, hemorrhage, and rash. The objective response rates were 7% (one patient) in the single and 13% (two patients) in the combined arm, respectively. There was a minor trend toward improved PFS in

the combined arm: 3.21 weeks (95% CI 3.0–22.0) for docetaxel and 9 weeks (95% CI 5.86–18.1) for docetaxel plus vandetanib. The lack of clinical benefit led to early closure of this study.

Pazopanib

Pazopanib is an oral angiogenesis inhibitor. It targets VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, and stem-cell factor (c-Kit). Its use is approved for the treatment of advance renal cell carcinoma and metastatic soft tissue sarcomas. In a preclinical study, pazopanib was evaluated in combination with radiation therapy using in vivo models (Meredith et al. 2014). In this study, non-small cell lung cancer cell line and head and neck cancer cell line were used to establish xenografts in female athymic nude mice. Tumor-bearing mice were treated with pazopanib and/or escalating doses of radiation therapy. Compared with either agent alone, the combination of pazopanib and radiation therapy resulted in a trend of enhanced tumor growth inhibition.

Two studies are currently evaluating pazopanib in the treatment of HNSCC (Table 2). A phase I study evaluates the combination of cetuximab and pazopanib in the treatment of recurrent and/or metastatic HNSCC (NCT01716416). A phase II study evaluates pazopanib as a single agent in patients refractory to a platinum-based chemotherapy with recurrent and/or metastatic HNSCC (NCT01377298).

Axitinib

Axitinib is an oral VEGFR protein tyrosine kinase inhibitor. Its use is approved for the treatment of metastatic renal cell cancer. A nonrandomized phase II study using axitinib in monotherapy was recently published in the treatment of recurrent and/or metastatic HNSCC (Scwiecicki et al. 2015). Thirty evaluable patients were enrolled and received a monotherapy of axitinib with planned dose escalation based on tolerability. The treatment was well tolerated with no grade 3–4 bleeding adverse event, but only 19 patients received the full planned dose. The overall response rate was 6.7% with two patients who presented a partial response, and the disease control rate was 76.7%. The median progression-free survival was 3.7 months with an overall survival of 10.9 months. Based on those data, further clinical trials evaluating axitinib should be considered.

Mechanisms of Resistance to VEGF-/VEGFR-Targeted Therapy

Resistance to anti-angiogenic therapy is a major issue. Two types of resistance are reported: intrinsic resistance, i.e., the tumor fails to respond from the onset of treatment, and acquired resistance for which tumor initially responds but progresses later on treatment. Several mechanisms could explain resistance to anti-angiogenic therapies (Vasudev and Reynolds 2014):

- Heterogeneity of tumor blood vessels: VEGF-targeted therapies are more effective in suppressing growth of newly formed tumor vessels than of more established tumor vasculature.
- Alternative pro-angiogenic signaling pathways: blocking VEGF pathway by using anti-angiogenic therapy can induce activation of other pro-angiogenic pathways that can induce blood vessel growth and survival.
- Stromal cell infiltration: in preclinical models the infiltration of the tumor by various stromal cell types (e.g., endothelial progenitor cells or fibroblasts) can induce resistance to anti-angiogenic therapy.
- Adaptation of tumor cells to conditions of stress: it was demonstrated in preclinical data that tumor cells can adapt to survive under conditions of reduced supply of nutrients and hypoxia.
- VEGF-independent mechanisms of tumor vascularization: it could be possible that tumor stem cells could differentiate into endothelial cells; the tumor can also recruit vessels from the normal surrounding tissue.
- Increased tumor aggressiveness: some preclinical data report that VEGF-targeted therapy could suppress tumor growth but increased tumor invasion and metastasis.

All of these mechanisms of resistance must be explored to better understand tumor biology and improve treatment strategies.

Conclusion

Treatment of HNSCC, especially in the recurrent and/or metastatic setting, remains a challenge. Improvement in understanding the molecular biology of cancer has led to new promising research strategies, including the development of molecular-targeted therapies like anti-angiogenic therapies. Bevacizumab in combination with other therapeutic modalities has generated interesting results, and several clinical studies are ongoing or completed, with results pending. TKIs targeting VEGFR indicate that these agents are generally not effective as monotherapy, warranting their exploration in combination with radiation therapy, chemotherapy, or other targeted therapies, knowing possible limits of their combinability. Furthermore, toxicity profile of anti-angiogenics implicates a carefully selection of patients for a safe clinical use, especially regarding bleeding events. Further clinical trials evaluating angiogenesis inhibitors in HNSCC should carefully select patients to minimize the risk of severe bleeding adverse events. Unfortunately there is currently no validated biomarker for appropriately selecting patients with cancer for anti-angiogenic therapy. In conclusion, the use of anti-angiogenics remains currently experimental in the treatment of HNSCC and should be investigated carefully in prospective trials only.

Cross-References

- Anti-angiogenics and Radiation Therapy
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis
- Cytotoxics and Anti-angiogenics: Metronomic Therapies
- Combination of Anti-angiogenics and Other Targeted Therapies
- Mechanisms of Tumor Angiogenesis

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Part XIV

Anti-angiogenics in Lung Cancer

Inhibition of Tumor Angiogenesis in the Treatment of Lung Cancer

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Contents

Introduction 498

Bevacizumab in Addition to First-Line Chemotherapy of Advanced NSCLC 499

Bevacizumab in the Treatment of Epidermal Growth Factor Receptor Mutation Positive Cases 502

Other Anti-angiogenic Drugs Tested as First-Line Treatment of Advanced NSCLC 502

Anti-angiogenic Drugs in the Second-Line Treatment of Advanced NSCLC 503

The Attempt of Targeting Angiogenesis in the Adjuvant Treatment of NSCLC 504

Anti-angiogenic Drugs in the Treatment of Small Cell Lung Cancer 505

Bevacizumab as Part of First-Line Treatment of Malignant Pleural Mesothelioma 506

Conclusion 506

Cross-References 507

References 507

Abstract

Several anti-angiogenic drugs have been investigated in patients with thoracic malignancies. In patients with advanced, non-squamous, non-small-cell lung cancer (NSCLC), the anti-Vascular Endothelial Growth Factor (VEGF) monoclonal antibody bevacizumab is approved in combination with first-line platinum-based chemotherapy. The use of bevacizumab has been restricted to patients with non-squamous tumors, due to safety reasons related to the

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relevant risk of life-threatening bleeding in squamous tumors. Consistently with the schedule tested in pivotal trials, bevacizumab is administered concomitant with chemotherapy, followed by single-agent maintenance therapy until disease progression or unacceptable toxicity. More recently, bevacizumab has also shown efficacy when added to erlotinib as first-line treatment of cases with activating mutations of Epidermal Growth Factor receptor (EGFR). Both the anti-VEGF receptor (VEGFR) monoclonal antibody ramucirumab (in squamous as well as non-squamous tumors) and the small molecule tyrosine kinase inhibitor nintedanib (in patients with adenocarcinoma) have shown efficacy in addition to docetaxel as second-line treatment of advanced NSCLC, after failure of platinum-based chemotherapy. Unfortunately, to date, similarly to other tumors, in patients with advanced NSCLC no useful predictive factors of efficacy have been validated for anti-angiogenic drugs. In completely resected early stage NSCLC, bevacizumab in combination with chemotherapy has been also tested as adjuvant treatment, but no impact on outcome was demonstrated. In small cell lung cancer, no anti-angiogenic drug has produced successful results. Treatment options for patients with malignant pleural mesothelioma have been recently enriched by the addition of bevacizumab to cisplatin plus pemetrexed as first-line therapy, as reported in one phase III study.

Keywords
NSCLC · SCLC · Malignant-pleural mesothelioma · Bevacizumab · Ramucirumab · Nintedanib · Sunitinib · Sorafenib · Cediranib · Motesanib · Vandetanib · VEGF · Angiogenesis · Randomized-controlled trials

Introduction

In lung cancer, similarly to other solid tumors, the development of a vascular supply is essential to allow the growth of tumor mass, which – in the absence of neo-angiogenesis – could probably not exceed a diameter of few millimeters (Folkman 1990). In addition, the development and the growth of new vessels allow the migration of tumor cells into the systemic circulation, representing the first step for the onset of distant metastases (Folkman 1971). Similarly, to most solid tumors, lung cancer cells can produce several pro-angiogenic factors and substances acting as endothelial cells mitogens, and this can have a substantial impact on the natural history of the disease (Hicklin and Ellis 2005; Herbst et al. 2005).

In the last 20 years, several studies in patients with lung cancer (Fontanini et al. 1997; Ushijima et al. 2001; Meert et al. 2002; Trivella et al. 2007) have reported the prognostic role of microvessel density and tumor vascularity, with partially conflicting results. Fontanini et al. (1997) analyzed the relationship between tumor angiogenesis and survival in a series of 407 patients with non-small-cell lung cancer (NSCLC), treated with potentially curative surgery. Microvessel counts were divided in five categories, and a highly significant trend toward worse prognosis was observed with the increase of tumor vascularity. At multivariable analysis, tumor microvessel count was the most important prognostic factor (hazard ratio [HR] of death for the highest microvessel counts 8.38, 95% confidence interval [CI] 4.19–16.78). Ten years later, however, a meta-analysis based on individual patients data did not confirm an independent prognostic role of microvessel density in patients with non-metastatic surgically treated NSCLC (Trivella et al. 2007).

In a systematic review and meta-analysis of the prognostic role of factors members of the Vascular Endothelial Growth factor (VEGF) family, Zheng et al. (2015) found that the expression of VEGFA (particularly in squamous cell cancer and early stage NSCLC), VEGFC, and VEGFR1 is associated with a worse outcome in patients with NSCLC. Furthermore, coexpression of VEGFA and VEGFR2, and coexpression of VEGFC and VEGFR3 were associated with a significantly worse prognosis. Another recent meta-analysis, published by Hu and colleagues (2016), reported in patients with operable NSCLC and SCLC a

significant prognostic role of the overexpression of basic Fibroblast Growth Factor (bFGF), one of the factors associated with the stimulation of angiogenesis, although the same negative impact of bFGF overexpression was not demonstrated in advanced NSCLC.

Beyond the partially conflicting results of the studies analyzing the prognostic role of angiogenesis in lung cancer, there has been a growing interest in angiogenesis as a potential therapeutic target (Chu and Otterson 2016) in thoracic malignancies. Unfortunately, the vast majority of the performed studies has produced negative results, leading to interruption of the development of many drugs in this setting, but positive results have also been obtained, leading to the authorization for the use in clinical practice of some anti-angiogenic agents, followed by their inclusion in the recommendations within the official clinical practice guidelines.

Bevacizumab in Addition to First-Line Chemotherapy of Advanced NSCLC

The first anti-angiogenic drug associated with a significant clinical benefit in patients with advanced NSCLC was bevacizumab.

Bevacizumab is a monoclonal antibody, directed against VEGF. VEGF-A is considered to be one of the key molecular players of tumor angiogenesis. Since its initial approval for patients with advanced colorectal cancer, bevacizumab has been successfully tested and incorporated into clinical practice guidelines for a large number of different solid tumors. On the basis of a promising randomized phase II trial (Johnson et al. 2004), bevacizumab added to first-line platinum-based chemotherapy for patients with advanced NSCLC has been tested within two randomized phase III trials (Sandler et al. 2006; Reck et al. 2009). The first trial (ECOG 4599) was conducted in the USA (Sandler et al. 2006), while most of the patients included in the second trial (AVAiL) were from Europe, Australia, and Canada (Reck et al. 2009). In both studies, the monoclonal antibody was administered concomitantly with first-line chemotherapy and, after the completion of the planned six cycles of chemotherapy, patients who were progression-free continued bevacizumab as single-agent maintenance treatment, until disease progression or unacceptable toxicity.

In the above mentioned randomized phase II trial, the addition of bevacizumab to chemotherapy was associated with a significantly increased risk of bleeding (Johnson et al. 2004). Of note, the risk was reported to be higher among patients with squamous-cell carcinoma. If the risk was due to squamous histology itself, or if the bleeding was secondary to tumor necrosis, cavitation or its location adjacent to major blood vessels was not completely clear. However, for safety concerns, both the pivotal phase III trials were then conducted in a selected population, with the exclusion of patients with squamous tumors and of those patients with major risk factors for bleeding: history of gross hemoptysis, history of documented hemorrhagic diathesis or coagulopathy, presence of metastases in the central nervous system, therapeutic anticoagulation, regular use of aspirin, nonsteroidal anti-inflammatory agents, or other agents known to inhibit platelet function. Furthermore, in the AVAiL trial, tumors invading or abutting major blood vessels were also excluded, because those tumors were considered at higher risk of bleeding, independently from tumor histology (Reck et al. 2009).

The ECOG 4599 study tested in detail the addition of bevacizumab to carboplatin plus paclitaxel (Sandler et al. 2006). The primary endpoint of the trial was overall survival (OS), and 878 patients were enrolled. In patients assigned to the experimental arm, bevacizumab was administered at the dose of 15 mg/kg every 3 weeks. A significant OS improvement was observed in the experimental arm: median OS was 12.3 months versus 10.3 months for patients assigned to chemotherapy alone (HR 0.79; 95%CI 0.67–0.92; $p = 0.003$). The addition of bevacizumab produced an improvement also in progression-free survival (PFS, median PFS was 6.2 versus 4.5 months, HR 0.66; 95%CI 0.57–0.77; $p < 0.001$) and in the objective response rate (35% versus 15%, $p < 0.001$). Bevacizumab was well tolerated

in the majority of patients, but – despite the selective exclusion criteria – it was still associated with increased risk of clinically significant bleeding (4.4% versus 0.7%, $p < 0.001$). There were 15 treatment-related deaths in the group receiving chemotherapy plus bevacizumab, including five cases from pulmonary hemorrhage.

In the AVAiL study, bevacizumab was tested at two different doses in combination with cisplatin plus gemcitabine (Reck et al. 2009). The protocol was initially designed to test superiority for OS, but a subsequent amendment shifted to PFS as primary endpoint (Di Maio et al. 2008). Eligible patients were randomized to: chemotherapy plus placebo (347 patients), chemotherapy plus bevacizumab 7.5 mg/kg (345 patients), or chemotherapy plus bevacizumab 15 mg/kg (351 patients). For both bevacizumab arms, the improvement in PFS was statistically significant compared to placebo arm, but the difference was quite negligible in absolute terms: in detail, median PFS was 6.1, 6.7, and 6.5 months in the chemotherapy alone, chemotherapy plus bevacizumab 7.5 mg/kg, and chemotherapy plus bevacizumab 15 mg/kg arms, respectively. Corresponding hazard ratios for progression were 0.75 and 0.82 compared to control, for the lower and higher dose of bevacizumab, respectively. No differences in OS were observed (Reck et al. 2010). Toxicity was acceptable, with low rates of clinically relevant bleeding and pulmonary hemorrhage.

A systematic review and meta-analysis, including four randomized trials, for a total of 2200 patients, that tested the addition of bevacizumab to first-line treatment of patients with locally advanced or metastatic NSCLC was presented by Botrel and colleagues (2011). The addition of bevacizumab to chemotherapy (both at the dose of 7.5 mg/kg and at the dose of 15 mg/kg) was associated with a statistically significant improvement in the objective response rate (risk ratio 0.58 for the lower dose and 0.53 for the higher dose), and with a statistically significant prolongation of PFS (HR 0.78 for the lower dose and 0.72 for the higher dose). The difference in terms of OS was statistically significant for the dose of 15 mg/kg in the fixed effects model analysis, but the significance was not confirmed at the random effects model and in any case the magnitude of benefit was quite negligible (HR 0.89 and 0.90 at the fixed effects and random effects model, respectively). As for toxicity, this meta-analysis confirmed the increase in the occurrence of severe hematologic toxicities, neutropenia, and febrile neutropenia in patients receiving bevacizumab in addition to chemotherapy. A subsequent, similar meta-analysis confirmed the limited magnitude of the OS benefit associated with the addition of bevacizumab to chemotherapy (Soria et al. 2013).

The results of the above mentioned randomized trials prompted several remarks (Di Maio et al. 2008; Gridelli et al. 2010). When added to cisplatin and gemcitabine, bevacizumab was associated with a clinically modest benefit in a surrogate endpoint (PFS), without confirming the prolongation of survival reported in addition to carboplatin plus paclitaxel. These results did not solve the doubt about the potential difference in efficacy of bevacizumab according to the type of chemotherapy used as first-line treatment. In particular, the two pivotal trials did not test the addition of bevacizumab to cisplatin and pemetrexed, which is currently the most used combination for patients with non-squamous NSCLC. After the pivotal trials, several studies (Patel et al. 2013; Barlesi et al. 2013) have tested different combinations of platinum-based combinations followed by maintenance (with bevacizumab and/or pemetrexed and bevacizumab), but none of these trials evaluated the efficacy of bevacizumab itself in addition to platinum and pemetrexed.

Another important issue is that, in both pivotal trials, bevacizumab was administered per protocol as single-agent maintenance until progression, based on the rationale that the withdrawal of anti-VEGF treatment could lead to a quick vascular regrowth. In the two phase III studies there was no arm testing bevacizumab only concomitant to chemotherapy, without maintenance. More recently, the AVAPERL trial tested the addition of maintenance pemetrexed to maintenance bevacizumab after a first-line treatment with bevacizumab, cisplatin, and pemetrexed (Barlesi et al. 2013). However, the trial evaluated the

efficacy of maintenance pemetrexed in addition to bevacizumab, but the anti-VEGF agent was administered in both arms, and the demonstration of its efficacy was not among the aims of the trial.

Last, but not least, the identification and validation of predictive factors of efficacy would allow an optimization in the use of the drug, but to date – not only in NSCLC but in all solid tumors where the drug has been approved – we have no conclusive data in this direction.

In October 2006, U.S. Food and Drug Administration (FDA) approved bevacizumab for the first-line treatment of patients with advanced, non-squamous NSCLC, in combination with carboplatin plus paclitaxel. In August 2007, also European Medicines Agency (EMA) approved the drug for the same treatment setting, but in combination with any platinum-based chemotherapy. According to the 2015 update of the American Society of Clinical Oncology (ASCO) clinical practice guidelines, the addition of bevacizumab to first-line treatment with carboplatin plus paclitaxel is recommended, in the absence of contraindications, with an intermediate quality of evidence and a moderate strength of recommendation (Masters et al. 2015). According to the 2016 edition of the European Society of Medical Oncology (ESMO) clinical practice guidelines, the addition of bevacizumab to platinum-based chemotherapy may be considered in eligible patients with non-squamous NSCLC and performance status 0–1 (Novello et al. 2016a).

Beyond the recommendations included in clinical practice guidelines, some caution in patient selection for treatment with bevacizumab is needed. Coherently with inclusion criteria of the two randomized trials, the addition of bevacizumab to first-line chemotherapy should be considered as a treatment option only in patients with non-squamous histology, good performance status (ECOG 0 or 1), without history of clinically significant bleeding or hemoptysis, without tumor cavitation or invasion of major blood vessels, without uncontrolled hypertension, without use of anticoagulants, and without recent arterial thrombotic events. The presence of brain metastases was an exclusion criterion in randomized trials, but treatment with bevacizumab has been shown to be acceptably safe in NSCLC patients with treated brain metastases, and currently there is no more an absolute contraindication to its administration.

In the subgroup analysis of elderly patients enrolled in the ECOG 4599 trial, the addition of bevacizumab to carboplatin plus paclitaxel was associated with a higher incidence and severity of adverse events (Ramalingam et al. 2008). In detail, severe toxicities (grade 3–5) occurred in 87% of elderly patients randomized to bevacizumab, compared to 61% of those assigned to the control arm. Compared with younger patients, elderly patients had higher incidence of severe neutropenia, bleeding, and proteinuria. In the same subgroup analysis performed in the AVAil trial, the addition of bevacizumab to chemotherapy in elderly patients was associated with an improvement in the outcome similar to the results obtained in the intent-to-treat study population, and bevacizumab-based therapy was well tolerated (Leighl et al. 2010).

Many of the patients with non-oncogene addicted and non-squamous NSCLC are currently treated with a combination of cisplatin and pemetrexed, followed by pemetrexed maintenance. Unfortunately, there is no clear evidence of the added value of a bevacizumab-based regimen compared to cisplatin plus pemetrexed. The randomized trial PRONOUNCE by Zinner et al. (2015) compared the efficacy and safety of pemetrexed plus carboplatin followed by pemetrexed versus paclitaxel plus carboplatin plus bevacizumab followed by bevacizumab in patients with advanced non-squamous non-small-cell lung cancer (NSCLC). The primary objective was PFS without grade 4 toxicity. The study results did not show any significant difference between treatment arms, neither in the primary endpoint nor in other endpoints, including OS. Another randomized trial, conducted in Italy (Galetta et al. 2015), compared cisplatin plus pemetrexed versus carboplatin plus paclitaxel and bevacizumab, with quality of life as primary endpoint. The study did not demonstrate any significant difference between treatment arms in quality of life and in OS, although the sample size was underpowered to exclude potentially relevant differences.

Bevacizumab in the Treatment of Epidermal Growth Factor Receptor Mutation Positive Cases

Preclinical studies have shown promising results with the combination of anti-Epidermal Growth Factor Receptor (EGFR) and anti-angiogenesis drugs in different tumor models, including NSCLC. Several clinical trials have been performed to test the efficacy of the combined approaches (Di Maio et al. 2014). The largest body of evidence has been produced evaluating the combination of erlotinib plus bevacizumab (Herbst et al. 2007, 2011), or erlotinib plus a multitargeting receptor tyrosine kinase inhibitor, namely sunitinib (Scagliotti et al. 2012a) or sorafenib (Gridelli et al. 2011; Spigel et al. 2011a). Furthermore, the efficacy of several dual inhibitors, targeting both EGFR and VEGFR, has been explored, with the greatest body of evidence produced with vandetanib (Heymach et al. 2008; Natale et al. 2011; Herbst et al. 2010; de Boer et al. 2011; Lee et al. 2012). However, despite the intriguing preclinical background, the combination strategy has not produced clinically relevant results. Several phase III trials showed an improvement in PFS, underlining some activity of drugs targeting both pathways concurrently, but to date no impact on OS has been demonstrated. As a matter of fact, the vast majority of trials conducted in this setting were performed without any selection criteria based on molecular characteristics, compromising the chance of detecting a potentially relevant benefit in selected subgroup of patients (Di Maio et al. 2014).

After the approval in addition to chemotherapy for patients with advanced, metastatic, or recurrent NSCLC without specific molecular alterations, bevacizumab has been tested also in the subpopulation of cases with activating mutations of Epidermal Growth Factor Receptor (EGFR). According to current clinical practice guidelines, standard first-line treatment for these patients is an EGFR tyrosine kinase inhibitor (gefitinib, erlotinib, or afatinib). A randomized, multicenter phase II study, conducted in Japan, tested the addition of bevacizumab to erlotinib in this setting (Seto et al. 2014). The study included Japanese patients with stage IIIB/IV or recurrent non-squamous NSCLC with activating *EGFR* mutations, Eastern Cooperative Oncology Group performance status 0 or 1, chemo-naive (only adjuvant or neoadjuvant therapy was allowed). Patient assigned to experimental arm received erlotinib 150 mg/day plus bevacizumab 15 mg/kg every 3 weeks, while patients assigned to control arm received erlotinib 150 mg/day as single-agent. In both arms, treatment was continued until disease progression or unacceptable toxicity. The primary endpoint was PFS, and 154 patients were randomized. The addition of bevacizumab to erlotinib was associated with a significant improvement in PFS: median PFS was 16.0 months in patients receiving bevacizumab plus erlotinib, versus 9.7 months in patients receiving erlotinib alone (hazard ratio 0.54, 95% confidence interval 0.36–0.79; $p = 0.0015$). This improvement in disease control was observed with an acceptable safety profile, consistent with the expected toxicity of bevacizumab: The addition of the anti-VEGF antibody produced an increase in the occurrence of hypertension (60% versus 10%) and proteinuria (8% versus 0%), but the incidence of serious adverse events was not significantly different between the arms (24% with erlotinib plus bevacizumab versus 25% with erlotinib alone).

On April 2016, the European Medicines Agency approved the use of bevacizumab, in combination with erlotinib, as first-line treatment of adult patients with unresectable advanced, metastatic, or recurrent non-squamous NSCLC with EGFR activating mutations.

Other Anti-angiogenic Drugs Tested as First-Line Treatment of Advanced NSCLC

Many tyrosine kinase inhibitors, targeting tumor angiogenesis, have been tested, in addition to platinum-based chemotherapy, in the setting of first-line treatment of advanced NSCLC. Among these drugs were sorafenib (Scagliotti et al. 2010; Paz-Ares et al. 2012), sunitinib (Socinski et al. 2010), cediranib (Dy et al. 2013; Laurie et al.

2014), and motesanib (Scagliotti et al. 2012b; Novello et al. 2014). Many of these trials showed an unacceptable increase in toxicity with no significant prolongation of OS for the combination approach compared to chemotherapy alone. Consequently, differently from bevacizumab, none of these tyrosine kinase inhibitors has been approved in the first-line setting.

Anti-angiogenic Drugs in the Second-Line Treatment of Advanced NSCLC

On average, prognosis of patients with advanced NSCLC experiencing disease progression after first-line treatment is poor (Di Maio et al. 2010). However, around two thirds of those who have failed first-line therapy are still fit and eligible for further treatment. Since 2000, single-agent docetaxel has been the standard second-line chemotherapy for patients with advanced NSCLC.

In this setting, several anti-angiogenic agents have been tested in combination with docetaxel: Among these, the monoclonal antibody ramucirumab and the small molecule tyrosine kinase inhibitor nintedanib have reached positive results in a randomized phase III trials, leading to authorization for use in clinical practice.

Ramucirumab is a human IgG1 monoclonal antibody, directed against the extracellular domain of Vascular Endothelial Growth Factor receptor 2 (VEGFR-2). The multicenter, double-blind, randomized phase III trial REVEL was conducted to test the efficacy of ramucirumab plus docetaxel, compared to placebo plus docetaxel, as second-line treatment for patients with stage IV NSCLC, after failure of first-line platinum-based treatment (Garon et al. 2014). Both squamous and non-squamous NSCLC were eligible. In both arms, docetaxel was administered at the standard dose of 75 mg/m^2, every 3 weeks. In the experimental arm, ramucirumab was administered at the dose of 10 mg/kg every 3 weeks, until disease progression or unacceptable toxicity. The primary endpoint was OS, and 1253 patients were randomized. The addition of ramucirumab produced a significant improvement in the primary endpoint of the study: median OS was 10.5 months in the experimental arm, compared to 9.1 months in the control arm (HR 0.86, 95%CI 0.75–0.98; $p = 0.023$). Median PFS was 4.5 months versus 3.0 months (HR 0.76, 95%CI 0.68–0.86; $p < 0.0001$). The combination was associated with an increase of severe neutropenia (49% versus 40%), febrile neutropenia (16% versus 10%), and hypertension (6% versus 2%), without significant difference in the number of toxic deaths. Health-related quality of life (QoL) analysis, assessed by the Lung Cancer Symptom Scale and by clinician-reported functional status, showed that changes in quality of life were similar between the two arms (Pérol et al. 2016) meaning that no improvement in QoL was shown with the addition of ramucirumab to docetaxel but, at least, this combination did not impair patient QoL, symptoms, or functional status compared to single-agent docetaxel.

More recently, a phase II randomized study conducted in a population of Japanese patients assessed efficacy and safety of ramucirumab plus docetaxel compared to placebo plus docetaxel in the same setting of the REVEL study (Yoh et al. 2016). The primary endpoint was PFS, and 160 patients were randomized. Median PFS was 5.22 months with ramucirumab plus docetaxel compared to 4.21 months with placebo plus docetaxel (hazard ratio 0.83, 95%CI 0.59–1.16). Median OS was 15.15 months with ramucirumab-docetaxel versus 14.65 months in the control arm (hazard ratio 0.86, 95%CI 0.56–1.32). The authors concluded that, in Japanese patients, second-line ramucirumab plus docetaxel improved PFS, similarly to the results previously obtained in the REVEL trial, with an acceptable safety profile.

On December 2014, U. S. Food and Drug Administration approved ramucirumab in combination with docetaxel, for the treatment of patients with metastatic NSCLC with disease progression during or after platinum-based chemotherapy (patients with Epidermal Growth Factor Receptor mutation or ALK translocation can be considered for ramucirumab only after disease progression on FDA-approved therapy for these aberrations). Also the European Medicines Agency has approved ramucirumab, in combination with docetaxel, for the treatment of adult patients with locally advanced or metastatic NSCLC

with disease progression after platinum-based chemotherapy.

Nintedanib is a small molecule tyrosine kinase inhibitor, targeting vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR). The randomized phase III LUME-Lung 1 study assessed the efficacy and safety of the addition of nintedanib to docetaxel as second-line treatment of patients with advanced NSCLC (Reck et al. 2014). Eligible patients could have received bevacizumab as part of first-line treatment. Both squamous and non-squamous NSCLC were eligible. Patients assigned to experimental arm received docetaxel at the standard dose of 75 mg/m^2 every 3 weeks and nintedanib at the dose of 200 mg orally twice daily, until disease progression or unacceptable toxicity. Patients assigned to control arm received docetaxel plus placebo. Investigators and patients were masked to assignment. The primary endpoint was PFS by independent central review. OS was a secondary endpoint, analyzed according to a prespecified hierarchic order: first, in patients with adenocarcinoma who progressed within 9 months after start of first-line therapy, then in all patients with adenocarcinoma, then in the whole study population. Overall, 1314 patients were randomized: 655 assigned to experimental arm and 659 assigned to control. PFS was significantly longer in patients who received docetaxel plus nintedanib compared to the control group (median 3.4 months versus 2.7 months; HR 0.79, 95%CI 0.68–0.92, p = 0.0019). The addition of nintedanib to docetaxel was associated with a significant improvement in OS in the prespecified analysis of patients with adenocarcinoma histology who progressed within 9 months after the beginning of first-line treatment (median OS 10.9 months versus 7.9 months; HR 0.75, 95% CI 0.60–0.92, p = 0.007). A significant improvement in OS was confirmed also in all patients with adenocarcinoma histology (median 12·6 months versus 10.3 months; HR 0.83, 95%CI 0.70–0.99, p = 0.04), but not in the overall population including all the histologic subtypes (median 10.1 months versus 9.1 months; HR 0.94, 95%CI 0.83–1.05, p = 0.27). The administration of nintedanib in addition to docetaxel was associated with an increase in the occurrence of severe diarrhea (6.6% versus 2.6%), increases in alanine aminotransferase (7.8% versus 0.9%) and in aspartate aminotransferase (3.4% versus 0.5%). Similarly to the QoL results reported with ramucirumab, the addition of nintedanib to docetaxel did not produce substantial differences in terms of patients' self-reported quality of life (Novello et al. 2015).

Based on the results of the LUME-Lung 1, nintedanib has been approved by the European Medicines Agency, in combination with docetaxel, for use in adult patients with locally advanced, metastatic, or locally recurrent NSCLC of adenocarcinoma histology, after the failure of first-line chemotherapy.

According to European Society of Medical Oncology clinical practice guidelines (Novello et al. 2016a), nintedanib combined with docetaxel is a treatment option in patients with adenocarcinoma, especially in those progressing within 9 months from the start of first-line chemotherapy, while ramucirumab combined with docetaxel is a therapeutic opportunity in patients with NSCLC progressing after first-line chemotherapy with performance status 0–2.

The role of these drugs in clinical practice will be necessarily influenced by the results recently obtained with immune checkpoint inhibitors (as nivolumab, pembrolizumab, atezolizumab).

The Attempt of Targeting Angiogenesis in the Adjuvant Treatment of NSCLC

Based on the significant improvement in life expectancy shown by several randomized trials and meta-analyses, cisplatin-based chemotherapy is recommended as adjuvant treatment for patients with stage II and stage III NSCLC after surgery. However, the absolute benefit associated with the administration of adjuvant chemotherapy is limited, and most of the treated patients experience recurrent disease, mainly within the first 2 years after surgery. Based on

the results obtained with bevacizumab when added to chemotherapy in patients with advanced disease, the drug has been tested also in the early stages as part of the adjuvant treatment. In the E1505 phase III trial, patients with stage IB, II, or IIIA NSCLC, from 6 to 12 weeks after radical surgery, were randomized to receive platinum-based chemotherapy plus bevacizumab, or doublet chemotherapy alone (Wakelee et al. 2015). In detail, chemotherapy consisted of four cycles of cisplatin, administered every 3 weeks, in combination with vinorelbine or docetaxel or gemcitabine or pemetrexed (the latter only for patients with non-squamous histology). Patients assigned to the experimental arm received bevacizumab at the dose of 15 mg/kg, starting with the first cycle of chemotherapy, and for a maximum of 1 year. Also patients with squamous tumor were eligible: Squamous histology represents a contraindication for the use of bevacizumab in patients with advanced disease, due to the risk of bleeding, but in the adjuvant setting it should not be intrinsically associated with increased risk after surgical removal of the tumor. Overall, 1501 patients were randomized: stage was IB in 26.2% of the patients, II in 43.8%, and IIIA in 30.0%. About 28% of patients had squamous cell histology. The addition of bevacizumab did not produce any improvement, neither in disease-free survival nor in OS. Quite disappointingly, the hazard ratio for OS, the primary endpoint was 0.99 ($p = 0.93$), and the hazard ratio for disease-free survival was 0.98 ($p = 0.75$). Consequently, in clinical practice, to date, there is no room for bevacizumab or for other anti-angiogenic drug, as part of adjuvant treatment.

Anti-angiogenic Drugs in the Treatment of Small Cell Lung Cancer

Small cell lung cancer (SCLC) is characterized by a rapid clinical evolution and a poor prognosis, despite the initial chemosensitivity allows obtaining an objective response to first-line treatment in more than half of the patients. Standard first-line treatment is represented by cisplatin (or carboplatin) in combination with etoposide, and no relevant improvement has been recorded in the last decades. After disease progression, SCLC is characterized by a poor response to treatments. Median OS for patients with extensive stage is around 10–12 months.

Unfortunately, all clinical trials testing of anti-angiogenic drugs in patients with SCLC have produced disappointing results (Stratigos et al. 2016). Sunitinib was tested as maintenance treatment in the Cancer and Leukemia Group B (CALGB) 30504 randomized phase II trial: patients with extensive-stage SCLC, without progression after four to six cycles of standard chemotherapy with cisplatin or carboplatin plus etoposide, were randomized to sunitinib (37.5 mg daily) or placebo, until disease progression (Ready et al. 2015). The primary endpoint of this study was PFS, and the study was formally positive. However, the toxicity of the experimental arm was not negligible, and this preliminary result has never been confirmed within a phase III trial.

Bevacizumab has been tested in combination with chemotherapy in several trials (Spigel et al. 2009, 2011a; Horn et al. 2009; Pujol et al. 2015). Recently, a randomized trial conducted in patients with extensive SCLC testing the addition of bevacizumab to first-line treatment with cisplatin (or carboplatin) and etoposide has been presented (Tiseo et al. 2017). The combination was associated with a statistically significant improvement in PFS (median PFS was 5.7 versus 6.7 months; HR 0.72, 95% CI 0.54–0.97; $p = 0.030$), with an acceptable toxicity profile. However, the difference in terms of OS, although numerically in favor of the addition of bevacizumab, was not statistically significant (median OS was 8.9 versus 9.8 months; HR 0.78, 95%CI 0.58–1.06; $p = 0.113$). The interpretation of these negative data is complicated because of the small sample size, and the authors concluded that further research in the area of angiogenesis therapy of patients with SCLC is not denied by these results. To date, no anti-angiogenic drug has received regulatory approval for the treatment of SCLC.

Bevacizumab as Part of First-Line Treatment of Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is strongly linked to occupational asbestos exposure and is characterized by a poor prognosis (Novello et al. 2016b). Standard systemic treatment is currently represented by the combination of cisplatin and pemetrexed.

VEGF signaling has a relevant role in the physiopathology of malignant pleural mesothelioma (Ohta et al. 1999; Strizzi et al. 2001). In this setting, several anti-angiogenic drugs have been tested, as single agents, but the results have been disappointing (Dubey et al. 2010; Garland et al. 2011; Nowak et al. 2012; Buikhuisen et al. 2013). The anti-VEGF monoclonal antibody bevacizumab has been tested in combination with cisplatin and gemcitabine within a randomized phase II trial (Kindler et al. 2012). Unfortunately, that trial did not show a significant improvement with the addition of anti-VEGF antibody to chemotherapy, neither in terms of PFS nor in OS. However, the study was characterized by a limited sample size and a small statistical power, which could potentially explain the negative result. Furthermore, cisplatin plus gemcitabine is not the standard chemotherapy regimen for patients with MPM. On the contrary, the addition of bevacizumab to standard first-line chemotherapy for these patients has been tested in a multicenter, randomized, open-label phase 3 trial conducted in France (Zalcman et al. 2016). The trial recruited patients with unresectable disease, chemo-naive, with age between 18 and 75 years, an Eastern Cooperative Oncology Group performance status of 0–2, and without substantial cardiovascular comorbidity. Patients assigned to control arm received cisplatin plus pemetrexed for a maximum of six cycles, while patients assigned to experimental arm received the same chemotherapy plus bevacizumab, at the dose of 15 mg/kg, and, after the completion of 6 cycles, maintenance administration of bevacizumab until disease progression or unacceptable toxicity was allowed. The primary endpoint of the trial was OS, and 448 patients were randomized (223 assigned to experimental arm and 225 assigned to control arm). Of note, the addition of bevacizumab to chemotherapy was associated with a significant improvement in OS: In detail, median OS was 18.8 months in experimental arm and 16.1 months in control arm (hazard ratio 0.77, 95% confidence interval 0.62–0.95, $p = 0.0167$). Patients receiving bevacizumab obtained also an improvement in PFS: median PFS was 9.2 months versus 7.3 months (adjusted hazard ratio 0.61, 95% confidence interval 0.50–0.75, $p < 0.0001$). The addition of bevacizumab was not detrimental in terms of health-related QoL, but produced an increase in the occurrence of grade 3–4 toxicity (71% of patients versus 62%): As expected, patients receiving the anti-angiogenic drug had a higher incidence of severe hypertension (23% versus 0%) and thrombotic events (6% versus 1%). However, the authors judged acceptable and manageable this increase in toxicity, suggesting that the addition of bevacizumab to pemetrexed plus cisplatin should be considered as a new treatment option for patients with newly diagnosed MPM, if eligible to receive bevacizumab and not candidates for curative-intent surgery. Unfortunately, also in this setting no predictive factor is available: The exploratory analysis of baseline serum VEGF concentration did not show any significant interaction between levels of VEGF and efficacy of bevacizumab.

Conclusion

Based on a strong preclinical background, several anti-angiogenic drugs have been tested in patients with thoracic malignancies. Following the results of two randomized phase III trials, the anti-VEGF monoclonal antibody bevacizumab is currently reimbursed in combination with first-line platinum-based chemotherapy for patients with advanced non-squamous NSCLC. Bevacizumab has also shown efficacy when added to erlotinib as first-line treatment of patients with activating mutations of Epidermal Growth Factor receptor. Both the anti-VEGF receptor monoclonal antibody ramucirumab (in squamous as well as non-squamous tumors) and the small molecule

tyrosine kinase inhibitor nintedanib (in patients with adenocarcinoma) have shown efficacy in addition to docetaxel as second-line treatment, after failure of platinum-based chemotherapy. Recently, bevacizumab has also proven efficacy in addition to first-line chemotherapy with cisplatin plus pemetrexed in patients with malignant pleural mesothelioma.

Unfortunately, to date, no useful predictive factors of efficacy have been validated for anti-angiogenic drugs in patients with thoracic malignancy.

Cross-References

- Combination of Anti-angiogenics and Other Targeted Therapies
- Cytotoxics and Anti-angiogenics: Metronomic Therapies
- Mechanisms of Anti-angiogenic Therapy
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part XV

Anti-angiogenics in Gynecological Tumors

The Value of Anti-angiogenics in Breast Cancer Therapy

Malgorzata Banys-Paluchowski, Tanja Fehm, and Volkmar Müller

Contents

Introduction 516

Clinical Relevance of Anti-angiogenics in Metastatic Breast Cancer 516
Bevacizumab 516
Sorafenib 519
Sunitinib 520
Metronomic Chemotherapy 520

Clinical Relevance of Anti-angiogenics in Nonmetastatic Breast Cancer 521
Bevacizumab 521
Sorafenib 522

Predictive Factors for Treatment Efficacy of Angiogenesis Inhibitors 522

Conclusion 522

Cross-References 523

References 523

Abstract

Tumor-induced angiogenesis supplies the tumor with nutrients and oxygen necessary to grow and provides tumor cells with a possibility to intravasate into blood vessels as first step of metastatic spread. In the last two decades, evidence has been accumulating that controlling tumor-associated angiogenesis might be a promising strategy against cancer growth. In breast cancer, a number of angiogenesis inhibitors have been investigated in clinical trials. The antibody against vascular endothelial growth factor (VEGF) bevacizumab has been approved for treatment of metastatic breast cancer in Europe. Although the mechanism of action is still under study, bevacizumab was also tested in other clinical settings of breast cancer treatment such as neoadjuvant and adjuvant therapy, as maintenance therapy, and in

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combination with both chemotherapy and other targeted agents. Other anti-angiogenic agents, such as oral tyrosine kinase inhibitors sorafenib, sunitinib, and pazopanib, were tested and have not yielded as promising results. In this chapter, we will review the current evidence and clinical relevance of anti-angiogenic treatment in early and metastatic breast cancer.

Keywords
Angiogenesis · Bevacizumab · Breast Cancer · Metastatic Cascade · Anti-angiogenic Therapy · Tyrosine Kinase

Introduction

Breast cancer is the most common cancer type in women; its mortality is primarily due to distant metastatic growth. Hematogenous spread of cells, shed from the primary tumor into the blood stream, was first described in the nineteenth century (Ashworth 1869; Paget 1889). In this complex cascade of events, from tumor cells confined to a primary site at an early stage to the development of distant metastasis, one crucial step is the invasion of cancer cells into the lumen of a nearby blood vessel (Pantel and Brakenhoff 2004). The mechanisms of intravasation are dependent on factors stimulating the formation of tumor-associated blood vessels. Thus, the process of neo-angiogenesis not only provides the tumor with an ample blood supply but enables cancer cells to enter blood circulation as well.

The development of a vascular bed requires a variety of factors collectively inducing a pro-angiogenic microenvironment (Ziyad and Iruela-Arispe 2011). Beyond this, tumor cells take over the local vasculature as they expand, resulting in a mixture of co-opted "old" and tumor-initiated "new" vessels. In contrast to normal angiogenesis, tumor-induced blood vessels are characterized by thin walls, absent pericytes, and abnormal dilation, and they are exceptionally permeable due to the lack of a functioning basement membrane (Papetti and Herman 2002). Among the soluble mediators regulating tumor-associated angiogenesis are the vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), heparanase, interleukins (especially interleukin-8), and matrix metalloproteinases (MMP), especially MMP-2. These factors, and their respective pathways, have been investigated extensively in recent decades, with the aim of developing agents targeting and suppressing tumor-initiated neo-angiogenesis. In breast cancer, several angiogenesis inhibitors have been studied in phase I–III trials, such as bevacizumab, sunitinib, and sorafenib, the first of these being the only anti-angiogenic drug approved for breast cancer treatment (Table 1).

In this chapter, we will review the current evidence regarding angiogenesis in breast cancer (BC) with a special focus on the clinical use of angiogenesis inhibitors in the treatment of early and metastatic BC.

Clinical Relevance of Anti-angiogenics in Metastatic Breast Cancer

Bevacizumab

Bevacizumab (Avastin®) is a monoclonal antibody designed to block the action of VEGF-A and has proved to be effective in a variety of cancers, such as glioblastoma, non-small cell lung cancer, renal cell carcinoma, and ovarian and colorectal cancer. The initial phase I–II trial of bevacizumab in breast cancer was a single-agent dose-escalation study in 75 patients with previously treated metastatic breast cancer (MBC) (Cobleigh et al. 2003). Patients received bevacizumab in different doses administered every 2 weeks. The overall response rate at the 10-mg/kg dose ($n = 41$) was 12%, including two patients with a complete remission. Based on these preliminary data, a phase III randomized trial was undertaken to evaluate bevacizumab in women with heavily pretreated MBC. In total, 462 patients were randomized to receive bevacizumab plus capecitabine or only capecitabine (Miller et al. 2005). The combination

Table 1 Angiogenesis inhibitors and their clinical relevance in breast cancer

Drug	Mode of action	Metastatic breast cancer	Primary, nonmetastatic breast cancer	Relevance in other tumor entities
Bevacizumab	Monoclonal antibody against VEGF-A; intravenous administration	Approved for first-line therapy in combination with paclitaxel or capecitabine in Europe (FDA approval revoked in 2011) Addition of bevacizumab to chemotherapy prolongs PFS but not OS in first-line therapy (significant OS benefit only in patients previously treated with taxanes) (Miles et al. 2013)	Addition of bevacizumab to standard neoadjuvant chemotherapy improves pCR rate (Cao et al. 2015) Contradictory survival results; in most neoadjuvant/adjuvant trials, addition of bevacizumab to chemotherapy did not improve DFS/OS; improved OS in one trial (NSABP B-40) (Bear et al. 2015)	Approved for treatment of metastatic colorectal cancer, non-small cell lung cancer, ovarian cancer, advanced cervical cancer, metastatic renal cell carcinoma and recurrent glioblastoma
Sorafenib	Oral inhibitor of various tyrosine kinases: VEGF receptor (VEGFR) 2, fms-like tyrosine kinase 3 (FLT3), PDGFR, fibroblast growth factor receptor (FGFR) 1	No survival benefit when added to chemotherapy, endocrine therapy, or bevacizumab	Limited data available. Concurrent therapy with paclitaxel not feasible due to high toxicity (Gradishar et al. 2013)	Approved for treatment of advanced hepatocellular carcinoma, renal cell carcinoma, and thyroid carcinoma
Sutinib	Oral inhibitor of various tyrosine kinases: c-kit, VEGFR1–3, PDGFR-alpha and beta, FLT3, CSF-1R	No survival benefit when added to chemotherapy or administered as a single-agent Possible efficacy in combination with HER2-targeted treatment (Bachelot et al. 2014)	Limited data available	Approved for treatment of advanced renal cell carcinoma, gastrointestinal stromal tumor, and pancreatic neuroendocrine tumors
Pazopanib	Oral inhibitor of various tyrosine kinases: VEGFR1-3, PDGFR-alpha and beta, FGFR1/3, c-kit	Limited data from phase II studies available. Possible efficacy in combination with lapatinib; the use of the combination limited due to higher toxicity (Johnston et al. 2013)	Limited data available. Concurrent therapy with paclitaxel not feasible due to high toxicity (Tan et al. 2015)	Approved for treatment of advanced renal cell carcinoma and soft tissue sarcoma
Suramin	Intravenous VEGF inhibitor (originally developed as an antiparasitic drug)	Limited data available Low activity in combination with paclitaxel (Lustberg et al. 2012)	Limited data from preclinical studies available.	Not approved for oncological therapy. FDA approval for metastatic prostate cancer rejected

(*continued*)

Table 1 (continued)

Drug	Mode of action	Metastatic breast cancer	Primary, nonmetastatic breast cancer	Relevance in other tumor entities
Ramucirumab	Monoclonal antibody against VEGFR2; intravenous administration	No survival benefit when added to docetaxel in HER2-negative MBC (Mackey et al. 2015)	Limited data from preclinical studies available	Approved for treatment of metastatic non-small cell lung cancer, gastric cancer, and colorectal cancer
Lenvatinib	Oral inhibitor of various tyrosine kinases: VEGFRs, RET, FGFR	Limited data from preclinical studies available.	Limited data from preclinical studies available	Approved for treatment of advanced renal cell carcinoma and thyroid cancer
Cediranib	Oral inhibitor of various tyrosine kinases: VEGFR1-3, PDGFR-alpha and beta, FGFR1, c-kit	Limited data available Possible efficacy in combination with fulvestrant; the use of the combination limited due to higher toxicity (Hyams et al. 2013) Discouraging results in combination with olaparib in triple-negative MBC (Liu et al. 2013)	Limited data from preclinical studies available	Not approved for oncological therapy. Promising results in recurrent platinum-sensitive ovarian cancer as a single agent (ICON-6 trial) or in combination with olaparib (Ledermann et al. 2016; Liu et al. 2014)

of bevacizumab with capecitabine resulted in a doubling of the response rate (19.8% vs. 9.1%; $p < 001$). However, the primary endpoint of that trial, the progression-free survival (PFS), was statistically identical in the two arms (4.2 vs. 4.9 months). Possibly, the vasculature of the tumors in heavily pretreated patients in this trial was more established, and therefore strategies aimed at VEGF and blockade of these pro-angiogenic signals were less likely to be effective. This explanation resulted in the evaluation of bevacizumab as first-line treatment of MBC. In the ECOG 2100 trial (NCT00028990), paclitaxel given weekly was administered. An almost doubling in the response rate (36.9% vs. 21.2%) and a doubling in the PFS (median 11.8 vs. 5.9 months) was observed in patients receiving bevacizumab. The final analysis of this study, however, failed to show an improvement in overall survival (OS) (Miller et al. 2007). An updated report about the results of this study with independent review of the radiology assessments was published with very similar results (Gray et al. 2009). The bevacizumab/paclitaxel combination was approved in many countries for first-line treatment of MBC. Another important study in the first-line metastatic setting is the avastin and docetaxel (AVADO) trial. Unlike E2100 this was a double-blind, placebo-controlled study, which examined adding two different doses of bevacizumab (or a placebo) to every-3-week docetaxel. The PFS was improved by less than one month with bevacizumab, a difference which was statistically significant, but less impressive than the ECOG 2100 results. Again, no OS benefit for bevacizumab has been observed. As patients in the placebo arm were allowed to cross over to bevacizumab at progression, it is unlikely that an OS benefit could have been seen with bevacizumab in this study. It is unknown, whether the results are a reflection of less synergy between docetaxel and bevacizumab, a smaller anti-angiogenic effect with docetaxel dosed every 3 weeks as compared to weekly paclitaxel, or other factors (Pivot et al. 2011; Miles et al. 2010). These results led to different reactions of the approval authorities. While the conditional approval in the USA was revoked, the European Medical Agency maintained the approval of

bevacizumab combined with paclitaxel and also of the combination bevacizumab and capecitabine.

To examine the effect of different chemotherapeutic agents in combination with bevacizumab, the Regimens in Bevacizumab for Breast Oncology (RIBBON-1) study was initiated (NCT00262067). RIBBON-1 was an international randomized phase III trial comparing first-line chemotherapy for MBC (capecitabine, taxane, or anthracycline combinations) with or without bevacizumab. The addition of bevacizumab to either chemotherapy improved overall response rates and PFS with again no difference seen for OS (Robert et al. 2011a). The combination of capecitabine with bevacizumab is approved in Europe based on these results. The addition of vinorelbine to this combination did not provide a relevant advantage (Welt et al. 2016). Also, the combination of bevacizumab with endocrine therapy was examined in a phase III trial (Dickler et al. 2016). In this trial, the addition of bevacizumab to letrozole improved PFS in hormone receptor-positive MBC, but this benefit was associated with a markedly increased risk of grade 3 to 4 toxicities.

In the second-line treatment of MBC, the phase III study RIBBON-2 compared the efficacy and safety of bevacizumab combined with standard chemotherapy regimens versus chemotherapy alone in patients with HER2-negative MBC (NCT00281697) (Brufsky et al. 2011). Median PFS increased from 5.1 to 7.2 months. Nevertheless, this approach was not approved in the USA or Europe. According to the first reports, it seemed that patients benefit from continuing bevacizumab until progression when chemotherapy is stopped (Fumoleau et al. 2008). The potential role of continuing bevacizumab throughout multiple lines of treatment was evaluated in the TANIA-trial (NCT01250379) (von Minckwitz et al. 2014b). In this open-label, randomized, phase III trial, patients who had HER2-negative, locally recurrent, or metastatic BC that had progressed after receiving 12 or more weeks of first-line bevacizumab plus chemotherapy were included. Patients were randomized to receive second-line single-agent chemotherapy either alone or with bevacizumab. PFS was significantly longer for patients treated with bevacizumab plus chemotherapy than for those receiving chemotherapy alone (median: 6.3 vs. 4.2 months). The IMELDA trial evaluated a different concept: patients with no progression after an induction with bevacizumab and docetaxel were randomly assigned to receive either bevacizumab and capecitabine or bevacizumab only (NCT00929240). Despite prematurely terminated accrual, both progression-free and overall survival were significantly improved with bevacizumab and capecitabine as compared to bevacizumab alone as maintenance treatment (Gligorov et al. 2014). These results might inform future maintenance trials and current first-line treatment strategies in MBC.

Sorafenib

Sorafenib is an oral multi-kinase inhibitor that has been shown to suppress tumor cell proliferation by interfering with several tyrosine kinases both on the tumor cells and cells in the vascular bed (Gadaleta-Caldarola et al. 2015). Sorafenib is currently approved for metastatic renal cell carcinoma as well as advanced hepatocellular and thyroid cancer. In MBC, sorafenib has been tested both as a single agent and in combination with other drugs. Two phase II trials investigated the efficacy of monotherapy with sorafenib in advanced BC (Moreno-Aspitia et al. 2009; Bianchi et al. 2009). No significant activity was observed in this setting: no patient experienced a complete response and the rate of partial responses was 0% (Moreno-Aspitia et al. 2009) and 2% (Bianchi et al. 2009), respectively. Stabilization of disease for 6 months or more was achieved only in 10% and 13%, respectively. Based on these results, further research focused on the combination of sorafenib with chemotherapy or endocrine therapy rather than single-agent treatment. Four double-blind phase II randomized trials were included in an international multicenter research program TIES (Trials to Investigate the Effects of Sorafenib in HER2-negative BC): the SOLTI-0701 trial aimed at evaluating sorafenib in combination with capecitabine, NU07B1 with paclitaxel, and AC01B07 with gemcitabine or

capecitabine in patients who progressed after bevacizumab-based therapy and FM-B07-01 with docetaxel and/or letrozole (Gradishar et al. 2013; Baselga et al. 2012, 2013; Schwartzberg et al. 2013). Of these trials, two (FM-B07-01 and NU08B1) did not demonstrate an improvement in PFS in the combination arm, the AC01B07 trial showed modestly improved PFS, and the promising results of the SOLTI-0701 trial led to the initiation of a confirmatory phase III study with a modified dose regime. In the SOLTI-0701 trial, the addition of sorafenib to capecitabine resulted in a significantly longer PFS versus capecitabine alone (6.4 vs. 4.1 months) but the combination led to unacceptable toxicity in many patients (Baselga et al. 2012). Therefore, in the phase III RESILIENCE trial, patients were treated with a reduced dose of sorafenib (600 mg instead of 800 mg per day) (Baselga et al. 2013). However, this trial failed to show a survival benefit when sorafenib was added to capecitabine, and no subgroup could be identified that benefitted from the combination therapy (Baselga et al. 2014). Another cytotoxic agent, ixabepilone, was tested as a combination partner for sorafenib in a phase II trial (NCT00825734) (Yardley et al. 2016). The combination therapy was poorly tolerated as first-line treatment in MBC, and its activity was similar to the activity reported for ixabepilone monotherapy. Several studies explored the possibility of combining sorafenib with another anti-angiogenic agent (Azad et al. 2008). However, adding sorafenib to bevacizumab led to significantly increased toxicity in MBC patients and resulted in a premature termination of a phase II trial (Mina et al. 2013).

Sunitinib

Sunitinib is an oral inhibitor of multiple receptor tyrosine kinases approved for the treatment of renal cell carcinoma and relapsed gastrointestinal stromal tumors. It interacts with angiogenesis through inhibition of VEGF receptors. A number of phase III trials showed that sunitinib does not improve survival in HER2-negative MBC – neither in combination with chemotherapy nor as a single agent. In a phase III trial, addition of sunitinib to docetaxel for first-line therapy increased the overall response rate but did not prolong PFS and OS compared to docetaxel alone in HER2-negative MBC patients (Bergh et al. 2012). In another phase III trial aimed to evaluate paclitaxel in combination with sunitinib or bevacizumab for first-line treatment in HER2-negative disease, the sunitinib/paclitaxel regimen was clinically inferior to the bevacizumab/paclitaxel combination (Robert et al. 2011b). Further, bevacizumab/paclitaxel was better tolerated than sunitinib/paclitaxel due to a higher rate of grade 3/4 neutropenia in the sunitinib arm. Sunitinib monotherapy was shown to be clinically inferior and more toxic than capecitabine monotherapy (Barrios et al. 2010).

In HER2-positive disease, preclinical studies suggested that concurrent inhibition of VEGF and HER2 pathways may be more efficacious than suppression of either target alone (Konecny et al. 2004). Bachelot et al. treated 60 patients with HER2-positive advanced BC with a combination of trastuzumab and sunitinib in a phase II study and compared the efficacy of this combination to a historical cohort of MBC patients treated with trastuzumab monotherapy (Bachelot et al. 2014). Sunitinib/trastuzumab showed significant antitumor activity, reaching an overall response rate of 37% and a 1-year-survival rate of 91%.

Metronomic Chemotherapy

Metronomic treatment is based on the continuous administration of chemotherapy at low or very low doses, with the aim of reducing toxicity and enabling long treatment without breaks. This form of therapy has been investigated in preclinical and clinical studies in breast cancer, with a particular focus on the metastatic setting (Banys-Paluchowski et al. 2016). Animal-based studies demonstrated that metronomic chemotherapy may eliminate endothelial cells involved in tumor-initiated angiogenesis and thus lead to tumor regression (Browder et al. 2000). Several cytotoxic agents that can be administered in metronomic schedules, such as cyclophosphamide

and capecitabine, seem to have an anti-angiogenic component, inducing apoptosis of endothelial cells in the vascular bed of the tumor rather than tumor cells. Whether adding other anti-angiogenic agents, such as bevacizumab or pazopanib, to metronomic therapy provides a survival benefit remains unclear (Garcia-Saenz et al. 2008; Calleri et al. 2009; Di Desidero et al. 2015).

Clinical Relevance of Anti-angiogenics in Nonmetastatic Breast Cancer

Bevacizumab

A number of studies with bevacizumab in patients with primary, nonmetastatic breast cancer were conducted. It was assumed that this could be the most efficacious setting for bevacizumab, as the blood supply of micrometastases is much less established than that seen with visible metastatic disease and should theoretically be more sensitive to blockade. Beyond "classical" endpoints in adjuvant therapy trials, such as disease-free survival (DFS) and OS, the neoadjuvant setting provides additional endpoints, such as pathological complete response (pCR), that can be evaluated without long follow-up and potentially translate into significant improvements in survival (Cortazar et al. 2014). Data from four randomized phase III trials investigating bevacizumab in the neoadjuvant setting are available (GeparQuinto, NSABP B-40, ARTemis, and CALGB 40603) (Bertucci et al. 2016; Pierga et al. 2012; von Minckwitz et al. 2012, 2014a; Sikov et al. 2015; Bear et al. 2015; Earl et al. 2015). Most of the trials reported a significant improvement in pCR rates, which was highest in triple-negative patients (Cao et al. 2015; Sikov et al. 2015; Earl et al. 2015). However, contrary to the initially promising pCR-based results, long-term evidence from the GeparQuinto study showed no improvement in survival when bevacizumab was added to neoadjuvant anthracycline- and taxane-based chemotherapy (von Minckwitz et al. 2014a).

In the adjuvant setting, two large phase III trials examined the use of bevacizumab in HER2-negative patients. The BEATRICE trial randomized 2591 patients with triple-negative BC to either chemotherapy alone or in combination with bevacizumab (NCT00528567) (Cameron et al. 2013). The first report of the trial with a median follow-up of 31.5 months suggests that bevacizumab cannot be recommended as adjuvant treatment in unselected patients with triple-negative breast cancer since no significant advantage in long-term outcome was observed. Similar results were provided by the E5103 trial which recruited 4994 patients with HER2-negative disease and both hormone receptor-positive and hormone receptor-negative disease (NCT00433511). Again, no improvement in DFS or OS could be shown with the addition of bevacizumab for one year to adjuvant chemotherapy (Miller et al. 2014).

In contrast to the GeparQuinto study and the two large adjuvant trials, the recently published results of the neoadjuvant NSABP B-40 trial surprisingly reported a significant improvement in OS ($p = 0.004$) with the addition of neoadjuvant plus adjuvant bevacizumab, with a trend toward greater benefit in patients with hormone receptor-positive disease (Bear et al. 2015). A main difference between the NSABP B-40 trial and the other trials is the administration of bevacizumab both before and after surgical treatment.

The fact that an increased angiogenesis was observed in tumors with HER2 overexpression was the rationale for the use of both antibodies in the adjuvant BETH trial (NCT00625898). This phase III trial randomized 3509 patients with HER2-positve early BC to receive standard therapy (chemotherapy plus trastuzumab) with or without bevacizumab for one year. After a median follow-up of 38 months, adding bevacizumab to standard treatment failed to improve survival (Slamon et al. 2013). The so far contradictory or discouraging results in patients without metastatic disease could lead to the hypothesis that in contrast to the primary tumor, inhibition of neo-angiogenesis does not play a crucial role for the growth of micrometastases.

Sorafenib

Sorafenib has been investigated for adjuvant therapy in a multicenter phase II trial (Spigel et al. 2011). Forty five node-positive and/or high-risk BC patients received anthracycline- and taxane-based chemotherapy combined with oral sorafenib for a maximum of 12 months. Due to relatively high toxicity, only 31% of patients received maintenance sorafenib after completing combined paclitaxel/sorafenib therapy. The authors concluded that concurrent use with paclitaxel does not appear to be feasible since a high rate of patients could not receive all planned paclitaxel cycles due to the high dropout rate.

Predictive Factors for Treatment Efficacy of Angiogenesis Inhibitors

Established predictive markers of the efficacy of VEGF-targeted therapy are lacking. The association between VEGF-A plasma levels and response to bevacizumab was investigated in a recent meta-analysis including 1713 patients from three trials – one in the adjuvant and two in the first-line metastatic setting (Santos et al. 2015). Bevacizumab therapy improved both PFS and event-free survival of patients with above median VEGF-A plasma levels, but not of those with below median VEGF-A levels. The correlation between VEGF-A and overall survival benefit remains yet to be evaluated.

Since the genetic variability of VEGF has been linked to breast cancer risk, single-nucleotide polymorphisms (SNPs) in VEGF were evaluated as a possible predictive biomarker. Schneider et al. analyzed the ECOG 2100 study cohort and reported significantly improved OS in patients receiving bevacizumab who carried specific genotypes, with an additive effect for each allele (Schneider et al. 2008a, b). An association between SNPs in VEGF-A and achievement of pCR in the neoadjuvant setting was shown in the GeparQuinto trial (Hein et al. 2015). However, these findings need to be confirmed in prospective studies. In other tumor entities, retrospective analyses have suggested that the development of hypertension is predictive for therapy outcome. However, this observation could not be confirmed in the AVADO-trial (Chan et al. 2008).

Other studies focused on factors involved in tumor-induced angiogenesis such as matrix metalloproteinases (MMP). In a small cohort of patients with HER2-positive inflammatory BC treated with bevacizumab−/trastuzumab-based neoadjuvant chemotherapy in the BEVERLY-2 trial, high bMMP2 and low bMMP9 serum levels were identified in patients with better survival (Tabouret et al. 2016). Whether MMP2 might be useful for selecting patients most likely to benefit from bevacizumab therapy remains to be evaluated. In another study, multiple angiogenesis- and hypoxia-related proteins, such as VEGF-A, soluble VEGFR2, angiopoietin 2, interleukins 6 and 8, and carbonic anhydrase 9, were prognostic for PFS and OS in patients treated with first-line bevacizumab-based chemotherapy (Lam et al. 2016).

Conclusion

Since tumor-induced angiogenesis has become an attractive target for cancer therapy, a number of angiogenesis inhibitors have been developed and evaluated in clinical trials. The most promising of those, the anti-VEGF antibody bevacizumab, prolongs progression-free survival in metastatic breast cancer patients and is approved in Europe in this disease setting, while its use in early breast cancer has not been associated with a clear survival benefit. Clinical studies involving other anti-angiogenic agents, such as sorafenib, sunitinib, and ramucirumab, provided discouraging results despite promising performances in the preclinical setting. Future research should aim at identifying biomarkers for prediction of response to anti-angiogenic treatment.

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Cross-References

- Biomarkers for Anti-angiogenic Therapy
- Combination of Anti-angiogenics and Other Targeted Therapies
- Cytotoxics and Anti-angiogenics: Metronomic Therapies
- Mechanisms of Anti-angiogenic Therapy
- Mechanisms of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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The Value of Anti-angiogenics in Ovarian Cancer Therapy

Sven Mahner and Fabian Trillsch

Contents

Introduction 530

Anti-angiogenic Treatment with Bevacizumab 530
Bevacizumab in First-Line Therapy 531
Bevacizumab in Recurrent, Platinum-Sensitive Ovarian Cancer 532
Bevacizumab in Recurrent, Platinum-Resistant Ovarian Cancer 532

Inhibition of Angiogenesis by Multi-kinase Inhibitors 534
Pazopanib 535
Nintedanib 536
Sorafenib 536
Cediranib 538
Sunitinib 538

Other Anti-angiogenic Treatment Strategies 539
Trebananib 539

Future Directions 540
Additional Combination Partners: PARP Inhibitors 540
Additional Combination Partners: Immunotherapies 541

Conclusion 541

Cross-References 541

References 541

Abstract

Introducing the monoclonal vascular endothelial growth factor (VEGF) antibody bevacizumab to first-line treatment was the first implementation of a targeted therapy for ovarian cancer patients. Until then, standard of care for more than 10 years had been the combination chemotherapy of six cycles of carboplatin and paclitaxel every 3 weeks. Two phase III trials on bevacizumab proved

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efficacy of anti-angiogenic therapy for first-line setting in 2011 leading to approval in several countries. Since then, bevacizumab has become available for different therapeutic indications although the treatment effect is still restricted to progression-free survival in the target groups. Therefore, further tailored treatment strategies have been studied or are still under investigation to improve efficacy and possibly reduce toxicity. Combinations of anti-angiogenic therapies with other effective drugs to overcome resistance are further promising approaches being currently tested in clinical trials.

Keywords
Ovarian cancer · Anti-angiogenic therapy · First-line treatment · Recurrent disease · Bevacizumab · Multi-kinase inhibitors · Pazopanib · Nintedanib · Sorafenib · Cediranib · Sunitinib · Trebananib · PARP inhibitors · Combination therapies

Introduction

Therapeutic concepts in ovarian cancer concentrated on classical chemotherapeutics for many years. More than a decade, the standard treatment was the combination chemotherapy of carboplatin and paclitaxel every 3 weeks for six cycles since publication of the corresponding studies in 2003 (du Bois et al. 2003; Ozols et al. 2003; Mahner et al. 2013). Since then, several phase III trials intended to extend therapeutic options by adding further chemotherapeutics without cross-resistance (e.g., epirubicin, pegylated liposomal doxorubicin, gemcitabine, and topotecan), by triplet combinations, or by dose-dense treatment plans. Besides increased toxicities, no prognostic improvements could be achieved so that the scientific scope concentrated more and more on alternative drugs. Targeted therapies addressing specific tumorbiologic pathways have been established and are now being tested to prove efficacy in ovarian cancer patients.

Due to high tumor load, early tumor cell dissemination, vascular permeability, and frequent presence of ascites, ovarian cancer biology appears very promising for anti-angiogenic approaches. Over the past years, several phase III trials have been conducted with different drugs focusing on inhibition of neo-angiogenesis.

In this context, the humanized monoclonal antibody of vascular endothelial growth factor (VEGF) bevacizumab specifically addresses the VEGF pathway. To achieve a broader affinity against different VEGF receptors and further intracellular pathways, multi-kinase inhibitors were developed and implemented in clinical trials introducing pazopanib, nintedanib, cediranib, sunitinib, as well as sorafenib. In contrast, the peptibody trebananib was designed to target the angiopoietin cascade, a VEGF-independent signaling pathway. Although most experience exists for bevacizumab, current evidence and clinical data for the different anti-angiogenic drugs will be elucidated in the following chapter.

Anti-angiogenic Treatment with Bevacizumab

VEGF and VEGF receptors can be detected on ovarian cancer cells. Increased VEGF expression has been linked to malignant ascites formation and tumor progression (Graybill et al. 2015). The first report of antitumor activity by bevacizumab in ovarian cancer was published in March 2005. In a patient with high-grade ovarian cancer and a total of 11 previous lines of chemotherapy, an objective response to single-agent bevacizumab at 15 mg/kg (IV) every 3 weeks could be achieved over more than 5 months (Monk et al. 2005). Since then, bevacizumab has become the most intensively studied targeted therapy in ovarian cancer. Following preclinical investigations, different phase I and phase II studies, two prospective randomized phase III trials focusing on first-line therapy, were published in December 2011 (Table 2).

Bevacizumab in First-Line Therapy

In GOG 218, a placebo controlled trial with three treatment arms, the effect of bevacizumab 15 mg/kg q3w was investigated in addition to standard chemotherapy of carboplatin AUC 6 and paclitaxel 175 mg/m^2 q3w (Burger et al. 2011). The control group received placebo every 3 weeks over a total of 22 cycles and a corresponding time period of 15 months. The second group received bevacizumab during the six cycles of chemotherapy followed by placebo from cycle 7 to 22, while the third group received bevacizumab throughout the 22 cycles. Therefore, this study enabled to compare bevacizumab-initiation therapy with a subsequent maintenance therapy. The results of this trial demonstrated a significantly improved progression-free survival (PFS) by the addition of bevacizumab but only for the maintenance therapy group. For these patients, median PFS of 14.1 months was significantly improved compared to 10.3 months in the control group with a corresponding hazard ratio (HR) of 0.72 (95% confidence interval [CI] 0.63–0.82). In contrast, in the bevacizumab-initiation therapy group without maintenance, no statistical difference was reached with a median PFS of 11.2 months. Despite the positive effect on PFS, prognostic impact of the bevacizumab-maintenance arm on overall survival (OS) was non-significant with 43.8 versus 40.6 months (HR 0.87, 95% CI 0.74–1.03) (Burger et al. 2011). Nevertheless, GOG 218 could prove that bevacizumab is effective for ovarian cancer patients but, however, requires a maintenance therapy to achieve a significant prolongation of PFS. Regarding adverse events, patients in the maintenance arm had higher rates of hypertension with grade $\geq$2 in 23% of patients versus 7% in the chemotherapy-only arm. Other parameters revealed only minor differences. Of note, quality of life analyses demonstrated that anti-angiogenic therapy caused mildly but significantly impaired quality of life during the six cycles of chemotherapy, which was then unchanged during the following maintenance therapy (Burger et al. 2011).

Specific interest deserves the fact that the PFS of 10.3 months in the standard treatment arm of this study is remarkably low compared to other published trials for first-line ovarian cancer therapy in which 16–17 months following standard chemotherapy are usually achieved. This reflects an important selection bias within GOG 218 in which only patients with postoperative residual tumor were included with consecutively impaired prognosis.

In AGO-OVAR 11/ICON 7, the second trial published in the same issue of the *New England Journal of Medicine*, the lower dose of 7.5 mg/kg of bevacizumab was investigated in addition to the same standard chemotherapy of six cycles of carboplatin AUC 5/6 and paclitaxel 175 mg/m^2 q3w and a subsequent maintenance phase for a total period of 15 months with bevacizumab versus placebo (Perren et al. 2011). In this trial on 1528 patients, subjects with complete macroscopic tumor resection (microscopic disease) as well as 10% of patients with high-risk early-stage disease had also been included. As in GOG 218, PFS was significantly improved in the experimental arm with 19.8 months compared to 17.4 months in the control arm and a corresponding HR of 0.87 (95% CI 0.77–0.99). For bevacizumab-treated patients, hypertension was significantly more frequent with grade $\geq$2 in 18% compared to 2% of patients. The rate of thromboembolic events was higher with 11% versus 6%, but apart from this, no increased toxicity was registered. Of note, this also applied for the risk of gastrointestinal perforations which had been one of the major concerns following the previous phase II trials (Perren et al. 2011).

Recent publication of the mature OS data did also fail to reveal a significant impact on OS for these patients (44.6 vs. 45.5 months; $p = 0.85$). This could only be noted in a subgroup of "high-risk patients," defined as patients with postoperative residual tumor in FIGO stage III or patients in FIGO stage IV. This explorative analysis of the subgroup with 502 patients demonstrated a significant OS prolongation of 39.3 versus 34.5 months ($p = 0.03$) (Oza et al. 2015).

Consequently, both these two large, prospective randomized controlled phase III trials with almost 3400 patients could demonstrate a

significant improvement of PFS with possible pronounced survival benefits in patients with postoperative residual tumor. Effectivity of anti-angiogenic therapies in ovarian cancer could be proven although several questions for clinical routine remain unanswered. Apart from selecting patients who benefit the most, dosage and duration of therapy need also be further investigated. In this context, the AGO-OVAR17 trial challenges the question if an extended maintenance therapy (30 vs. 15 months of bevacizumab 15 mg/m^2 q3w) could be beneficial for selected patients. Results of this trial are expected in 2018.

Interestingly, interpretation of the described data was different depending on the side of the ocean. In Europe, bevacizumab 15 mg/kg q3w as combination for first-line chemotherapy with a subsequent maintenance of 15 months was approved for ovarian cancer patients in FIGO stage IIIB to IV. Contrarily, the US Food and Drug Administration (FDA) refrained from approving this first targeted therapy which reflects a different approach to new treatment strategies in evaluating patients' benefits, cost-effectiveness, and safety.

Bevacizumab in Recurrent, Platinum-Sensitive Ovarian Cancer

Shortly after approval of bevacizumab for first-line therapy, clinical data of the OCEANS trial on platinum-sensitive recurrent disease raised further attention in 2012 (Aghajanian et al. 2012) (Table 2). In this international phase III trial, bevacizumab 15 mg/m^2 every 3 weeks was added to second-line chemotherapy in patients without previous anti-angiogenic therapy. The chemotherapy in this protocol was the combination of carboplatin AUC 4 and gemcitabine 1000 mg/m^2 day 1 and 8 every 3 weeks with a following maintenance therapy of bevacizumab versus placebo until progression, death, or intolerable toxicity. As seen in first-line setting, bevacizumab significantly prolonged PFS with a 6-month benefit of 12.4 months versus 8.4 months (HR 0.48, 95% CI 0.38–0.60) (Aghajanian et al. 2012). The toxicity within this trial was again tolerable. Previous concerns regarding higher rates of gastrointestinal perforation and fistula could not be confirmed as no cases were noted in the bevacizumab-treated patients during treatment phase. Two cases of gastrointestinal perforation were, however, registered short after study treatment discontinuation. Of note, this trial did also fail to reach a significant improvement of overall survival (Aghajanian et al. 2015). Nevertheless, the PFS benefit was again estimated to be clinically relevant so that the European Medical Association approved bevacizumab for the first platinum-sensitive relapse in patients without previous anti-angiogenic therapy. This was again contrary to the FDA who refused approval due to missing impact on patients' OS.

Further clinical information is expected from the AGO-OVAR 2.21 trial in which an alternative combination chemotherapy is evaluated with bevacizumab. In this study, bevacizumab has been applied together with carboplatin and pegylated liposomal doxorubicin (PLD) and was compared to the "OCEANS combination" carboplatin/gemcitabine/bevacizumab. In addition to this novel combination, this study might also answer the question if re-induction of bevacizumab may be beneficial, since the study protocol did not exclude patients with previous anti-angiogenic therapy. So far, only very sparse literature on this topic is available, and it is not clear if second application of bevacizumab is still effective or if it then may cause intolerable toxicity.

Bevacizumab in Recurrent, Platinum-Resistant Ovarian Cancer

The third indication, in which bevacizumab was tested in a large prospective phase III study for ovarian cancer, was platinum-resistant disease within the AURELIA trial (Pujade-Lauraine et al. 2012) (Table 2). In this study, 361 patients were randomized to mono-chemotherapy with or without bevacizumab 15 mg/m^2 every 3 weeks until progression or intolerable toxicity. Possible treatment schedules for chemotherapy were paclitaxel 80 mg/m^2 day 1, 8, 15, and 22 q4w; topotecan

Table 1 Overview of approved indications for bevacizumab therapy according to the authorities (European Medicines Agency [EMA] and US Food and Drug Agency [FDA])

Europe (EMA)	Indication	United States (FDA)
✓	First-line therapy with subsequent maintenance phase (carboplatin/paclitaxel+bevacizumab)	–
✓	Platinum-sensitive disease (carboplatin/gemcitabine+bevacizumab)	–
✓	Platinum-resistant disease (PLD/paclitaxel/topotecan+bevacizumab)	✓

4 mg/m^2 day 1, 8, and 15 q4w (or 1.25 mg/m^2 day 1–5 weekly); or PLD 40 mg/m^2 day 1 q4w.

In the experimental arm, bevacizumab significantly improved PFS, the primary study endpoint, from 3.4 up to 6.7 months with a corresponding HR of 0.48 (95% CI 0.38–0.60). As patient with platinum-resistant disease are frequently characterized by high tumor volume and impaired quality of life due to symptoms from disease, inclusion criteria of the AURELIA study protocol specifically addressed the suspected risk of bowel perforations and fistula. Consequently, patients with history of bowel obstruction related to underlying disease, a history of abdominal fistula, gastrointestinal perforation, or intra-abdominal abscess was excluded. Maybe, due to this pre-defined exclusion criteria, toxicity was again comparable to the previous data from first-line treatment and especially rates for bowel perforation, intra-abdominal fistulas and abscesses were fortunately low (2.2% vs. 0.0%) (Pujade-Lauraine et al. 2012). Therefore, other clinical trials adopted study plans and included the AURELIA criteria into their study protocols. Of note, prospectively envisaged quality of life analyses revealed a significant improvement for bevacizumab-treated patients regarding abdominal and gastrointestinal symptoms which deserves specific consideration given the palliative therapeutic aspects of this indication. As a consequence, not only the European but also the US authorities gave bevacizumab the approval for the treatment of recurrent, platinum-resistant ovarian cancer (Table 1).

In an exploratory subgroup analysis of the AURELIA cohort, it could be demonstrated that the time frame in which patients develop platinum resistance may translate to different clinical as well as biological behavior (Trillsch et al. 2016). Patients who had responded to at least one chemotherapy with a progression-free interval of at least 6 months and secondarily developed platinum-resistant disease (secondary platinum resistance, SPR) had a better prognosis and response to bevacizumab than patients who already had initial recurrence within 6 months (primary platinum resistance, PPR). This significant difference could be demonstrated in univariate as well as in multivariate analyses. Consequently, SPR versus PPR should be considered as stratification factor in future clinical trials on anti-angiogenic therapy for platinum-resistant ovarian cancer (Trillsch et al. 2016).

Another interesting aspect could be noted in a further exploratory subgroup analysis in which treatment efficacy of bevacizumab was compared among the different chemotherapeutic agents (paclitaxel vs. PLD vs. topotecan) (Poveda et al. 2015). In this analysis, paclitaxel appeared to have best synergistic effects which supports the experience with metastatic breast cancer in which bevacizumab combined with weekly paclitaxel appeared to be more active than other chemotherapeutic backbones. For the design and clinically evaluation of future trials on anti-angiogenic agents, these facts need to be taken into account. However, it is important to note that these retrospective subgroup analyses should only be considered as hypothesis generating and should not yet be the base for clinical decisions (Table 2).

Inhibition of Angiogenesis by Multi-kinase Inhibitors

In contrast to bevacizumab, multi-kinase inhibitors exhibit their potential via intracellular blockade of signal transduction pathways targeting receptor tyrosine kinases. Despite this appealing concept, broader activity against more targets may lead to additional toxicity. Due to oral administration of most multi-kinase inhibitors, improved convenience can be considered as an advantage, but contrarily inconsistent bioavailability and inflexibility in dosing may be drawbacks.

Some multi-kinase inhibitors already proved efficacy in other tumor entities. For ovarian

Table 2 Overview of ovarian cancer trials with bevacizumab

Trial name (author)	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase III – first-line treatment					
GOG 218 (Burger et al. 2011)	6× carboplatin AUC6 + paclitaxel 175 mg/m^2 q3w + bevacizumab 15 mg/kg q3w over a total of 15 months versus 6× carboplatin AUC6 + paclitaxel 175 mg/m^2 q3w and bevacizumab 15 mg/kg during chemotherapy, subsequent placebo versus 6× carboplatin AUC6 + paclitaxel 175 mg/m^2 q3w + placebo q3w over a total of 15 months	1873 (1:1:1)	14.3 months HR 0.72 (95% CI 0.63–0.82; $\boldsymbol{p < 0.001}$) versus 11.2 months HR 0.91 (95% CI. 0.80–1.04; $p = 0.16$) versus 10.3 months	43.8 months versus 40.6 months HR 0.87 (95% CI 0.74–1.03)	All included patients with postoperative residual tumor
AGO-OVAR 11/ICON 7 (Perren et al. 2011; Oza et al. 2015)	6× carboplatin AUC 5/6 + paclitaxel 175 mg/m^2 q3w + bevacizumab 7.5 mg/kg q3w for 15 months versus 6× carboplatin AUC 5/6 + paclitaxel 175 mg/m^2 q3w + placebo q3w for 15 months	1528 (1:1)	16.9 months versus 19.3 months HR 0.86 (95% CI 0.75–0.98; $\boldsymbol{p = 0.019}$)	44.6 months versus 45.5 months; $p = 0.85$	OS benefit only in high-risk patients (postoperative residual tumor in FIGO stage III or patients in FIGO stage IV)
Phase III – recurrent disease					
OCEANS (Aghajanian et al. 2011, 2015)	Carboplatin AUC 4 + gemcitabine 1000 mg/m^2 d1+8 q3w+ bevacizumab 15 mg/kg q3w versus carboplatin AUC 4 + gemcitabine 1000 mg/m^2 d1+8 q3w + placebo q3w	484 (1:1)	12.4 months versus 8.4 months HR 0.48 (95% CI 0.30–0.61. $\boldsymbol{p < 0.001}$)	33.6 months versus 32.9 months; HR 0.95 ($p = 0.65$)	Patients with first platinum-sensitive relapse without prior anti-angiogenic therapy
AURELIA (Pujade-Lauraine et al. 2012)	Standard mono-chemotherapy (PLD, paclitaxel, topotecan) + bevacizumab 15 mg/kg q3w versus standard mono-chemotherapy (PLD, paclitaxel, topotecan) + placebo q3w	361 (1:1)	6.7 months versus 3.4 months HR 0.48 (95% CI 0.38–0.60. $\boldsymbol{p < 0.001}$)	16.6 months versus 13.3 months HR 0.85 (95% CI 0.66–1.08. $p = 0.174$)	Patients with platinum-resistant disease, 1 or 2 prior treatment regimens

Table 3 Overview of ovarian cancer trials with pazopanib

Trial name (author)	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase III – first-line treatment					
AGO-OVAR 16 (du Bois et al. 2014)	Pazopanib 800 mg orally daily versus placebo orally daily subsequent to first-line chemotherapy up to 24 months	940 (1:1)	17.9 months versus 12.3 months HR 0.77 (95% CI 0.64–0.91. ***p* = 0.002**)	Immature data	Pure maintenance therapy subsequent to first-line chemotherapy
Phase II – recurrent disease					
Friedlander et al. (2010)	Pazopanib 800 mg daily following complete CA-125 response to initial platinum-based chemotherapy and subsequent rise	36	–	–	Overall response rate (ORR): 18% in patients with measurable disease at baseline
Pignata et al. (2015)	Paclitaxel 80 mg/m^2 + pazopanib 800 mg daily versus paclitaxel 80 mg/m^2	74 (1:1)	Median 6.35 months (95% CI 5.36–11.02) versus 3.49 months (95% CI 2.01–5.66); HR 0.42 (95% CI 0.25–0.69); ***p* = 0.0002**	–	–

cancer, promising data has been published although this did not lead to approvals for clinical routine so far.

Pazopanib

Pazopanib is an oral tyrosine kinase inhibitor targeting three different protein kinases (VEGFR, PDGFR, c-KIT). Following promising data in phase II trials (Friedlander et al. 2010; Pignata et al. 2015), a randomized, double-blind phase III study (AGO-OVAR 16) with 940 patients was initiated by the Arbeitsgemeinschaft Gynaekologische Onkologie (AGO) for first-line treatment (Table 3). This trial addressed for the first time a solely anti-angiogenic maintenance therapy. Pazopanib versus placebo subsequent to standard chemotherapy with carboplatin and paclitaxel was administered orally in a dose of 800 mg. A significant improvement in PFS of 5.6 months for patients in the pazopanib arm was noted (median 17.9 vs. 12.3 months, HR 0.77, 95% CI 0.64–0.91, $p = 0.002$) (du Bois et al. 2014). However, no difference in overall survival was seen. A significant higher rate of grade 3 or 4 adverse events, mainly hypertension (30.8%), neutropenia (9.9%), liver-related toxicity (9.4%), and diarrhea (8.2%), was reported in the pazopanib group. In 33% of patients of the pazopanib arm, treatment was discontinued due to adverse events, while this rate was only at 6% in the placebo arm (du Bois et al. 2014).

As previously seen with dose-dense treatment schedule in context of ovarian cancer first-line therapy, data on pazopanib further supports that ethnical aspects have to be considered when new treatment strategies are evaluated. In pooled analyses of the AGO-OVAR16 and an East Asian study solely focusing on 354 East Asian patients, the PFS improvement of pazopanib could not be confirmed, and, moreover, regarding OS even a detrimental effect among 209 patients from AGO-OVAR16 was noted (Kim et al. 2015).

Currently, pazopanib is investigated in other phase II studies (e.g., NOGGO-TOPAZ, an ongoing phase II study for patients with platinum-resistant recurrence: pazopanib 400 mg/d orally vs. placebo in combination with topotecan 4 mg/m^2 weekly) which may eventually prove the effect of pazopanib for further indications.

Table 4 Overview of ovarian cancer trials with nintedanib

Author	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase III – first-line treatment					
AGO-OVAR 12 (du Bois et al. 2016)	Carboplatin AUC 5/6 q3w + paclitaxel 175 mg/m^2 q3w + nintedanib 200 mg orally BID up to 120 weeks versus carboplatin AUC 5/6 q3w + paclitaxel 175 mg/m^2 q3w + placebo orally BID up to 120 weeks	911 (2:1)	17.3 months versus 16.6 months HR 0.84 (95% CI 0.72–0.98; ***p* = 0.024**)	Immature data	Oral anti-angiogenic therapy parallel to chemotherapy with subsequent maintenance phase
Phase II – recurrent disease					
Ledermann et al. (2011)	Nintedanib 250 mg BID versus placebo BID continuously for 36 weeks as maintenance	83 (1:1)	Thirty-six–week PFS rates: 16.3% versus 5.0% HR 0.65 (95% CI, 0.42–1.02; $P = 0.06$)	–	–

Nintedanib

Following results from a positive phase II study in relapsed ovarian cancer (Ledermann et al. 2011; McCormack 2015), nintedanib, another oral triple angiogenic kinase inhibitor (BIBF 1120) targeting VEGFR, PDGFR, and FGFR, was studied for first-line therapy of ovarian cancer patients (Table 4). In the positive prospective, randomized phase III study (AGO-OVAR 12/LUME-Ovar 1) with a total of 1366 patients, nintedanib 200 mg BID versus placebo was given parallel to chemotherapy with carboplatin AUC 5/6 and paclitaxel 175 mg/m^2 followed by a maintenance phase for a maximum duration of 120 weeks (du Bois et al. 2016). Median PFS, the primary endpoint, was prolonged from 16.6 to 17.3 months (HR 0.84; 95% CI 0.72–0.98; $p = 0.024$). Of note, treatment-related toxicity was significantly increased in the nintedanib arm with predominantly hematologic and gastrointestinal adverse events (Grad $\geq$3 22% vs. 2%). So far, no significant effect on overall survival was noted (du Bois et al. 2016).

With regard to previous results from the bevacizumab trials, subgroup analyses of the AGO-OVAR 12/ LUME-Ovar 1 raised attention. Here, PFS seemed to be most improved among patients of the low-risk group with small residual tumor after surgery which is conflicting to the previous bevacizumab experience (du Bois et al. 2016). So far, no sufficient explanation for this fact could be identified, but it underlines that despite the same overall target mechanism, anti-angiogenic agents seem to differ in terms of treatment effect as well as predictive subgroups. Currently, approval of nintedanib is not expected. However, important information can be retrieved from this data when future studies addressing patient selection and optimized tolerability will be planned and executed.

Sorafenib

Sorafenib is also an oral multi-targeting tyrosine kinase inhibitor, blocking VEGFR2, VEGFR3, as well as PDGFR beta, Flt-3, and c-kit (Wilhelm et al. 2006). In addition to these targets, sorafenib has partial inhibitory effects on the RAS/RAF/MEK/ERK signaling pathway which is known to play a central role in ovarian cancer

Table 5 Overview of ovarian cancer trials with sorafenib

Author	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase II – first-line treatment					
Herzog et al. (2013)	Sorafenib 400 mg BID versus placebo maintenance in patients with complete remission after first-line chemotherapy	246	Median 12.7 versus 15.7 months; HR 1.09 (95% CI 0.72–1.63)	–	–
Phase II – recurrent disease					
Matei et al. (2011)	Sorafenib 400 mg orally BID	71	Patients with at least 6 months PFS: 24% (90% CI, 15% to 35%)	–	Overall response rate (ORR): 3.4%
Sehouli et al. (2016)	Six cycles of topotecan 1.25 mg/m^2, d1–5, q21d +sorafenib 400 mg BID d6–15 and maintenance sorafenib 400 mg BID for 1 year versus placebo	172 (1:1)	6.7 versus 4.4 months; HR 0.6, (95% CI of 0.43–0.83, $\boldsymbol{p = 0.002}$)	Overall survival: 17.1 versus 10.1 months (HR 0.65, 95% CI of 0.45–0.93, $\boldsymbol{p = 0.017}$)	First randomized trial in platinum-resistant ovarian cancer with a significant OS benefit by targeted therapy

development, especially of low-grade tumors (Wilhelm et al. 2006).

In a phase II study of 71 patients concentrating on recurrent ovarian cancer, a modest antitumor effect could be demonstrated for sorafenib maintenance treatment at dose of 400 mg twice a day following chemotherapy. However, this impact was achieved at the expense of significant toxicity (Matei et al. 2011). Comparable results were revealed by a randomized phase II trial of 246 patients with complete remission after first-line chemotherapy in which no significant difference between treatment with sorafenib 400 mg twice a day versus placebo could be demonstrated for PFS (median 12.7 vs. 15.7 months; hazard ratio 1.09; 95% CI 0.72–1.63) (Herzog et al. 2013). Of note, high rates of dose reductions (67.5% vs. 30.1%) and early discontinuations were observed in the sorafenib arm interfering with the efficacy analysis. The most common grade $\geq$3 adverse events were hand-foot skin reaction (39.0% vs. 0.8%) and rash (14.6% vs. 0%). The authors concluded that sorafenib therefore should not be recommended as maintenance therapy for patients with OC experiencing complete remission (Herzog et al. 2013). Nevertheless, recent presentation of a clinical phase II trial (NOGGO-TRIAS) raised attention as this study proved a statistically significant improvement in PFS as well as in OS (Sehouli et al. 2016) (Table 5). Within the study protocol, monochemotherapy of topotecan was compared with the combination of topotecan and sorafenib. PFS of the experimental arm was significantly improved with 6.7 versus 4.4 months (HR 0.6, 95%CI of 0.43–0.83, $p = 0.002$) as well as OS with 17.1 versus 10.1 months (HR 0.65, 95%CI of 0.45–0.93, $p = 0.017$). Of note, the observed effects remained significant even in more pre-treated patients undergoing second-, third-line or higher therapy with still acceptable toxicity. Addition of sorafenib led to more hand-foot-skin reactions (13.3% vs. 0%; $p < 0.001$) and alopecia grade 2 (28.9% vs. 13.5%; $p = 0.015$) but no additional severe toxicity grade 3/4 (Sehouli et al. 2016). Despite these promising results, no phase III trial for this indication is currently open, and approval for this agent has not been requested so far. Nevertheless, as this was the first trial for platinum-resistant ovarian cancer achieving an OS benefit by targeted therapies, this data needs to be discussed and considered in future executive boards planning the implementation of additional drugs to systemic therapy.

Table 6 Overview of ovarian cancer trials with cediranib

Author	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase III – recurrent disease					
ICON6 (Ledermann et al. 2016)	Cediranib 20 mg orally daily during platinum-based chemotherapy and followed up to 18 months versus cediranib 20 mg orally daily during platinum-based chemotherapy and followed by placebo up to 18 months versus placebo with platinum-based chemotherapy	456 (3:3:2)	11.0 months versus 8.7 months; HR 0.68; $\boldsymbol{p < 0.0001}$	20.3 months versus 17.6 months; HR 0.70; $p = 0.042$	Platinum-sensitive recurrent ovarian cancer; first study with targeted therapy and effect on OS
Phase II – recurrent disease					
Matei et al. (2011)	Cediranib 45 mg daily, subsequently 30 mg daily (single arm)	46	–	–	Overall response rate (ORR): 17% (95% CI, 7.6%–30.8%)

Cediranib

The oral tyrosine kinase inhibitor cediranib is a potent inhibitor of all three VEGF receptors (VEGFR-1, VEGFR-2, VEGFR-3) and c-kit with pronounced selectivity for VEGFR-2. It demonstrated activity in an open-label phase II trial among 46 patients with recurrent disease although the dose of cediranib had to be reduced from 45 to 30 mg/day due to significant toxicity, such as hypertension, fatigue, and diarrhea (Matulonis et al. 2009). Based on these results, the randomized, double-blind, placebo-controlled phase III trial ICON6 was initiated to evaluate cediranib in 456 patients with platinum-sensitive recurrent disease (Raja et al. 2011) (Table 6). The original study design promised more reliable evidence, but instead of randomly assigning roughly 2000 participants, the study underwent a major revision as the drug supplier ceased commercial development of cediranib during the course of the study accrual owing to negative findings in phase III studies on different cancers. However, patients could still be randomized to receive six cycles of carboplatin AUC 5 or 6 plus paclitaxel 175 mg/m^2 q3w with either placebo (reference), cediranib 20 mg per day, followed by placebo (concurrent), or cediranib 20 mg per day, followed by cediranib (concurrent plus maintenance). In this further reduced dosage, treatment was sufficiently well tolerated during initial toxicity assessment (Raja et al. 2011).

In the final analysis of the corrected endpoints and the corrected sample size, the results significantly improved PFS in the cediranib concurrent and maintenance arm compared to placebo (11.0 vs. 8.7 months; HR 0.56; $p<0.0001$) (Ledermann et al. 2011) (Table 6). The most common cediranib-related adverse events included diarrhea, nausea, and fatigue. Although first presentation of the ICON6 results suggested an additional significant effect on OS, this has not been yet confirmed in the final publication due to so far immature data for this endpoint.

Sunitinib

A further multi-kinase inhibitor targeting VEGF receptors, PDGF receptors, stem cell factor receptor (KIT), and FMS-like tyrosine kinase-3 (FTL3) has also been included in phase II studies for ovarian cancer and recurrent disease (Mendel et al. 2003) (Table 7). Initially, sunitinib as single agent was investigated at a dose of 50 mg daily over 4 weeks of a 6-week cycle which was adopted to continuous 37.5 mg daily dosing in the second stage of accrual due to higher incidence of ascites or pleural effusions during off-treatment intervals (Biagiet al. 2011). Although sunitinib exhibited modest activity

Table 7 Overview of ovarian cancer trials with sunitinib

Author	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase II – recurrent disease					
Biagi et al. (2011)	Sunitinib 50 mg daily (4 of 6 weeks) subsequently continuous 37.5 mg daily dosing	30	4.1 months	–	Overall response rate (ORR): 13.3%
Baumann et al. (2012)	Sunitinib 50 mg daily (4 of 6 weeks) versus continuous 37.5 mg daily dosing	73	4.8 (2.9–8.1) months versus 2.9 (2.9–5.1) months	–	Overall response rate (ORR): 16.7% versus 5.4%

in recurrent platinum-sensitive ovarian cancer, a dosage-dependent response was noted favoring the 50 mg intermittent schedule. Common adverse events included fatigue, gastrointestinal symptoms, hand-foot syndrome, and hypertension. No gastrointestinal perforation occurred during treatment period (Biagi et al. 2011).

The phase II AGO 2.11 study investigated single-agent sunitinib in 73 patients with platinum-resistant ovarian cancer in which moderate activity was noted. Included patients had received $\leq$3 prior chemotherapy regimens and were allocated to two treatment arms (arm 1: non-continuous treatment with 50 mg sunitinib daily orally for 28 days followed by 14 days off drug; arm 2: continuous treatment with 37.5 mg sunitinib administered daily). Patients receiving non-continuous treatment responded better to the systemic therapy regarding PFS (arm 1: 4.8 [2.9–8.1] months; arm 2: 2.9 [2.9–5.1] months), while the median OS (arm 1: 13.6 [7.0–23.2] months; arm 2: 13.7 [8.4–25.6] months) as well as the pattern of adverse events did not differ significantly (Baumann et al. 2012) (Table 7). So far, no phase III trial on sunitinib has been initiated so far.

Other Anti-angiogenic Treatment Strategies

Trebananib

The peptibody trebananib (AMG386) blocks the connection of the angiopoietins-1 (Ang1) and angiopoietins-2 (Ang2) to the Tie2 receptor and therefore addresses a VEGF-independent, parallel anti-angiogenic pathway. Ang1 and Ang2 are expressed on endothelial cells; Ang1 promotes vessel stabilization by increasing endothelial junctions and pericyte coverage; Ang2 blocks Ang1's blood vessel stabilizing action, increasing angiogenesis and vascularity in tumors.

Following promising phase II trials (Karlan et al. 2012), trebananib was investigated for recurrent ovarian cancer in the international, double-blind phase III TRINOVA-1 trial in which weekly paclitaxel 80 mg/m^2 was applied with trebananib 15 mg/kg i.v. weekly or placebo (Monk et al. 2014) (Table 8). In this trial 919 patients with recurrent ovarian cancer, a platinum-free interval $<$12 months and $\leq$3 prior therapies, were included. The trebananib arm had a significantly improved median PFS by 2.8 months (7.2 vs. 5.4 months; hazard ratio [HR] 0.66, 95% CI 0.57–0.77, $<$0.001) (Monk et al. 2014). Thus, the study met the primary endpoint, although no improvement in OS was noted (19.3 vs. 18.3 months, 0.95 95% CI, 0.81–1.11) (Monk et al. 2015). Compared to bevacizumab, a different profile of adverse events was noted. In general, treatment was well tolerated with reported edema, ascites, and pleural effusions but less traditional VEGF-associated effects (hypertension, proteinuria, thromboembolic events). An envisaged study for patients with platinum-sensitive recurrent disease has not been completed so far. For first-line setting, a double-blind, phase III trial comparing chemotherapy of carboplatin/paclitaxel with trebananib in combination with chemotherapy followed by a subsequent weekly trebananib maintenance therapy versus placebo (AGO-OVAR 18, TRINOVA-3) has completed recruitment and is currently under follow-up.

Table 8 Overview of ovarian cancer trials with trebananib

Author	Study design	n (rel.)	Results PFS median	Results OS median	Further aspects
Phase III – recurrent disease					
TRINOVA-1 (Monk et al. 2014)	Paclitaxel 80 mg/m^2 q1w + trebananib 15 mg/kg q1w versus paclitaxel 80 mg/m^2 q1w + placebo q1w	919 (1:1)	7.2 months versus 5.4 months	19.3 months versus 18.3 months	Recurrent ovarian cancer with <12 months platinum-free interval
			HR 0.66 (95% CI 0.57–0.77; $p < 0.001$)	HR 0.95 (95% CI, 0.81–1.11; $p = 0.52$)	
Phase II – recurrent disease					
Karlan et al. (2012)	Trebananib 10 mg/kg + paclitaxel 80 mg/m^2 q1w versus trebananib 3 mg/kg + paclitaxel 80 mg/m^2 q1w versus placebo + paclitaxel 80 mg/m^2 q1w	161 (1:1:1)	7.2 months (95% CI, 5.3–8.1) versus 5.7 months (95% CI, 4.6–8.0) versus 4.6 months (95% CI, 1.9–6.7)	–	Overall response rate (ORR): 37% versus 19% versus 27%

Future Directions

Although anti-angiogenic treatment has been the first targeted approach approved and included in guidelines for ovarian cancer patients, several questions for planning and execution of this treatment strategy remain unanswered.

So far, reliable tests and predictors which may predict response to the anti-angiogenic agents are lacking. Patients benefiting the most from a specific treatment regimen need to be identified to avoid unnecessary toxicity, deteriorated quality of life of nonresponding patients, as well as costs for the healthcare providing system. This needs to be addressed more specifically and ideally prospectively in future trials.

Especially regarding maintenance therapies, patient-reported outcomes to assess the overall quality of life as well as interpretation of significant adverse events will become progressively relevant.

Other strategies focus on the identification of other effective combinations with further targeted therapies to overcome resistance toward anti-angiogenic drugs during the clinical course. Novel combinations appear to have promising potential to improve treatment effects for ovarian cancer patients.

Additional Combination Partners: PARP Inhibitors

In this context, the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib as combination partner for anti-angiogenic therapies seems to be a promising new option. In a randomized phase II trial, the combination of cediranib 30 mg daily and the olaparib 200 mg BID versus olaparib 400 mg BID alone has been tested in 90 women with recurrent platinum-sensitive ovarian cancer and a deleterious germline BRCA1 or BRCA2 mutation. The chemotherapy-free experimental arm of cediranib and olaparib significantly improved PFS from 9.0 to 17.7 months (HR 0.42, 95% CI 0.23–0.76, $p = 0.005$), while OS data is not mature yet (Liu et al. 2014). Drug-related adverse events were more common in the cediranib plus olaparib arm (70% of patients with grade 3 or higher event) than in olaparib monotherapy (11%) so that further envisaged phase III studies need to account for tolerability of this novel combination (Liu et al. 2014). The international randomized phase III PAOLA-1 trial was recently initiated by the French Groupe d'Investigateurs Nationaux pour l'Étude des Cancers Ovariens (GINECO) to investigate the combination of chemotherapy, anti-angiogenic therapy, and PARP inhibitors in the first-line setting for ovarian cancer patients.

Accounting for the approval status in Europe, bevacizumab instead of cediranib was chosen for combination with olaparib- and platinum-based chemotherapy.

For recurrent disease, the AGO-OVAR 2.28 phase III study is in preparation, which will also examine the combination of anti-angiogenic and anti-PARP treatment.

Additional Combination Partners: Immunotherapies

Over the past years, immunotherapy has become another very interesting treatment strategy in cancer therapy. Programmed death-1 (PD-1) is a co-inhibitory immune signal receptor expressed on T cells which binds to PD-1 ligand (PD-L1) which are both key regulators for antitumor immunity. For some tumor entities like metastatic malignant melanoma or lung cancer, convincing data on efficacy could be demonstrated with significant prognostic improvements and durable remissions for some of these patients. For ovarian cancer, so far encouraging efficacy data of anti-PD-1 and anti-PDL-1 from small, early-phase II trials is available, but reliable data from larger phase II/III is still lacking. However, preclinical and early-phase trial results suggest a possible option to combine anti-PD-L1 or PD-1 agents with chemotherapy and bevacizumab (De Felice et al. 2015; Monk et al. 2016). Synergistic effects of VEGF signaling could be an increase antitumor T cells like CD8+ and CD4+ central memory cells and a consecutive decrease of pro-tumor immune populations like myeloid-derived suppressor cells and regulatory T cells (Kusmartsev et al. 2008; Monk et al. 2016).

This has been the rationale for preparation of clinical, randomized trials in which anti-angiogenic agents and immune checkpoint inhibitors are combined. One example will be the randomized phase III ATALANTE trial comparing the PD-L1 antibody atezolizumab versus placebo in addition to a platinum-based combination chemotherapy and bevacizumab in recurrent, platinum-sensitive ovarian cancer patients.

Conclusion

Following the results of clinical trials on anti-angiogenic therapies over the past years, this treatment approach has become well established in ovarian cancer therapy. According to the approval of bevacizumab for different indications in Europe, almost all patients with epithelial ovarian cancer will receive an anti-angiogenic agent over their clinical course. However, an increase in cure rates of the disease could not be achieved; so far patients' benefits solely concentrate on progression-free survival. Despite, further, promising results for other anti-angiogenic compounds including multi-kinase inhibitors, the efficacy still appears modest without significant impact on overall survival and concurrent considerable toxicity. In this context, future perspectives focusing on predictive biomarkers and combination strategies with other interesting drugs generate optimism.

Identifying agents with a well-tolerated dosage and dosing schedule, optimal combination partners, and a selection process for patients with expected high response rates will be major aims for future investigations in ovarian cancer.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Combination of Anti-angiogenics and Other Targeted Therapies
- ▶ Cytotoxics and Anti-angiogenics: Metronomic Therapies
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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The Value of Anti-angiogenics in Cervical Cancer Therapy

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Contents

Introduction 546
Screening for Cervical Cancer 546
Preinvasive Changes 546
Risk Factors for Cervical Cancer 547
Treatment Options 547
Angiogenesis in Cervical Cancer 548

Clinical Relevance of Anti-angiogenics in Cervical Cancer 552
Bevacizumab (Avastin®) 553
Other Anti-angiogenic Drugs 558

Future Directions 560
VEGF-Pathway 560
Other Angiogenic and Non-angiogenic Mechanisms 560

Conclusion 561

Cross-References 562

References 562

Abstract

Uterine cervical cancer is the most common gynecologic malignancy in women worldwide in both prevalence and mortality. In 2012 an estimated 527,600 cancer cases and 265,700 related deaths were registered. Over 99% of cervical carcinomas are caused by the human papillomavirus (HPV).Different components of the HPV, especially E6 and E7 oncoproteins, are responsible for the early transformation of the stratified squamous epithelia of the cervix and subsequent carcinogenesis. Concurrently, these translated oncoproteins promote angiogenesis in cervical cancer. The unique characteristic of HPV-driven angiogenesis is the early onset of the angiogenic switch in carcinogenesis. As this mechanism underlies the vascular endothelial growth factor (VEGF-) pathway, anti-angiogenic therapeutics targeting VEGF or its transmembrane tyrosine kinase receptor are of increasing interest.

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Until 2014 bevacizumab was investigated in a phase III trial and proved significant efficacy without simultaneous alarming toxicity. Bevacizumab was approved by the US Food and Drug Administration in the therapy of persistent, recurrent, or metastatic cervical cancer in combination with cisplatin or topotecan and paclitaxel. This outstanding innovation paved the way for further anti-angiogenic drugs. Cediranib, pazopanib, and sunitinib have recently undergone phase I and II trials with variable success. However, with the introduction of Bevacizumab to cervical cancer therapy and the knowledge that angiogenic mechanisms appear much earlier in cervical cancer than most other entities because of HPV infection, cervical cancer therapy has taken a new direction.

Keywords
Cervix uteri · Cervical cancer · HPV infection · HPV oncoproteins · Angiogenesis · Angiogenesis inhibitors · Anti-angiogenic therapy · VEGF inhibitor · Bevacizumab · Tyrosine kinase inhibitors · Cediranib · Pazopanib · Sunitinib

Introduction

Cervical cancer is the third most common gynecologic malignancy in the United States as well as in other developed countries (Ferlay et al. 2015). Prevalence is higher in the developing countries, where women have no or limited access to screening programs and the vaccination against the human papillomavirus (HPV). Thanks to the increased use of vaccination and detection of early disease stages, incidence and mortality rates have declined (Quinn et al. 1999). Further reduction of the prevalence is expected as the effect of the vaccination of adolescents comes into action.

At the time of the diagnosis, the majority of patients are between 40 and 59 years of age. The median age of affected patients is 53 years and has gone down by 15 years in the last 25 years (RKI Krebs in Deutschland 2009/2010). However, the mean age of patients with a premalignant disease is only 34 years (RKI. Krebs in Deutschland 2009/2010 2013). In the years 2009 and 2010, most patients were diagnosed in stage T1 (62%) but 14% in stage T3 or T4 (RKI. Krebs in Deutschland 2009/2010 2013).

Screening for Cervical Cancer

Breast cancer aside, cervical cancer is the only entity of gynecologic malignancies for which screening programs are available. This includes regular cytology smears of the cervix (Papanicolaou [PAP] test) and testing for high-risk types of HPV. It is reliable and valid both for squamous cell carcinomas and adenocarcinomas. The benefits of the screening are large and established beyond doubt as early stages of cervical cancer are often asymptomatic. Symptoms of cervical cancer include irregular bleeding, vaginal discharge, and bleeding after intercourse. In advanced stages, patients may present with hydronephrosis due to ureteral strictures or distant metastases.

The screening initiation recommendations vary between countries. It is generally between 20 and 25 years, in the United States 21 years. The time of the first sexual intercourse is usually not relevant (Wilt et al. 2015). The intervals between subsequent screenings of asymptomatic patients may be stretched to up to 3 or even 5 years. Here, again, guidelines vary between developed countries. In asymptomatic patients, screening may be stopped at 65 years of age. However, screening in immunocompromised women (e.g., HIV infection), where clearance rates of HPV may be lower, is recommended every year (Madeddu et al. 2014).

Preinvasive Changes

The development of cervical cancer via preinvasive changes is well understood. As a prerequisite, in $> 99\%$ of cases, there is an infection with HPV at the cervical epithelium at the transformation zone (Schiffman et al. 2007; Walboomers

et al. 1999). This may persist or be transient, not causing alterations in the epithelium. In the case of persistence, preinvasive changes occur first. Invasive carcinoma may follow. Generally, one can assume a time period of approximately 15 years between the HPV infection and the development of invasive cancer (Collins et al. 2006). The prevalence of preinvasive changes is higher than that of cervical cancer but is expected to decrease thanks to the effect of vaccination (van Kriekinge et al. 2014).

Risk Factors for Cervical Cancer

A number of risk factors have been identified for the development of cervical cancer. The most important one is the infection with HPV, in particular the high-risk types 16 and 18. However, the infection persists only in 5–10% of women, and only about 3% of those infected develop cervical cancer (Schiffman et al. 2011). HPV also contributes to the development of other cancers including (but not limited to) anal and vulvar cancer (Forman et al. 2012).

Cervical intraepithelial neoplasias are a frequent precondition of uterine cervical cancer triggered by HPV infection. These cellular changes are known to show a high risk for malignant transformation. Therefore, preventive medical examinations are conducted in form of regular PAP smears, followed by a colposcopy and a biopsy. In case of an intraepithelial neoplasia, a cervical excision or ablation is performed. If the margins are not clear, a hysterectomy may be recommended. After uterine-preserving therapy, patients should be followed up by routine PAP smears. Further risk factors include smoking (>15/d), immune suppression (drug induced or HIV), early start of cohabitation, frequent changes of sexual partners, other genital infections, and low socioeconomic status (Berrington de González and Green 2007). Interestingly, the use of combined oral contraceptives plays a role as well, obviously by changing the environment (Appleby et al. 2007). However, this observation may be a confounder. After stopping contraceptives, the risk is reduced to normal (Appleby et al. 2007).

Treatment Options

The staging of cervical cancer according to FIGO (Fédération Internationale de Gynécologie et d'Obstétrique) is a clinical procedure, not including the involvement of lymph nodes. MRI of the pelvis is recommended in order to determine the exact size and location of the tumor. Further, evaluation and excision of pelvic and para-aortic lymph nodes mainly by laparoscopy are crucial for planning the further therapy. Options for treatment of cervical cancer include surgical therapy and/or radiochemotherapy in the curative situation, depending on the stage of the tumor. In an early stage cervical cancer, modified radical hysterectomy and pelvic lymphonodectomy are recommended.

Early stages include the FIGO stages IA and IB1, defined as <4 cm in diameter. Today, surgeries are mainly performed either by laparoscopy or robotic surgery. In certain cases, fertility-sparing therapy (trachelectomy) may be an option (Plante 2013). In high-risk situations (positive surgical margins, pathologically confirmed involvement of the pelvic lymph nodes, microscopic involvement of the parametrium), adjuvant radiotherapy accompanied by administration of cisplatin is recommended (Monk et al. 2005). Patients with advanced stages of cervical cancer are mainly treated with radiochemotherapy considering the high risk of recurrence after the surgery. Furthermore, surgery plus radiotherapy is likely to cause severe morbidity.

A small portion of patients present with metastatic disease at the time of initial diagnosis. For systemic therapy, platinum-based regimens are used, possibly combined with paclitaxel. As mentioned above, the majority of cases can be detected at an early stage with a good prognosis. However, in patients with advanced stages, effects on the health-related quality of life are extensive. One must differentiate between locoregional problems, including bleeding from the tumor and fistulas. Bleeding from the tumor may require tamponades or embolization of the tumor. On the other hand, there may be distant metastases, mainly in the lungs. In these situations, anti-angiogenic substances are part of the

treatment options which will be further discussed in this chapter.

Angiogenesis in Cervical Cancer

During the process of carcinogenesis, tumor growth, invasion, and metastasis, angiogenesis is essential for guaranteeing the availability of nutrients and oxygen (Hanahan and Weinberg 2000; Kerbel 2008). Therefore, all neoplastic cells need to develop the ability of driving independent angiogenesis. It has been proven that hypoxia or low tissue oxygen levels in general are the major conditions leading to the formation of new blood vessels in many solid neoplasias including uterine cervical neoplasm (Brat et al. 2003; Ravi et al. 1999).

Like in other cancer types, cervical cancer runs a molecular pathologic mechanism between the hypoxic condition and final angiogenesis comprising the activity of several angiogenic agents. Among these agents, tumor suppressor p53, hypoxia-inducible factor 1α (HIF-1α), retinoblastoma protein (pRb), and especially vascular endothelial growth factor (VEGF) play a key role (Alldredge and Tewari 2016).

Importantly, cervical epithelium exhibits one unique feature, namely, HPV-induced tumor angiogenesis. After HPV infection, it shows HIF-1α stabilization, elevated VEGF expression, and increased microvessel density (MVD) already in early carcinogenesis and also in normal stratified cervical epithelia as signs of chronic hypoxia of the tissue (Smith-McCune et al. 1997; Smith-McCune and Weidner 1994). In other cancer types, angiogenesis usually occurs later as the disease progresses (Krill and Tewari 2015). Additionally, a variety of trials conducted in the last three decades demonstrated that HPV infection modulates the VEGF pathway by influencing single agents involved.

Since HPV infection represents the origin in the vast majority of cervical cancers and additionally triggers angiogenic mechanisms in early stages of the disease, focusing the underlying pathologic pathway and the role of HPV in it is worth consideration. This helps determine the value of possible anti-angiogenic therapies.

In cervical cancer, there are two well-described external triggers of pathologic angiogenesis. Hypoxia represents one of these elicitors. In general, during tumor growth as well as the formation of metastases, neoplastic cells inevitably sustain external hypoxic stress leading to the generation of an angiogenic phenotype. This step in turn provides the ultimate basis for the formation of new blood vessels in order to guarantee tumor cell survival and therefore cancer progression (Ravi et al. 1999).

Like in most other malignant diseases, in cervical cancer, the central mechanism of the subsequent angiogenic cascade is the VEGF pathway that is potentiated and primarily triggered by the upregulation of the transcription factor HIF-1α (Nakamura et al. 2009). Furthermore, the downregulation of the tumor suppressor p53 has also been reported to favor proangiogenic VEGF alterations under hypoxic conditions (Ravi et al. 1999). VEGF is a signaling molecule activating angiogenesis when binding to its receptor tyrosine kinase VEGFR, since VEGF mainly stimulates its receptor on endothelial cells. This leads to the proliferation of cells with vascular endothelial differentiation. The VEGF pathway, however, is not specific to cervical cancer and has been proven principle of tumor genesis, tumor progression, and metastasis in general.

Nevertheless, there are few unique characteristics of these mechanisms in uterine cervical cancer, described in the following paragraphs. Figure 1 shows the general and cervical cancer-specific pathologic pathways of angiogenesis.

Cervical cancer has been reported to be strongly induced by high-risk HPV infections (Bosch et al. 2002; Muñoz et al. 2003; Walboomers et al. 1999). Several oncogenic HPV subtypes could be successfully identified by Nobel laureate zur Hausen et al. in the 1980s (Dürst et al. 1987). Especially the high-risk HPV subtypes 16 and 18 favor the development of cervical intraepithelial neoplasia and therefore facilitate invasive cancer of the uterine cervix (Muñoz et al. 2003). It is estimated that 10% of the women carrying an oncogenic HPV subtype are likely to develop precancerous lesions and that among these 8% progress into noninvasive

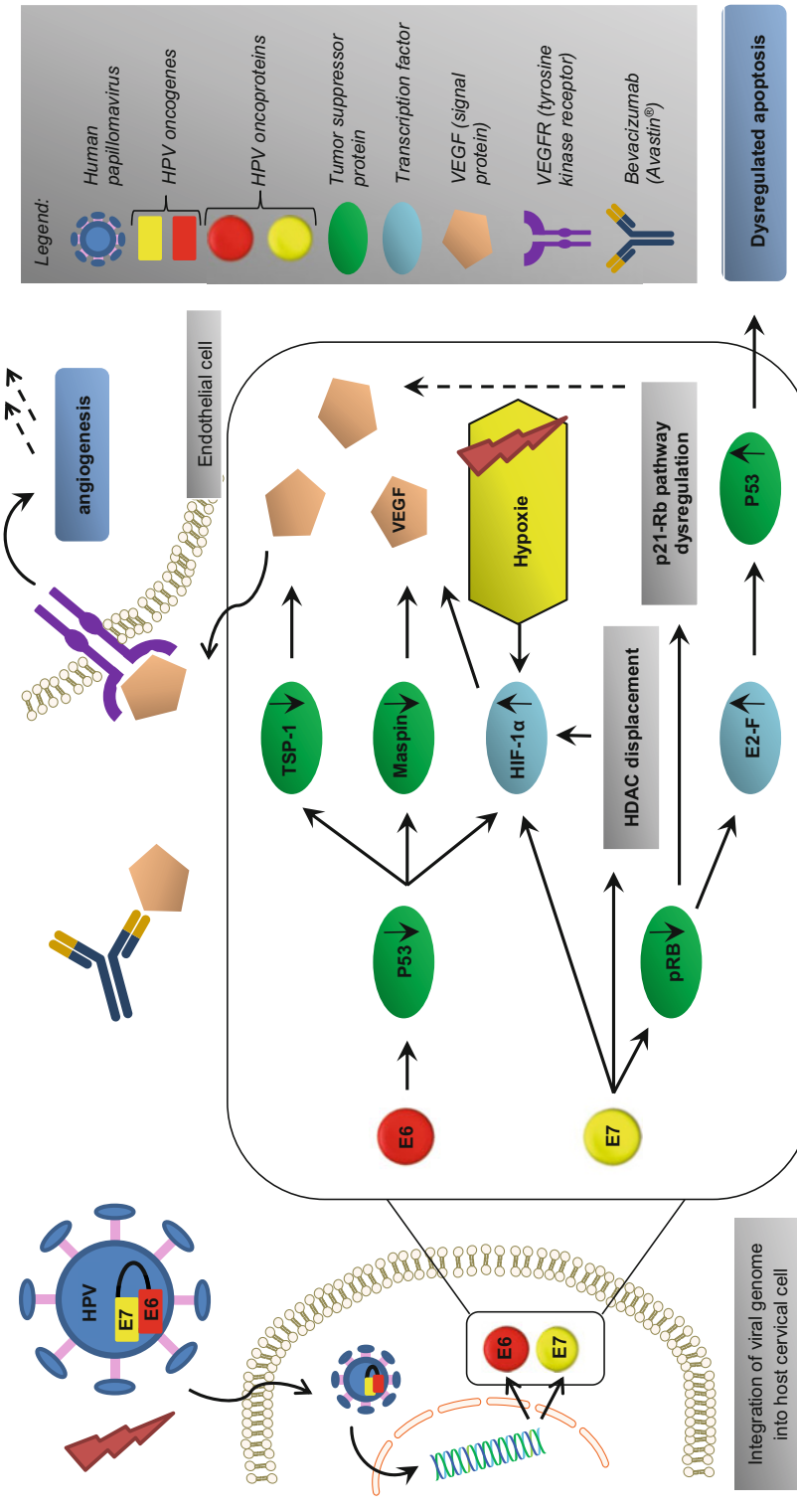

Fig. 1 VEGF-angiogenic pathway in cervical cancer and its alterations after HPV-infection

carcinoma in situ of the cervix. However, those patients who eventually develop invasive disease exhibit HPV infection in 99.7% (Frazer 2014; Yim and Park 2005). It is evident that HPV occupies an essential role in the formation of uterine cervical cancer. HPV belongs to the subgroup of DNA viruses that tend to infect keratinocytes of the human body (Zur Hausen 1999). Its genome consists of eight genes (HPV E1-E8) in form of double-stranded circular DNA. Among these, HPV E6 and HPV E7 encode oncoproteins which are the primary causes of malignant transformation of cervical cells. They have pleiotropic functions and are responsible for the generation of a malignant phenotype by becoming independent from tumor controlling cellular signals and by developing the capability of self-controlled angiogenesis (Yim and Park 2005). After an infection with the two high-risk HPV subtypes 16 and 18, E6 and E7 oncogenes and their products are steadily present in inconspicuous uterine cervical cells (von Knebel Doeberitz et al. 1994). Their steady influence triggers the dysregulation of the genome of cervical host cells which paves the way for the integration of the viral oncogenes into the hosting cell's genome (Melsheimer 2004). It is suggested that persistent infection and herewith continuous expression of E6 and E7 are crucial to the transformation of normal epithelial into malignant epithelial cervical cells.

With the influence of their gene products, among other things disturbance of cell cycle regulation and induction of multiple mitotic aberrations take place (Mantovani and Banks 2001). As a result, chromosomal instability arises in form of aneuploidization leading to increasingly profound structural changes of cervical epithelial stem cells (Duensing et al. 2000; Duensing and Münger 2002; Zur Hausen 1999). Subsequently, it is possible that the HPV genome is integrated into the genome of the hosting cells. In the next steps, viral oncogenes are transcribed regularly, in part because the intrinsic viral repressors of gene expression encoded by HPV E2 are switched off (Baker et al. 1987; Romanczuk and Howley 1992). Two severe changes take place: firstly, increasing tumor growth mediated by integrate-derived papillomavirus oncogene transcripts (iPOTS) and, secondly, the uncontrolled formation of new vessels due to enhanced E6 and E7 oncoprotein expression (Alldredge and Tewari 2016; Melsheimer 2004). Resulting, the pathologic mechanism behind HPV-induced uterine cervical cancer not only consists of dysregulated tumor cell proliferation.

HPV infection is also closely related to pathologic angiogenesis in the development of this disease. The reported infection rates and the molecular pathologic relation between HPV genome and angiogenesis make it indispensable to know the detailed pathway in between.

More precisely, the influence of E6 and E7 oncoproteins on angiogenesis comprises alterations of several angiogenic agents as well as direct impact on the VEGF pathway (Krill and Tewari 2015). To simplify this mechanism, it can be divided into three steps: first, direct modification of p53 and pRb by external stressors; second, subsequent alteration of expression levels of the direct VEGF targeting proteins HIF-1α, Thrombospondin-1 (TSP-1), and Maspin, and finally VEGF upregulation (Kodama et al. 2001). Both HPV E6 and E7 oncoproteins are able to influence the tumor suppressor gene p53 directly or indirectly. As a result, they affect the VEGF pathway and angiogenesis. Interestingly, their impact on p53 shows opposite effects: E6 leads to a downregulation, whereas E7 leads to an upregulation of the tumor suppressor gene (Bodily et al. 2011; Nakamura et al. 2009).

After HPV infection, the expression of the oncoprotein E6 leads to p53 degradation in the hosting cervical cells (Scheffner et al. 1990; Lopez-Ocejo et al. 2000). p53 is a tumor suppressive protein encoded by the tumor suppressor gene p53. The function of this protein is DNA repair and initiation of cell cycle arrest as well as apoptosis. Additionally, it plays an important role in the formation of new blood vessels. In the downstream angiogenic mechanism, p53 is linked to TSP-1 and Maspin. TSP-1 is a multifunctional glycoprotein secreted by different cell types including tumor cells. As an angiogenesis inhibitor, it takes a significant role in the angiogenic switch of tumor tissue (Kodama et al. 2001; Wu

et al. 2004). Maspin is another angiogenesis suppressor and part of the angiogenic pathway in cervical cancer (Liu et al. 2014).

Under normal cellular condition, the p53 protein is unstable, and subsequently TSP-1 expression and VEGF-driven angiogenesis are not activated. In case of an incidental DNA defect, p53 is stabilized and TSP-1 is increased. As a consequence, elevated p53 expression levels initiate cell cycle arrest, and TSP-1 upregulation leads to inhibition of angiogenesis. However, under pathologic conditions like hypoxia and HPV infection, p53 is degraded and cannot exhibit its function in cell cycle arrest and DNA repair (Bodily et al. 2011; Münger et al. 2004). This is achieved by the generation of a trimeric structure between E6, p53, and the ubiquitination enzyme E6-AP causing proteolysis of the ubiquitinylized p53-protein (Yim and Park 2005). Furthermore, TSP-1 and Maspin expression levels are low in cervical cells with E6 and E7 expression. On the contrary, VEGF expression is augmented (Krill and Tewari 2015). A third angiogenic promoter regulated by p53 is HIF-1α. E6 expression leads to HIF-1α upregulation and subsequent angiogenesis via degradation of p53 (Ravi et al. 1999).

To sum up, these effects enable endothelial cells to replicate in an uncontrolled manner and lead to a loss of differentiation. Due to the degradation of p53 in uterine cervical cells and the subsequent malfunctioning cell cycle arrest and the DNA repair as well as apoptosis, E6 is responsible for an uncontrolled tumor cell proliferation and eventually for angiogenic switch in tumor tissue. In contrast, HPV E7 oncoprotein stimulates VEGF via two alternative pathways. On the one hand, it indirectly boosts p53 activity by the inactivation of pRB which in turn culminates in dysregulated apoptosis (Toussaint-Smith et al. 2004). pRB plays a major role in the repression of replication enzyme genes. It is a tumor suppressor that binds to several transcription factors of the E2-F-family. E2-F transcription factors in their active form are responsible for initiating transcription and therefore finally lead to cell proliferation. Herein lies the tumor suppressive activity of pRb, as it favors the inactive form of E2-F by binding it. HPV E7, however, binds to pRb and forms an inactive complex. E2-F is enabled to exist in its active form contributing to increasing gene transcriptions, among which p53 is strongly represented (Chellappan et al. 1992; Lipinski and Jacks 1999). Interestingly, HPV E7 expression in cervical cells leads to an upregulated effect on the p53 levels compared to HPV E6 expression which degrades p53. Thus, both reactions eventually lead to one common cellular response, namely, angiogenesis. This originates from the fact that p53 unfolds its impact concentration dependent. In case of its degradation, it leads to an angiogenic switch via upregulation of VEGF. However, in case of increased p53 levels over a certain cutoff point, it induces apoptosis. Beyond that, the pRb inactivation and the subsequent p21-Rb pathway dysregulation are reported to enhance VEGF expression levels via other molecular pathways. These, however, are not completely delineated (Krill and Tewari 2015).

Another pathogenic characteristic of HPV E7 consists in its ability to influence HIF-1α activity (Tang et al. 2007). HIF-1α is a transcription factor that, under sufficient oxygen conditions, is hydroxylated and subsequently degraded via the van Hippel-Lindau/proteasomic pathway. Hypoxia inhibits its hydroxylation and degradation. As a result, HIF-1α accumulates and triggers the transcription of its target genes, including a range of proangiogenic factors (Bárdos and Ashcroft 2005; Nakamura et al. 2009). Moreover, histone deacetylases (HDACs) are crucial for the regulation of HIF-1α activity.

Under normal conditions, they bind to HIF-1α and inhibit its influence as angiogenic transcription factor. In case of any disruption of this linking, however, HIF-1α starts to unfold its impact on VEGF expression levels. After HPV E7 presentation to cervical cellular components, HIF-1α is activated because of the displacement of the histone deacetylases HDAC1, HDAC4, or HDAC7. This dislocation is due to a linking of HPV E7 to the HIF-1α inhibiting HDACs. As a consequence, HIF-1α is given the opportunity to influence the VEGF expression levels (Bodily et al. 2011).

The bottom line of all beforementioned HPV E6- and HPV E7-driven pathways is the

upregulation of VEGF expression levels. After its activation, downstream mechanisms follow the same principles like in other tumors, starting that VEGF proteins bind to their transmembrane receptor tyrosine kinases (Prewett et al. 1999). At present, five VEGF glycoproteins (VEGF A-E) and three VEGF receptors (VEGFR 1–3) have been identified (Karkkainen and Petrova 2000; Neufeld et al. 1999). VEGFR-2 turned out to be the leading mediator in angiogenesis, and VEGF A, VEGF C, and VEGF E show the ability to bind it (Hicklin and Ellis 2005). The subsequent downstream mechanism comprises dimerization and autophosphorylation of the receptor tyrosine kinase VEGFR-2. This activates several intracellular signal transduction molecules whose activity leads to gene expression and cell proliferation as well as cell survival and cell migration of endothelial cells. As a result, endothelial cells spread and form new blood vessels (Matsumoto and Claesson-Welsh 2001).

In summary, there are three well-investigated mechanisms that enhance angiogenesis under pathologic conditions in cervical cancer: the p53/TSP-1-pathway, the pRb/p21/p53-pathway, and the HIF-1α-pathway. All of them eventually lead to an elevated VGFR expression. However, it is the combination of uncontrolled cell proliferation and dysregulated apoptosis of VEGF-positive endothelial cells that contributes to the formation of new vessels in neoplastic tissue. The origin of these malignant alterations is E6 and E7 expression after HPV infection. It has been stated that only approximately 10% of all HPV infections progress into the premalignant conditions of the cervical tissue and even fewer of them into invasive cervical cancer. This suggests the existence of additional angiogenic agents and mechanisms that still need to be investigated. Among them, several proteins verified significant participation in recent studies. Still, the results are not consistent enough to clearly determine their role in angiogenesis and their possible value for anti-angiogenic therapy, but they should be known as elements of blood vessel formation in cervical cancer.

These findings theoretically illuminate the outstanding value of anti-angiogenic drugs in the therapy of uterine cervical neoplasms.

Clinical Relevance of Anti-angiogenics in Cervical Cancer

In the field of carcinogenesis and tumor progression, angiogenesis has been under investigation for decades. The discovery of its tremendous role in malignant transformation and tumor aggressiveness and the potential therapeutic benefit of the angiogenesis pathway inhibition have led to extensive investigation of the VEGF pathway and the development of anti-angiogenic therapies. Fumagillin and its analogon TNP-470 were the first substances which showed potential as inhibitors of this pathway. In several ensuing clinical trials, TNP-470 demonstrated promise as an anti-angiogenic agent in numerous tumor entities including colon, prostate, and breast cancer (Tanaka et al. 1995; Yamaoka et al. 1993). Trials for use in cervical cancer soon followed.

As mentioned before, despite sufficient screenings, diagnostics, and preventive vaccinations as new standard of care, especially women in resource-poor countries are still diagnosed with recurrent or metastatic disease. Additionally, heavily pretreated cervical cancer turned out to be comparatively refractory to chemotherapy, and women suffering recurrent disease after platinum-based chemoradiotherapy often have limited options only (Tewari and Monk 2014). Thus, the problem in cervical cancer therapy persists: early or locally advanced stages of the disease can be treated curatively with radical surgery and chemoradiation, whereas chemotherapy in women with metastatic and non-operable recurrent cervical cancer only has palliative intent. With evidence of HPV-triggered formation of cervical carcinomas and its role in the generation of blood vessels, uterine cervical cancer has demonstrated a dependency on angiogenesis for its development, growth, and malignancy. Therefore, TNP-470 was applied in a phase I trial of women with inoperable, recurrent, or metastatic cancer of the uterine cervix (Kudelka et al. 1997). Out of 18 patients, 1 patient went into complete remission, and in 3 patients the disease stabilized accompanied by moderate toxicity. When its data was published in 1997, the proof of significant anti-angiogenic efficacy led to further clinical

trials soon after. Since then, the long stagnant field of cervical cancer therapy has expanded in new directions.

Recently, various anti-angiogenic drugs are under investigation, and bevacizumab is the leading therapeutic in this field.

Bevacizumab (Avastin®)

Throughout the last decades, anti-angiogenics received attention and have broadened the field of targeted cancer therapy. Bevacizumab evolved as the central player among them. Clinical trials showed promising efficacy. Hence, it is included in the therapy protocols of many malignant diseases and has recently been approved as first-line therapy in the treatment of persistent, recurrent, and metastatic cervical carcinoma. Bevacizumab is a recombinant humanized monoclonal antibody which interacts with VEGF. It binds VEGF subtype A and thus inhibits signal transduction conferred by the two VEGF-receptor subtypes 1 and 2 located inside the cell wall of endothelial cells (Diaz-Padilla et al. 2013; U.S. Food and Drug Administration 2013). After the success of TNP-470 in uterine cervical cancer and first phase II trials of bevacizumab in various malignant diseases, a first retrospective analysis of its use in women suffering from advanced stage cervical cancer revealed possible efficacy (Wright et al. 2006). This study reviewed six cases and reported a clinical benefit of 67% as well as good tolerability.

In 2009, the Gynecology Oncology Group (GOG) started the first phase II trial GOG 227C with bevacizumab as monotherapy in advanced cervical cancer which proved its pharmacologic activity and acceptable safety with only manageable adverse events (Monk et al. 2009). Forty-six patients with persistent or recurrent disease were included. The primary outcome showed that the median progression-free survival (PFS) was 3.4 months (95% CI, 2.53–4.53 months). Secondarily, the study stated that median overall survival (OS) was 7.29 months (95% CI, 6.11–10.41 months). Noteworthy adverse events were those reported in previous studies: hypertension and other cardiovascular problems, thromboembolism, anemia, and gastrointestinal fistulas. Two more phase II clinical trials followed that also demonstrated the efficacy of bevacizumab in women with advanced cervical cancer. However, they reported different outcomes in terms of toxicity. Zighelboim et al. stated that in 27 investigated patients, the addition of bevacizumab to cisplatin and topotecan was effective but highly toxic (Zighelboim et al. 2013).

On the other hand, under the auspices of Schefter et al., the Radiation Therapy Oncology Group (RTOG) conducted a trial in which bevacizumab was combined with chemo- and radiotherapy in 49 patients with cervical cancer stages IB–IIIB. They found the combination to be effective and the toxicity acceptable (Schefter et al. 2014). Up until these two phase II trials, anti-angiogenic therapies only had experimental relevance in cervical as well as any other gynecologic malignancy.

In 2014, Tewari and colleagues published data based on a phase III trial investigating the effect of bevacizumab in combination with established chemotherapy in patients with persistent, recurrent, or metastatic squamous cell carcinoma, adenocarcinoma, or adenosquamous carcinoma of the cervix. This GOG study (GOG 240) compared the two therapeutic regimens cisplatin, paclitaxel, and bevacizumab and topotecan, paclitaxel, and bevacizumab with both chemotherapy regimens alone. They elucidated that bevacizumab in combination with cytotoxic therapy had a statistically significant therapeutic effect. This study was a hallmark in cervical cancer therapy, and it became the admission study of bevacizumab for the National Comprehensive Cancer Network (NCCN) that listed the Bevacizumab regimen as category 1 in their practice guidelines for cervical cancer ("Based upon high level evidence, there is uniform NCCN consensus that the intervention is appropriate") (National Comprehensive Cancer Network 2012). Subsequently, the US Food and Drug Administration approved the indication for bevacizumab in combination with chemotherapy in persistent, recurrent, or metastatic cervical cancer on August 14, 2014.

The GOG 240 study became the approval study because it proved the efficacy of bevacizumab in a sufficient number of patients. It enrolled 452 patients and demonstrated that the incorporation of bevacizumab into approved cytostatic regimens, for both the cisplatin or topotecan regimens, improved OS and PFS as well as response rates. The median OS advantage of the bevacizumab regimen compared to chemotherapy alone was 3.7 months (17 months vs. 13.3 months; HR for death, 0.71; 98% CI, 0.54–0.95); median PFS could be extended to 8.2 months compared to 5.9 without bevacizumab (8.2 vs. 5.9 months; HR for disease progression, 0.67; 95% CI, 0.54–0.82). The median response rate increased to 48% compared to 36% without anti-angiogenic drugs (relative probability of a response, 1.35; 95% CI, 1.08–1.68; $P = 0.008$, two-sided test) (Tewari et al. 2014). These results represent the first significant improvement of overall survival in any gynecologic malignancy after the addition of molecular targeted therapy (Alldredge and Tewari 2016). Additionally, both regimens showed efficacy in recurrent disease with prior pelvic radiotherapy and in women after cisplatin-based chemotherapy (Tewari et al. 2014). These two disease situations were previously treated with very limited success due to increasing chemoresistance. Moreover, comparing the outcome of the two different chemotherapy regimens, the results displayed no significant difference in both OS and response rates. However, the topotecan-paclitaxel regimen was associated with a significantly higher risk of progression (Tewari et al. 2014). The same study found that after adding bevacizumab to the preexistent therapy of advanced cervical cancer, there were neither unmanageable adverse events nor significant deterioration of patient-reported outcomes in either of the regimens.

As a result, the FDA extended its indications for bevacizumab usage to persistent, recurrent, and metastatic cervical carcinoma in combination with one of the two following cytostatic regimens: cisplatin and paclitaxel or topotecan and paclitaxel. In their drug information, they recommend a dosage of bevacizumab of 15 mg/kg body weight every 3 weeks as intravenous infusion (U.S. Food and Drug Administration 2013). Including the approved chemotherapeutics, two possible therapy protocols have been adopted as demonstrated in Tables 1 and 2.

The prescription information contains notable warnings and precautions. Many are based on the adverse events reported in the GOG 240 study. Other precautions derive from observations during bevacizumab treatment in other tumor entities. The most important side effects attributed to bevacizumab are gastrointestinal perforations, gastrointestinal and urogenital fistula, venous thromboembolic events, and hypertension.

Most importantly, gastrointestinal perforations and fistulas have been reported in women suffering persistent, advanced, or metastatic cervical cancer after bevacizumab application. With an incidence of 3.2%, cervical cancer patients are most likely across all investigated malignancies to develop gastrointestinal perforations (U.S. Food and Drug Administration 2013). Gastrointestinal fistulas occur even more often than perforation; 8.2% of the patients in the primary trial developed fistulas during and after bevacizumab therapy compared to 0.9% with the chemotherapy only treatment. These numbers are the highest regarding all approved indications. However, they must also be attributed to prior pelvic irradiation. Non-gastrointestinal fistulas are reported as well. In cervical cancer patients, vesical, vaginal, and other fistulas of the female genital tract are described but rare. Secondly, venous thromboembolic events (VTE) must be considered in patients with cervical cancer under bevacizumab therapy. VTE appeared in 10.6% of the treated women compared to 5.4% in patients with chemotherapy alone. Another common side effect of bevacizumab therapy is hypertension. In the phase III trial, 25% of patients had a hypertension severity of grade 2 and higher (Tewari et al. 2014). This makes it paramount to provide adequate blood pressure monitoring during and after bevacizumab application in order to react with appropriate antihypertensive therapy or even discontinue of Bevacizumab treatment in case of a hypertensive crisis. Reviewing all, there were two

Table 1 Chemotherapy protocol 1 for the treatment of persistent, recurrent, or metastatic cervical cancer approved by FDA as first-line therapy Bevacizumab + Cisplatin + Paclitaxel

Week 1	Day 1	Bevacizumab	15 mg/kg	i.v.	30–90 min
		Cisplatin	50 mg/m^2	i.v.	3 h or 24 h
		Paclitaxel	175 or 135 mg/m^2	i.v.	60 min
	Day 2	Here alternative Cisplatin application possible			
	Day 3	None			
	Day 4	None			
	Day 5	None			
	Day 6	None			
	Day 7	None			
Week 2	No application of chemotherapy				
Week 3	No application of chemotherapy				
Week 4	Day 1	Bevacizumab	15 mg/kg	i.v.	30–90 min
		Cisplatin	50 mg/m^2	i.v.	3 h or 24 h
		Paclitaxel	175 or 135 mg/m^2	i.v.	60 min
	Day 2	Here alternative Cisplatin application possible			
	Day 3	None			
	Day 4	None			
	Day 5	None			
	Day 6	None			
	Day 7	None			
Following	Chemotherapy application according to this protocol for maximum of 6 cycles or until disease progression or unacceptable toxicity				

Table 2 Chemotherapy protocol 2 for the treatment of persistent, recurrent, or metastatic cervical cancer approved by FDA as first-line therapy Bevacizumab + Topotecan + Paclitaxel

Week 1	Day 1	Bevacizumab	15 mg/kg	i.v.	30–90 min
		Topotecan	0,75 mg/m^2	i.v.	3 h
		Paclitaxel	175 mg/m^2	i.v.	30 min
	Day 2	Topotecan	0,75 mg/m^2	i.v.	3 h
	Day 3	Topotecan	0,75 mg/m^2	i.v.	3 h
	Day 4	None			
	Day 5	None			
	Day 6	None			
	Day 7	None			
Week 2	No application of chemotherapy				
Week 3	No application of chemotherapy				
Week 4	Day 1	Bevacizumab	15 mg/kg	i.v.	30–90 min
		Topotecan	0,75 mg/m^2	i.v.	3 h
		Paclitaxel	175 or 135 mg/m^2	i.v.	30 min
	Day 2	Topotecan	0,75 mg/m^2	i.v.	3 h
	Day 3	Topotecan	0,75 mg/m^2	i.v.	3 h
	Day 4				
	Day 5				
	Day 6				
	Day 7				
Following	Chemotherapy application according to this protocol for maximum of 6 cycles or until disease progression or unacceptable toxicity				

Table 3 List of adverse events (AE) under the treatment with chemotherapy plus Bevacizumab ($n = 218$) compared to chemotherapy alone ($n = 222$) and overall incidence of AE in chemotherapy plus bevacizumab regimen; subdivided according to frequency of occurrence; only AE with an incidence difference of ≥5% included

Adverse events	Incidence difference of AE (in %)	Overall AE incidence (in %)
AE of >10% increase		
Hypertension	23	29
Epistaxis	16	17
Weight loss	14	21
AE of >5% to 10% increase		
Hypomagnesemia	9	24
Hyponatremia	9	19
Headache	9	22
Loss of appetite	8	34
Infections of urinary tract	8	22
Hyperglycemia	7	26
Thrombosis (all)	7	10
Dysarthria	7	8
Lymphopenia	7	12
Anxiety	7	17
GI-fistulae	7	8
Proteinuria	7	10
Blood creatinine elevations	6	16
Anal fistulae	6	6
Neutropenia	6	12
Pelvic pain	6	14
AE of 5% increase		
Hypoalbuminemia	5	11
Fatigue	5	80
Infection	5	10
Stomatitis	5	15
Proctalgia	5	6
VET	5	10
AE decreasing under Bevacizumab		
Edema, peripheral	−7	15

Source: U.S. Food and Drug Administration (2013); Tewari et al. (2014)

elevated incidences of grade 3 or 4 adverse events in the bevacizumab group compared to the patients treated with chemotherapy alone: first, hypertension (11.5% vs. 0.5%) and second thrombosis (8.3% vs. 2.7%). However, the incorporation of bevacizumab into chemotherapy did not affect the incidence of fatal adverse reactions (U.S. Food and Drug Administration 2013). Additional adverse events that appeared related to bevacizumab treatment in other tumor types are postsurgical and wound healing complications, arterial thromboembolic events, hemorrhages of grade 3 and higher, posterior reversible encephalopathy syndrome, proteinuria, infusion reactions, and ovarian failure (U.S. Food and Drug Administration 2013). Although these did not primarily occur in patients with cervical cancer, they need to be considered. Table 3 provides a complete overview of all reported adverse events during or after bevacizumab application in women suffering persistent, recurrent, or metastatic cervical cancer. The FDA recommends immediate discontinuation of bevacizumab therapy in the case of any unmanageable gastrointestinal adverse event,

life-threatening VTE of grade four or higher, or a hypertensive crisis (U.S. Food and Drug Administration 2013).

Besides the manageable adverse events, another criterion should be incorporated into the evaluation of bevacizumab treatment in cervical cancer patients, namely, evaluation of quality of life (Qol). A downstream study of Penson et al. (2015) evaluated patient-reported outcomes after bevacizumab therapy in cervical cancer using three independent self-reported measures to determine a potential disruption in Qol.

In a cervical-specific functional assessment of cancer therapy, patient-reported scores confirmed that by adding bevacizumab to palliative chemotherapy, the health-related Qol remains unchanged (Cella 2015; Tewari et al. 2014). Furthermore, observations regarding neurotoxicity revealed that both bevacizumab regimens did not negatively affect neurotoxic symptoms compared to chemotherapy alone. Although neurotoxic symptoms were likely to increase during continuous therapy irrespective of the chemotherapeutics used, the trials revealed that the addition of bevacizumab to chemotherapy even showed the remarkable but insignificant tendency to develop less neurotoxic symptoms (Penson et al. 2015; Tewari et al. 2014). Finally, patient-reported scores from the brief pain inventory proved that both regimens are not associated to rising development or severity of pain (Penson et al. 2015).

Despite this satisfying combination of efficacy and manageable toxicity as well as maintenance of Qol, one thing needs to be kept in mind: the benefit of therapy containing bevacizumab and related quality of life depend highly on individual characteristics of each patient. First, quality of life at study entry is prognostic for survival (Penson et al. 2015). Second, age, performance status, race, squamous histologic type, prior platinum exposure, recurrent or persistent disease, and pelvic location of the target lesion represent individual prognostic factors (Tewari et al. 2014). Third and most questionable in the application of bevacizumab is whether women who are more symptomatic and in a less stable physical capacity will equally benefit from and tolerate this treatment. Since most of the women for whom a bevacizumab treatment could be considered suffer late stage cancer and underwent prior exhausting radiochemotherapy, this might be a problem for most of the patients. Focusing on these problems, Tewari et al. (2015) validated clinical prognostic factors that help stratify the risk and quantify the prognostic value of bevacizumab in the treatment of women suffering persistent, recurrent, or metastatic cancer of the cervix. The data for this study were collected from the GOG 240 trial.

According to this study, there are five factors, known as Moore criteria, which negatively affect the outcome after bevacizumab therapy: black race, a GOG performance status of 1, pelvic disease, prior treatment with platin-containing chemotherapeutics, and PFS of less than 1 year (Tewari et al. 2015). These five factors were pooled, and the three risk levels low-risk, medium-risk, and high-risk resulted, depending on how many factors were positive in each patient (0–1: risk is low, 2–3: risk is medium, 4–5: risk is high). After correlating the risk category with OS, Tewari et al. (2015) stated that women with medium- and high-risk status benefit the most from bevacizumab therapy. These findings are helpful for medical practice, since oncologists may use it as a tool to estimate the therapeutic risk and predict the individual benefit for each patient.

Summarizing, the present knowledge about bevacizumab makes it obvious that it defines the centerpiece of the anti-angiogenic treatment in cervical cancer. With its addition to chemotherapy, women suffering from persistent, recurrent, or metastatic disease are given the opportunity of survival prolongation in balance with acceptable adverse events and absence of Qol deterioration. Although these results justify the use of bevacizumab in advanced stages of cervical cancer, some guidelines rightfully criticize the low absolute advantage of survival accompanied by manageable but still increased morbidity. Moreover, the evaluation of the value of anti-angiogenic drugs in the therapy of cervical cancer must also comprise its practicability on a global scale. As explained previously, advanced cervical cancer stages mostly affect women without sufficient access to health care. While it has

proven efficacy, the therapeutic advantages of bevacizumab and its price point do not make its widespread use economically sound. This does not only account for developing countries but for the biggest part of the world, since the incremental cost-effectiveness ratio of US$ 155,000 per quality-adjusted life year is above the accepted limit in most countries (Cella 2015).

Other Anti-angiogenic Drugs

Beyond the success of bevacizumab, several other angiogenesis-inhibiting drugs were investigated as new targeted therapies for the management of cervical cancer. The demand for alternative VEGF and VEGF-receptor inhibitors for the treatment of women suffering persistent, recurrent or metastatic disease, in particular, dominates current clinical trials. However, the results of the analyzed therapeutics regarding activity, efficacy, and toxicity were of variable success. Of all of the potential anti-angiogenic drugs currently being studied, two substances stand out: cediranib and pazopanib. An overview of the present evidence of the anti-angiogenic therapy in cervical cancer is provided in Table 4.

Cediranib (Recentin®) is a multipotent tyrosine kinase inhibitor targeting VEGFR 1, VEGFR 2, and VEGFR 3 as well as c-KIT, which also plays a role in angiogenesis. Its ability to block the VEGF signaling pathway in endothelial cells and malignant tissue theoretically provides the opportunity to extend conventional chemotherapy in cervical cancer (Symonds et al. 2015). Recently, it demonstrated significant PFS and OS advantages in the treatment of recurrent ovarian cancer (Ledermann et al. 2016). These findings led to a randomized, controlled phase II clinical trial conducted by Symonds et al. (2015) which incorporated cediranib into the conventional chemotherapy of metastatic or locally recurrent cervical cancer. Sixty-nine patients were enrolled in this study. Compared to carboplatin and paclitaxel alone, PFS was 1.4 months longer (HR 0·58; 80% CI 0·40–0·85, one-sided $p = 0{\cdot}032$), and a cediranib-related elevation of the response rates could be secondarily observed. These advantages, however, came at the cost of quality of life as well as physical performance of the patients because of the significant toxicity. Among the many adverse events, febrile and afebrile neutropenia, diarrhea, and hypertension presented most often during the treatment. Cediranib application also resulted in decreased soluble VEGF-receptor concentrations in the plasma of treated women, which monitors and proves the effect of cediranib at a molecular level (Symonds et al. 2015). However, the lack of an overall survival advantage in this trial necessitates further investigations.

Pazopanib (Votrient®) is another oral multipotent tyrosine kinase inhibitor. It targets the two angiogenic agents VEGF receptor and c-KIT as well as platelet-derived growth factor receptor (PDGFR) and several other tyrosine kinases (U.S. Food and Drug Administration 2012). Its anti-angiogenic effect exhibited remarkable treatment advantages in various solid tumors, especially in advanced renal cell carcinoma for which it is FDA approved (Monk et al. 2010). Pazopanib has also been investigated in women with stage IV, persistent, or recurrent cervical cancer. In a randomized, open-label phase 2 clinical trial conducted by Monk et al. (2010), it was administered as a single drug or in combination with the tyrosine kinase inhibitor lapatinib. The results showed that Pazopanib alone was superior to the combination with lapatinib and that it improves PFS, response rates, and OS. Median PFS was 18.1 weeks, and median OS was 49.7 weeks (Monk and Pandite 2011). These data indicate that pazopanib is equal to the currently established chemotherapeutics of stage IV cervical cancer therapy (Monk et al. 2010). The reported toxicity was negligible with only rare grade 3 and 4 adverse events.

Sunitinib (Sutent®) also exhibited promising potential as anti-angiogenic drug. It is the third oral multi-targeted tyrosine kinase inhibitor investigated in a phase II trial with the purpose of improving the treatment options in persistent, recurrent, or metastatic cervical cancer. To name a few, it targets VEGF receptors, PDEGF receptors, c-Kit receptors, and Flt3 receptors, which enable it to influence angiogenesis. Recently, it

Table 4 Overview of the clinical evidence of anti-angiogenics in the treatment of cervical cancer

Drug	Study	Design	Results
TNP470	Kudelka et al. (1997)	Phase I trial; inoperable, recurrent or metastatic disease; TNP-470 monotherapy; ($n = 18$)	5.6% complete remission ($n = 1$) 16.7% stable disease ($n = 3$) Moderate toxicity
Bevacizumab	Wright et al. (2006)	Retrospective analysis; recurrent disease; Bevacizumab in combination with chemotherapy (5-FU or capecitabine) ($n = 6$)	17% complete response ($n = 1$) 17% partial response ($n = 1$) 33% stable disease ($n = 2$) Tolerable toxicity
	Monk et al. (2009) (GOG 227C)	Phase II trial; advanced disease; Bevacizumab monotherapy; ($n = 46$)	PFS: 3.4 months OS: 7.29 months Tolerable toxicity
	Zighelboim et al. (2013)	Phase II trial; Persistent or recurrent disease; Topotecan + Cisplatin + Bevacizumab ($n = 27$)	PFS: 7.1 months OS: 13.2 months Excessive toxicity
	Schefter et al. (2014) (RTOG 0417)	Phase II trial; Stage IB - IIIB disease; Bevacizumab + chemoradiotherapy; ($n = 49$)	DFS (3y): 68,7% OS (3y): 81,3% Minimal toxicity
	Tewari et al. (2014) (GOG 240)	Phase III trial; randomized, controlled; persistent, recurrent, metastatic disease; Cisplatin, paclitaxel + Bevacizumab vs. Topotecan, paclitaxel + Bevacizumab; ($n = 452$)	PFS prolongation: 2.3 months OS prolongation: 3.7 months Manageable toxicity
	Penson et al. (2015)	Assessment of Qol changes during/after Bevacizumab therapy ($n = 452$)	No significant deterioration in Qol
	Tewari et al. (2015)	Validation of prognostic factors of Bevacizumab therapy ($n = 452$)	Pooled Moore criteria mid-risk and high-risk patients benefit the most
Cediranib	Symonds et al. (2015)	Phase II trial; randomized, placebo-controlled; Metastatic or recurrent disease; Chemotherapy + Cediranib vs. chemotherapy + placebo; ($n = 69$)	PFS prolongation: 1.4 months Significant toxicity
Pazopanib	Monk et al. (2010) + Monk and Pandite, (2011)	Phase II trial; randomized, open-label; Stage IV, persistent or recurrent disease; Pazopanib-monotherapy vs. Lapatinib-monotherapy vs. Pazopanib-Lapatinib combination ($n = 228$)	Pazopanib monotherapy PFS: 18.1 weeks OS: 49.7 weeks Favorable toxicity
Sunitinib	Mackay et al. (2010)	Phase II trial Inoperable or metastatic disease; Sunitinib monotherapy ($n = 19$)	84% stable disease No objective response Unacceptable rates of fistulae ($> 25\%$)

Abbreviations: *OS* overall survival, *PFS* progression free survival, *DFS* disease free survival, *Qol* Quality of life, *5-FU* 5-Fluoruracil, *y* years, *n* number of patients investigated

was approved by the FDA as standard but second-line treatment option in metastatic renal cell carcinoma as well as in gastrointestinal stroma tumors, although only improved response rates but no clinical benefit could be demonstrated (U.S. Food and Drug Administration 2011). In cervical carcinoma, however, it was not as successful as in the aforementioned malignancies.

Mackay et al. (2010) analyzed the effect of sunitinib monotherapy in 19 patients with inoperable or metastatic cervical cancer in a single arm trial. Lack of sufficient activity and objective response accompanied by unacceptable rates of fistulae (>25%) did not lead to approval or even phase III trials. However, 84% of sunitinib-treated women showed stable disease (Mackay et al. 2010) which is why it should be investigated in a randomized controlled phase II trial in combination with chemotherapy.

Future Directions

The value of anti-angiogenic therapy in cervical cancer is increasing. Although only bevacizumab is presently approved for the treatment of cervical cancer and as such only in persistent, recurrent, or metastatic disease, there are numerous clinical trials with the potential to create new treatment options in the future. Cediranib, pazopanib, and sunitinib have been intensely investigated, with varying success. Concurrently, there are several other clinical as well as experimental approaches for targeted therapies in the field of advanced and metastatic cervical cancer. Among these, the VEGF-triggered angiogenesis pathway still plays a central role. Nevertheless, there are also VEGF-independent angiogenic pathways and therapies targeting other mechanisms of tumor genesis under investigation.

VEGF-Pathway

Since the VEGF pathway represents the central mechanism of angiogenesis in cervical cancer and its molecular processes as well as its triggers are precisely understood, targeting it has become an attractive therapeutic option in the management of diseased women. On the one hand, direct VEGF inhibitors are already used and evaluated in downstream phase III analyses to assess their safety and efficacy. On the other hand, recombinant tyrosine kinase inhibitors of the VEGF receptor are of interest. Just to name a few, brivanib, semaxanib, and nintedanib are under clinical evaluation in women with advanced or metastatic cervical cancer. Many of these came to the attention of researchers and clinicians since they demonstrated treatment advantages in other malignant diseases. Other tyrosine kinase inhibitors still have experimental status.

Other Angiogenic and Non-angiogenic Mechanisms

In addition to all VEGF pathway targeting agents, there are several approaches of targeted therapies directed against various pathways involved in carcinogenesis, tumor progression, and metastasis. First, alternative angiogenic pathways are of interest but are still unsought as therapeutic targets in the treatment of cervical cancer. The Tie-2 angiopoetin-2 pathway, for example, is reported to be essential for the formation of blood vessels and their maintenance (Hansen et al. 2010). The former intervenes in the process of vascular development, whereas vascular-disrupting agents (VDA) lead to necrosis of blood vessels in tumor tissue and therefore cut its blood supply. The VDA Vadimezan, for example, is currently investigated in non-small cell lung cancer (Tewari et al. 2014). In the future, both mechanisms could progress to be targeted in cervical cancer therapy. Furthermore, nonangiogenic signal transduction also attracted attention in targeted cancer therapy. Drugs with impact on other pathways crucial to carcinogenesis and cancer progression have already been evaluated in many other tumor types. Especially EGFR-based therapeutics such as cetuximab and erlotinib have already been analyzed in clinical trials for cervical cancer patients but showed disappointing activity and increased toxicity (Symonds et al. 2015). In contrast, lapatinib, a dual tyrosine kinase inhibitor targeting the ErbB family (EGFR and HER2/neu), proved to be a potent molecular targeting drug in cervical cancer but was inferior to VEGF-receptor inhibitors (Monk et al. 2010).

Immunotherapy is currently in trial as a treatment option in advanced or metastatic cervical cancer. This approach is comprised of three types of drugs. First, therapeutic vaccines can be used to

target HPV-infected cells. Since these vaccines are directed against HPV E6 and HPV E7 oncoproteins, all infected cells including preinvasive and invasive neoplasia of the cervix may be reached, and E6- and E7-mediated tumor angiogenesis can be blocked (Crafton and Salani 2016).

Second, immune checkpoint inhibitors such as nivolumab and pembrolizumab that stop the suppression of the immune system in tumor microenvironment need to be considered. All substances of this category were designed to counteract the T-cell-dependent development of pathologic tumor immune tolerance (Postow et al. 2015).

Last, adoptive T-cell therapy claimed its role in cervical cancer therapy as it can equally be administered to target E6 and E7 oncoproteins. Therefore, autologous tumor-reactive T-cells are transferred with the ability to immunologically fight HPV-infected cells (Stevanović et al. 2015).

Finally, PARP inhibitors (poly ADP-ribose isomerase inhibitors) like olaparib and veliparib are analyzed as targeted therapeutics in cervical cancer. Normally, PARP is involved in base excision DNA repair, cell replication, transcription, and differentiation. Two studies demonstrated activity of PARP inhibitors in cervical cancer patients when administered with cytotoxic therapy (Kunos et al. 2015; Thaker et al. 2015). Although advantages on PFS and OS were not consistent in the two studies, it is suggested that inhibiting this pathway in combination with chemotherapy benefits women suffering advanced cervical cancer. However, further phase II and III trials are needed to assess the ideal application protocols (Crafton and Salani 2016).

In summary, with the exception of bevacizumab, anti-angiogenics only play an experimental role in the treatment of cervical cancer patients. However, their status in clinical studies is currently in permanent flux and exhibits promising results in phase II trials. Pazopanib and cediranib in particular show promise.

The abovementioned experimental approaches to target tumorigenesis and especially angiogenesis in cervical cancer as well as other malignant diseases do not claim to fully summarize current research, but represent the most promising among them.

Conclusion

Considering epidemiology, the molecular pathologic background of blood vessel formation, and the present therapy of cervical cancer, the value of anti-angiogenics in this malignant disease remains contradictory. With the initiation of the anti-angiogenic drug bevacizumab into the therapy of persistent, recurrent, and metastatic cervical cancer, targeted therapy appeared for the first time in cervical cancer treatment protocols. However, its usage has only been approved for late stage disease in combination with established palliative chemotherapeutics. For these patients, it represents a meaningful treatment option, since it leads to an improvement of overall survival without generating unacceptable adverse events. For all other cervical cancer patients, the value is low.

In a global context, the value of bevacizumab for the treatment of late stage cervical cancer needs to be rated critically, because of the differing epidemiologic and economic situations in several parts of our world. Cervical cancer and especially its advanced stages pose a much bigger problem in resource-poor countries, where most women do not have appropriate access to cancer prevention and screening diagnostics. In these countries, the percentage of persistent, recurrent, and metastatic cervical cancer cases is much higher than in countries of high medical standard. Unfortunately, resource-poor countries, where bevacizumab therapy would be crucial for the treatment of cervical cancer, cannot stand its high economic strain. Moreover, its benefit in cervical cancer patients who are in bad shape remains to be elucidated. Both circumstances degrade the current value of bevacizumab to a large extent since only a very reduced number of patients can finally get the opportunity to profit by its use.

Meanwhile, the status of clinical trials investigating cediranib, pazopanib, sunitinib, and several other tyrosine kinase inhibitors with anti-angiogenic effect is promising but lacking practice-changing evidence. The de facto minor value of bevacizumab and other angiogenic drugs conflicts the unique and extensive role that angiogenesis plays in the development, growth, and

progression of cervical cancer. HPV infection obviously triggers angiogenesis over the VEGF pathway already in early formation of any cervical neoplasia. The detailed understanding of the HPV-triggered pathway displays the theoretical justification of anti-angiogenic therapy in cervical cancer patients. However, the high theoretical value of anti-angiogenics in cervical cancer therapy is not in accord with its actual use.

In comparison to other malignancies like breast or bladder cancer, anti-angiogenics still need to assure their position in cervical cancer therapy. For this purpose, investigations are currently in progress and will hopefully help identify potent therapeutics in the field of anti-angiogenics against cervical cancer.

Cross-References

- ▶ Cytotoxics and Anti-angiogenics: Metronomic Therapies
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part XVI

Anti-angiogenics in Urological Tumor Therapy

Anti-angiogenics in Kidney Cancer Therapy

Herbert Rübben and Andrej Panic

Contents

Introduction 570
Angiogenesis and Anti-angiogenics 570
TKI (Tyrosine Kinase Inhibitors) 572
Side Effects of Tyrosine Kinase Inhibitors (TKI) 573
Bevacizumab plus Interferon (IFN)-α 574
mTOR Inhibitors 574
Immunotherapy 574
Chemotherapy 575
Future Directions 575
References 576

Abstract

Metastatic renal cell carcinoma (RCC) remains largely incurable. Up to 30% of patients show metastasis at the time of the initial diagnosis. Prognostic criteria developed by the IMDC (International Metastatic Renal Cell Carcinoma Database Consortium) and MSKCC (Memorial Sloan Kettering Cancer Center) are used to classify patients based on certain pretreatment factors. The prognosis of patients with metastatic disease varies depending on these risk factors. Anti-angiogenic agents targeting the vascular endothelial growth factor (VEGF) and its receptors are standard treatments based on improved clinical outcomes in randomized phase III trials. Standard of care therapies now include multitargeted tyrosine kinase inhibitors (TKIs) such as sunitinib, axitinib, pazopanib, and cabozantinib, as well as the mTOR inhibitors temsirolimus and everolimus.

Tumor-associated PD-L1 expression has been detected in RCC and is associated with a worse prognosis. Immune checkpoint inhibitors such as the PD-1 inhibitor nivolumab have shown promising results in treatment of the metastatic disease. Future developments including novel combinations and attempts to

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find the optimal position of immunotherapy in the disease pathway are subject of ongoing clinical trials.

Keywords
Metastatic renal cell carcinoma (RCC) · Anti-angiogenics · Vascular endothelial growth factor (VEGF) · Tyrosine kinase inhibitors (TKIs) · mTOR inhibitors · Immune checkpoint inhibitors

Introduction

Renal cell cancer represents about 3% of all cancers and is largely incurable. In the European Union, there were approximately 84,400 new cases of RCC and 34,700 kidney cancer-related deaths in 2012 (Ferlay et al. 2013). There is a 1.5:1 male predominance, with a peak incidence between 60 and 70 years. Etiological factors include smoking, obesity, and hypertension (Bergström et al. 2001).

The number of incidentally diagnosed RCCs has increased due to increased detection of tumors by ultrasound as well as magnetic resonance imaging (MRI) and computed tomography (CT). These tumors are usually smaller and of lower stage (Tsui et al. 2000).

Renal cell carcinomas comprise a broad spectrum of histopathological entities. There are three main RCC types: clear cell (ccRCC), papillary (pRCC – type I and II), and chromophobe (chRCC). The RCC type classification has been confirmed by cytogenetic and genetic analyses (Moch et al. 2016). Other forms of kidney cancer tumors constitute the remaining 10–15% of renal cortical tumors, such as the very aggressive renal medullary carcinoma (<0.5% of all RCCs), acquired cystic disease-associated RCC (4% of patients), papillary adenoma, angiomyolipoma, renal oncocytoma, cystic renal tumors, sarcomatoid variants of RCC, and carcinoma of the collecting ducts of Bellini.

Up to 30% of patients show metastasis at the time of the initial diagnosis (Therasse et al. 2000). Patients with metastatic disease have a poor prognosis, with a 5-year survival rate of less than 20% (Itsumi and Tatsugami 2010). Recurrence occurs in approximately 40% of patients after treatment of a localized tumor. High levels of serum lactate dehydrogenase, low hemoglobin level, and high corrected level of serum calcium are the prognostic markers for metastatic RCC (Fig. 1). The average survival of patients with advanced RCC is approximately 12 months.

Angiogenesis and Anti-angiogenics

Drugs targeting angiogenesis are the primary treatment option for such patients. Angiogenesis is the physiological process of the growth of new blood vessels from preexisting blood vessels (Greenblatt and Shubik 1968). Angiogenesis is an important factor for tumor growth and metastasis in humans. Inactivation of the von Hippel-Lindau (VHL) gene is the most common genetic change present in clear cell RCC. In the absence of VHL, hypoxia-inducible factor (HIF) accumulates, leading to the production of several growth factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). One of the most important roles of hypoxia-inducible factor 1 α (HIF-1 α) in cancer is to induce angiogenesis through the synthesis of angiogenesis-related proteins (Kim and Kaelin 2004). HIF-1 α plays an important role in regulating cell cycle and apoptosis as well. The activity of these factors is associated with oncogenesis, growth, and the metastatic potential of RCC cells. Angiogenesis is a process that involves the formation of new blood vessels from the existing vasculature. In addition to its role in tumor growth, angiogenesis is an important step in tumor proliferation and metastasis that offers a route for tumor cells to spread to organs via the bloodstream.

Hypoxia-inducible factor (HIF) is a transcription factor that responds to reduced intracellular oxygen concentration. In the hypoxic condition, HIF accumulates in the cell and is transported to the nucleus where it induces the expression of a wide variety of target gene products such as growth factors, e.g., VEGF, fibroblast growth factor (FGF), transforming growth factor (TGF), etc.

Risk factors*	Cut-off point used
Karnofsky performance status	< 80%
Time from diagnosis to treatment	< 12 months
Haemoglobin	< Lower limit of laboratory reference range
Corrected serum calcium	> 10.0 mg/dL (2.4 mmol/L)
Absolute neutrophil count (neutrophilia)	> upper limit of normal
Platelets (thrombocytosis)	> upper limit of normal

* low risk, no risk factors; intermediate risk, one or two risk factors; high risk, three to six risk factors

Fig. 1 IMDC (International Metastastic RCC Database Consortium) Risk Score

These proteins in turn activate different signaling pathways, including PLCγ, PI3K, Smad, Src, etc., so that endothelial cell proliferation, vascular permeability, and cell migration are increased. Extracellular matrix proteases induce tissue matrix remodeling, and new tube formation occurs with the participation of the migrated endothelial cells. Various cytokines play key roles in the process. In addition to hypoxia, the PI3K and Ras pathways can also increase HIF expression by increasing HIF translation (Adams and Alitalo 2007). The growth of any tumor and its metastasis depend on the development of neovasculature in and around the tumor. Angiogenesis facilitates tumor growth progression by supplying adequate oxygen and nutrition to the tumor cells through several interrelated steps. The mechanism regulating angiogenesis is tissue specific (Hanahan et al. 1996). Angiogenic phenotype is regulated by the differential expression of growth factors and cytokines within the microenvironment of the organ.

RCC is one of the most vascular of the solid tumors, which suggests a prominent role for angiogenesis in the pathogenesis of RCC (Bard et al. 1986). VEGF is known as vascular permeability factor and stimulates endothelial cell proliferation in vitro and has got angiogenic activity in vivo. The second secreted angiogenic factor with a role in angiogenesis in RCC is PIGF (Maglione et al. 1993). Elevated VEGF expression is involved in the hypervascularity of RCC and plays an important role in determining the size, stage, and grade of carcinoma. VEGF, PIGF, and bFGF work together to increase angiogenesis in RCC; therefore they can be used as tumor markers, especially in the early stage of the disease (Atsushi et al. 1994).

Like many solid neoplasms, renal tumors are frequently characterized by hypoxic conditions due to local imbalance between oxygen (O_2) supply and consumption. Indeed, hypoxia and compensatory hyperactivation of angiogenesis are thought to be particularly important in RCC compared to other tumor types, given the highly vascularized nature of kidney tumors and the specific association of mutation in von Hippel-Lindau (VHL) gene with onset of RCC. Hypoxic signaling is mediated by the hypoxia-inducible factors (HIFs), which regulate the expression of over 200 genes involved in crucial pathways related to tumorigenesis including angiogenesis, invasion, and mitogenesis. In hypoxic RCC tumors, in the absence of VHL, HIF-α proteins remain constitutively expressed, thereby inducing vascular endothelial growth factor (VEGF) and other HIF targets. Increased expression of many of the HIF target genes is implicated in promoting cancer, inducing both changes within the tumor (cell-intrinsic) and changes in the growth of adjacent endothelial cells to promote blood vessel growth. The expression level of VEGF in RCC is known to strongly correlate with microvessel density, a measure of the degree of angiogenesis. A key step in angiogenesis is the upregulation of growth factor receptors on endothelial cells such as vascular endothelial growth factor receptors (VEGFR) and platelet-derived growth factor receptors (Bianconi et al. 2012). As

in many other tumors, targeting angiogenesis improves patients' outcome. Anti-angiogenic drugs targeting the VEGF pathway with proven benefit in RCC include inhibitors of VEGFRs sunitinib, sorafenib, pazopanib, axitinib, cabozantinib, and bevacizumab.

The family of VEGF and VEGFR is a very complex one. The VEGF family members are secreted, dimeric glycoproteins of 40 kDa, consisting of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor, and binding to specific receptors. The VEGF gene has several alternatively spliced isoforms, and the regulation of expression might differ between normal and tumor tissue. To find these differences, the attention focused on the expression of single-nucleotide polymorphisms (SNP), as it was the case in other carcinomas (Scartozzi et al. 2014). Interestingly, as all identified polymorphisms in VEGF are not in the coding region, alternative mechanisms for their role in gene expression have been proposed. Although many transcription factors bind to the promoter regions of VEGF, none occurs at the common polymorphic sites associated with VEGF expression. Nevertheless, SNPs have been reported to cause changes in VEGF expression levels (Pages and Puyssegur 2005). The SNPs in the VEGF and VEGFR genes have been also correlated with tumor neoangiogenesis through different biological mechanisms.

Sunitinib, pazopanib, and five other agents have been approved by the Food and Drug Administration for the treatment of clear cell, metastatic renal cell carcinoma. Among the tyrosine kinase inhibitors, pazopanib and sunitinib are first-line treatment options.

TKI (Tyrosine Kinase Inhibitors)

Sunitinib is an oral tyrosine kinase (TK) inhibitor with antitumor and anti-angiogenic activity. First-line monotherapy with sunitinib showed significantly longer PFS compared with IFN-α. Overall survival was greater in patients treated with sunitinib (26.4) versus INF-α (21.8 months) despite crossover (Motzer et al. 2009). Sunitinib as second-line monotherapy after cytokines in patients with metastatic renal cell carcinoma demonstrated a partial response in 34–40% and stable disease at >3 months in 27–29% of patients (Motzer et al. 2006). Sunitinib 50 mg/day (4 weeks on/2 weeks off) was compared in the EFFECT trial with continuous uninterrupted sunitinib 37.5 mg/day in patients with clear cell advanced RCC. Median time to progression with sunitinib 50 mg was numerically longer than the 37.5 mg arm (9.9 months vs. 7.1 months). There were no significant differences in overall survival. Toxicity was comparable in both arms. Because of the nonsignificant, but numerically longer time to progression with the standard 50 mg dosage, the authors recommended using this regimen (Motzer et al. 2012). Alternate scheduling of sunitinib (2 weeks on/1 week off) can be used to manage toxicity.

Pazopanib is an oral angiogenesis inhibitor targeting vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and c-Kit. The daily dose is 800 mg. In a trial of pazopanib versus placebo in treatment-naïve metastatic RCC patients and cytokine-treated patients, a significant improvement in progression-free survival and tumor response was observed (Sternberg et al. 2010). Median progression-free survival (PFS) with pazopanib compared with placebo was 9.2 versus 4.2 months in the overall study population, 11.1 versus 2.8 months for the treatment-naïve subpopulation, 7.4 versus 4.2 months for the cytokine-pretreated subpopulation. The COMPARZ trial, which compared pazopanib with sunitinib, established pazopanib as another first-line option. It showed that pazopanib was not associated with significantly worse PFS or overall survival compared to sunitinib. The two drugs had different toxicity profiles, and quality of life was better with pazopanib (Motzer et al. 2013a). The study was limited due to the fact that intermittent therapy (sunitinib) was compared with continuous therapy (pazopanib).

Axitinib is an oral selective second-generation inhibitor of VEGFR1, VEGFR2, and VEGFR3. Axitinib was first evaluated as second-line treatment. The daily dosage is 10 mg, to be taken as

5 mg twice per day. The AXIS trial compared axitinib to sorafenib in patients with previously failed cytokine treatment or targeted agents. The overall median PFS was greater for axitinib than sorafenib. The difference in PFS was greatest in patients in whom cytokine treatment had failed (Rini et al. 2011). In a randomized phase III trial of axitinib versus sorafenib in first-line treatment-naïve clear cell metastatic RCC, a significant difference in median PFS between the treatment groups was not demonstrated (Hutson et al. 2013). As a result of this study, axitinib is not approved for first-line therapy.

Sorafenib is an oral multi-kinase inhibitor. The recommended daily dose is 800 mg. Sorafenib improved progression-free survival in a trial which compared sorafenib and placebo after failure of prior systemic immunotherapy or in patients unfit for immunotherapy (Escudier et al. 2007a). A number of studies have used sorafenib as the control arm in sunitinib-refractory disease versus axitinib, dovitinib, and temsirolimus. None showed superior survival for the study drug compared to sorafenib.

Cabozantinib is an oral inhibitor of TK, including VEGF, and receptor tyrosine kinases MET and AXL. The recommended daily dose is 60 mg. A randomized phase III trial (METEOR) investigated cabozantinib versus everolimus in 658 patients with clear cell RCC failing one or more VEGF-targeted therapies. Cabozantinib delayed PFS compared to everolimus in VEGF-targeted therapy refractory disease by 42%. The median PFS for cabozantinib was 7.4 months versus 3.8 months for everolimus. The median OS was 21.4 months with cabozantinib and 16.5 months with everolimus in VEGF-resistant RCC. Grade 3 or 4 adverse events were reported in 74% with cabozantinib and 65% with everolimus and were managed with dose reductions. Discontinuation due to toxicity was not significantly different for the two drugs (Choueiri et al. 2015).

Lenvatinib is an oral multitarget TKI of VEGFR1, VEGFR2, and VEGFR3, with inhibitory activity against fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3, and FGFR4); platelet growth factor receptor α (PDGFRα), rearranged during transfection (RET); and receptor for stem cell factor (KIT). The recommended daily dose is 24 mg.

Tivozanib is a potent and selective TKI of VEGFR1, VEGFR2, and VEGFR3 and was compared in a phase III trial with sorafenib as initial targeted therapy in patients with mRCC (Motzer et al. 2013b). Tivozanib was approved by the European Medicines Agency in frontline mRCC. It can therefore be prescribed in the European Union. The recommended daily dose is 1.340 μg (3 weeks on/1 week off). The Panel of the European Urological Association feels that it remains an inferior option as compared to other TKIs in this setting without further randomized data; therefore other agents should be used in preference.

Side Effects of Tyrosine Kinase Inhibitors (TKI)

Treatment with anti-VEGFR tyrosine kinase inhibitors is limited by its tolerability, including skin toxicity, which has resulted in rates of discontinuation in some cases exceeding that of conventional cytotoxic chemotherapy (Prasad et al. 2014).

Dermatologic side effects include rash, alopecia, depigmentation, pruritus, xerosis, acneiform rashes, and mucositis (Kamba and McDonald 2007). For most patients the most troublesome cutaneous side effect is a hand-foot skin reaction (HFSR), which is characterized by painful, edematous, erythematous, and keratotic lesions on acral surfaces, particularly weight-bearing sites, usually occurring 1–6 weeks after therapy is initiated (Fischer et al. 2013). Acral dysesthesia and paresthesia commonly precede the lesions (Porta et al. 2007).

HFSR occurring with anti-VEGFR TKIs can be distinguished clinically from hand-foot syndrome (HFS) associated with "cytotoxic" chemotherapeutic agents (Janusch et al. 2006). While HFSR produces localized, hyperkeratotic plaques on acral sites (Yang et al. 2008), HFS is marked by symmetric, desquamative erythema and edema not typically extending beyond volar and plantar

surfaces (Lacouture et al. 2008). The mechanism of the HFSR is still poorly understood (Lipworth et al. 2009).

Besides dose reduction and drug discontinuation of oral agents, there are limited treatment options such as topical anti-inflammatory medications which include corticosteroid creams and topical pain relievers, such as lidocaine. These are used as a cream or a patch over painful areas in the palms and soles. There are also useful topical moisturizing exfoliant creams containing urea, salicylic acid, or ammonium lactate. Pain relievers such as ibuprofen, naproxen, and celecoxib can also be helpful.

Bevacizumab plus Interferon (IFN)-α

Bevacizumab is a humanized monoclonal antibody. The double-blind AVOREN study compared bevacizumab plus IFN-α with INF-α monotherapy in mRCC (Escudier et al. 2007b). Overall response was higher in the bevacizumab plus IFN-α group. Median PFS increased from 5.4 months with IFN-α to 10.2 months with bevacizumab plus IFN-α. An open-label trial of bevacizumab plus IFN-α versus IFN-α showed a higher median PFS for the combination group with the objective response rate being higher in the combination group. Overall toxicity was greater for bevacizumab plus IFN-α, with significantly more grade 3 hypertension, anorexia, fatigue, and proteinuria (Rini et al. 2010).

mTOR Inhibitors

Temsirolimus is a specific inhibitor of the mammalian target of rapamycin (mTor). Temsirolimus is an intravenous drug which interferes with the synthesis of proteins that regulate proliferation, growth, and survival of tumor cells. In the INTORSECT trial, temsirolimus versus sorafenib was investigated in patients who had previously failed sunitinib. Although no benefit in PFS was observed, a significant OS benefit for sorafenib was noted (Hutson et al. 2014). Based on these results, temsirolimus is not recommended in patients with VEFG TKI-refractory disease.

Everolimus is an oral mTOR inhibitor, established in the treatment of VEGF-refractory disease. The RECORD-1 study compared everolimus plus best supportive care (BSC) versus placebo plus BSC in patients with previously failed anti-VEGFR treatment (or previously intolerant of VEGF-targeted therapy). It showed a median PFS of 4.9 versus 1.9 months for everolimus and placebo, respectively, in the final analysis (Motzer et al. 2010). Subset analysis of PFS for patients receiving only one previous VEFG TKI was 5.4 months (Calvo et al. 2012). This included some patients who were intolerant rather than progressed on therapy (PFS was also 5.4 months). RECORD-1 included patients who failed multiple lines of VEGF-targeted therapy and received everolimus in a third- and fourth-line setting.

Immunotherapy

Interleukin-2 has been used to treat mRCC since 1985, with response rates ranging from 7% to 27% (McDermott et al. 2005). Complete and durable responses have been achieved with high-dose bolus IL-2; however IL-2 remains the only drug to date that can cure a small percentage of RCC patients (Yang et al. 2003). The toxicity of IL-2 is substantially greater than that of IFN-α.

Several studies showed that interferon (IFN)-α in metastatic RCC resulted in a response rate of 6–15%, a 25% decrease in tumor progression risk and a modest survival benefit compared to placebo (Motzer et al. 2002). However, patients with intermediate-risk disease failed to confirm this benefit (Negrier et al. 2007). Interferon-α may only be effective in some patient subgroups, including patients with ccRCC, favorable-risk criteria, as defined by the Memorial Sloan Kettering Cancer Center (MSKCC), and lung metastases only. Interferon-α has since been superseded by targeted therapy in clear cell metastatic RCC.

Chemotherapy

Chemotherapy should not be offered as first-line therapy in patients with clear cell metastatic RCC since it is moderately effective only if 5-fluorouracil (5-FU) is combined with immunotherapeutic agents (Stadler et al. 2003). In metastatic RCC, chemotherapy is otherwise not effective with the exception of gemcitabine and doxorubicin in sarcomatoid and rapidly progressive disease (Haas et al. 2012).

Future Directions

The introduction of newer immunotherapy, with the immune checkpoint inhibitors such as nivolumab, is a very promising new therapeutical option in kidney cancer treatment.

Immune checkpoint blockade with monoclonal antibodies targets and blocks the inhibitory T-cell receptor PD-1 or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) signaling to restore tumor-specific T-cell immunity (Ribas 2012). A phase III trial of nivolumab versus everolimus after one or two lines of VEGF-targeted therapy (CheckMate 025) reported a longer OS, better QoL, and fewer grade 3 or 4 adverse events with nivolumab than with everolimus (Motzer et al. 2015). Nivolumab has superior OS to everolimus in VEGF-refractory RCC with a median OS of 25 months for nivolumab and 19.6 months for everolimus. Patients who had failed multiple lines of VEGF-targeted therapy were included in this trial making the results broadly applicable.

The phase III trial CheckMate 214 investigated the combination of nivolumab and ipilimumab versus sunitinib in first-line treatment of treatment-naïve advanced or cc-mRCC. Results showed that a combination of ipilimumab and nivolumab was associated with a significant advantage for both RR and OS. A higher proportion of the patients treated with nivolumab plus ipilimumab achieved durable remissions, justifying their use in unselected patients (including favorable-risk disease). Health-related QoL assessment, based on the NCCN Functional Assessment of Cancer Therapy-Kidney Symptom Index (FKSI-19), was performed and favored the immunotherapy combination (Escudier et al. 2017).

Tumors which overexpressed the PD-L1 biomarker at baseline were associated with a better RR and PFS with nivolumab plus ipilimumab than sunitinib. This was not the case in the PD-L1-negative cohort, where PFS was almost identical. Therefore, the PD-L1 biomarker appears predictive for PFS. Nivolumab plus ipilimumab was associated with 15% grade 3–5 toxicity and 1.5% treatment-related deaths. It should therefore be administered in centers with experience of immune combination therapy and appropriate supportive care within the context of a multidisciplinary team. Nivolumab plus ipilimumab should not be offered outside of the first-line setting.

Patients who stop nivolumab plus ipilimumab because of toxicity should not be challenged with the same drugs in the future without expert guidance and support from a multidisciplinary team. Further combinations of VEGF-targeted therapy and immune therapy are being compared in phase III trials against sunitinib and may change treatment recommendations soon. These include pembrolizumab plus axitinib and lenvatinib plus everolimus or pembrolizumab.

The combination of nivolumab and ipilimumab is the standard of care in IMDC intermediate- and poor-risk patients (Fig. 2). Alternative agents such as sunitinib, pazopanib, and cabozantinib should be considered where nivolumab plus ipilimumab is not safe or feasible (EAU Guidelines). Sunitinib or pazopanib remains a preferable agent in favorable-risk patients due to the non-inferiority of pazopanib compared to sunitinib (COMPARZ). Key trials have established bevacizumab plus IFN-α as another first-line treatment option in treatment-naïve patients with cc-mRCC and a favorable-to-intermediate risk score. This has not been tested against nivolumab plus ipilimumab, and the evidence for subsequent therapies remains unclear. The same arguments apply for temsirolimus in poor-risk patients. It is therefore more appealing to treat patients with sunitinib or pazopanib, both

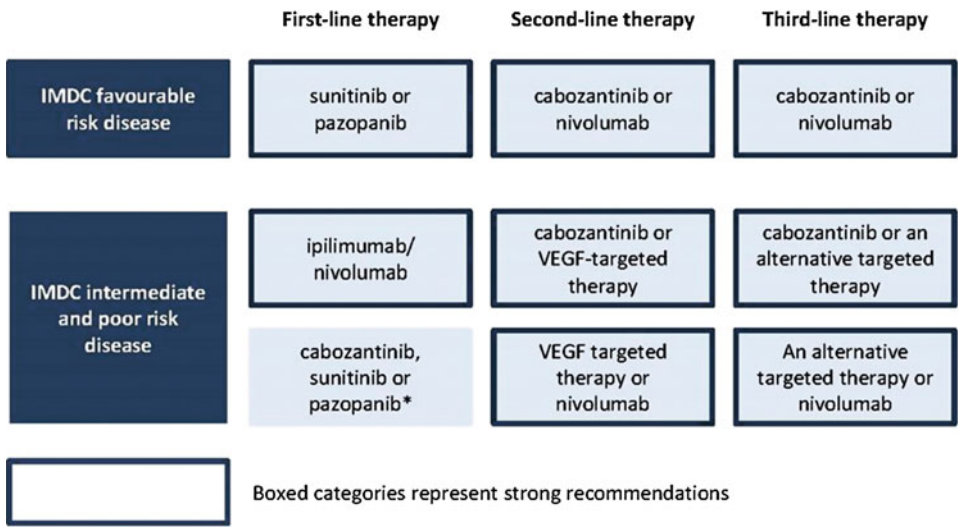

Fig. 2 EAU Guidelines recommendations for the treatment of first-line clear-cell metastatic renal cancer

of which were tested in all three risk groups in pivotal trials, where nivolumab plus ipilimumab is not safe or feasible.

Phase II data which compared sunitinib and cabozantinib for intermediate- and poor-risk RCC favored cabozantinib for RR and PFS, although not OS (Choueiri et al. 2017). It showcases the activity of cabozantinib, but due to missing of a randomized phase III study, it currently cannot be supported above other VEGF-TKIs such as pazopanib or sunitinib.

There is no evidence for sequencing of immune therapies, which remains within the realms of clinical trials. While data with the combination of VEGF-targeted therapy and immune checkpoint inhibition is promising, further randomized data is required prior to any recommendations.

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The Value of Anti-angiogenics in Prostate Cancer Therapy

Krisztian Homicsko and Dominik Berthold

Contents

Introduction 580

The Biology of Prostate Cancer Neo-angiogenesis 581

Proangiogenic Factors 582
Vascular Endothelial Growth Factors (VEGFs) 582
Platelet-Derived Growth Factors (PDGFs) 582
Fibroblast-Derived Growth Factors (FGFs) 583
Angiopoietins (Angs) 583

Anti-angiogenic Factors 583

Mechanisms of Resistance to Angiogenesis Inhibition 583
Adaptive Resistance 583
Innate Resistance 584

Anti-angiogenic Therapies of Advanced Prostate Cancer 584
Bevacizumab 584
Aflibercept 585

Tyrosine Kinase Inhibitors (TKIs): Sunitinib, Sorafenib, and Cabozantinib 585
Tasquinimod 586
Thalidomide 587
Lenalidomide 587

Conclusion 588

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Cross-References 589
References 589

Abstract

Prostate cancer, the most common cancer diagnosed in men, has been investigated extensively concerning the use of anti-angiogenics. There is a significant amount of preclinical and early clinical data about the potential value of this class of drugs as is the case with many other solid cancer types. Vascular endothelial growth factors and their receptors (VEGF/VEGFRs) seem to be key players in neo-angiogenesis and its expression can be regulated by androgen receptor signaling. Platelet-derived growth factor receptor alpha (PDGFR-α) is of lesser importance in primary prostate cancer; however, PDGFR-A might be involved in the formation of bone metastases. Other mechanisms of pro- and anti-angiogenic factors will be described herein.

The clinical development focused mainly on metastatic, castration-resistant prostate cancer. Phase I/II trials showed consistently interesting results in terms of response rates or reduction of tumor growth. Yet, randomized studies failed to demonstrate a significant overall survival benefit despite increased progression-free survival or clinical signs of activity, such as reduced need for analgesic drugs. This chapter will provide an overview of angiogenesis in prostate cancer and on the development of angiogenesis inhibitors, in particular bevacizumab, sunitinib, tasquinimod, lenalidomide, and cabozantinib.

Keywords

Castration-resistant · Prostate cancer · Angiogenesis · Bevacizumab · Sunitinib · Tasquinimod · Lenalidomide · Cabozantinib · Docetaxel

Introduction

Prostate cancer is the most common cancer in men in the more developed regions. There are an estimated 1.1 million men newly diagnosed with prostate cancer each year and over 300.000 deaths directly related to metastatic prostate cancer. Seventy percent of cases occur in developed regions of the world. Globally, there are over five million men living with prostate cancer (Ferlay et al. 2015).

Prostate cancer is suspected if prostate-specific antigen (PSA) is elevated during the screening of healthy men. About 70% of men in western countries are diagnosed following screening procedures. Other patients may be diagnosed because of specific symptoms, such as urinary irritation, obstruction, or bone pain. Most of the time, men with localized prostate cancer are cured, but those with locally advanced or metastatic prostate cancer frequently relapse, progress, and die. Upon relapse, the first manifestation is usually the rise of the PSA. In this scenario, hormonal treatment, such as medical or surgical castration, is offered to the patient.

Unfortunately, patient progress after months or years of castration leads to the development of castration-resistant prostate cancer (CRPC). In this scenario, several treatment options are available to prolong survival and/or improve quality of life. In particular, there are new androgen-targeting agents, such as abiraterone or enzalutamide; taxane-based chemotherapy drugs, like docetaxel or cabazitaxel; radionucleotides, including Radium 223 for bone metastases; or immunotherapies, such as that with Sipulocel-T (Crawford et al. 2015).

Treatments targeting angiogenesis appear to be potential regulators of tumor growth in cancers. The role of angiogenesis in tumor growth was first described as early as the mid-1940s by Algire (Algire and Chalkley 1945; Algire and Legallais 1948) followed by the work of others, including Greene and Folkman (Folkman et al. 1971; Greene 1950); the latter of whom was also considered to have been the first to postulate in 1971 that angiogenesis may prove to be a valuable target for anticancer drugs (Schweizer and Carducci 2013).

Despite the high incidence of prostate cancer, the role of angiogenesis has only been evaluated late in comparison to other cancer types. A first report in the 1970s linked blood vessel invasion of prostate cancer to poor prognosis (Kwart and Sims 1978). Further studies that focused on angiogenesis in prostate cancer were only published roughly 15 years later, which represents a delay of half a century compared to other cancers (Furusato et al. 1994; Wakui et al. 1992). Consequently, nowadays, we have 25 years of hindsight regarding prostate cancer angiogenesis.

Several anti-angiogenic therapeutics developed and approved for other types of cancers have been studied within the context of prostate cancer. To better understand these results, it is of importance to first comprehend the mechanisms involved in prostate angiogenesis. Therefore, in the first part of this chapter, we will review the state of the art regarding physiological condition of the prostate, angiogenesis in prostatic benign hyperplasia, and angiogenesis in prostate cancer. The findings of different trials focused on anti-angiogenics in prostate cancer will be described in the second part of the chapter.

The Biology of Prostate Cancer Neo-angiogenesis

The normal prostate gland is a well-vascularized organ; however, regional differences might be present (Scolnik et al. 1992). About 70% of prostate cancer initiates from the peripheral zone, while the transition zone is home to the other 30%. Prostate cancer virtually never originates from the central zone. In contrast, benign prostatic hyperplasia initiates from the transition and central zones. It is not known if regional differences in angiogenesis might contribute to these differences, but more hypoxic microenvironments may accelerate cancer development and in suppressing immune surveillance (Anastasiadis et al. 2002).

Once the development of prostate cancer has been initiated because of the acquisition of genetic changes, prostate cancer, similar to most other cancers, requires the initiation of neo-angiogenesis to sustain tumor growth as well as to go beyond local disease and metastasize (Folkman and Hanahan 1991). The Gleason score of prostate cancer, which defines both varying degrees of biological aggressiveness as well as prognostic value, has been shown to correlate with microvessel density and histological evaluation of angiogenesis (Mehta et al. 2001). Constitutively activated androgen signaling downstream from the androgen receptor (AR) is key for prostate cancer development and maintenance as reflected by robust initial efficacy of androgen deprivation therapy in advanced treatment of naive prostate cancer (Perlmutter and Lepor 2007). The AR functions as a nuclear transcription factor, and among other important targets, it has been demonstrated that expression of *FLT1* (FMS-related tyrosine kinase 1) encoding VEGFR-1 can be modulated by the AR (Sieveking et al. 2010). In addition, genetic polymorphisms in the AR-binding sites of the *FLT1* promoter correlated with overall survival in a cohort of 601 advanced prostate cancer patients treated by androgen deprivation therapy (Huang et al. 2012a).

Although heightened VEGFR-1 is fundamental to cancer neo-angiogenesis, many other factors play important roles in the establishment, adaptation, and maintenance of abnormal neo-vasculature (Welti et al. 2013). In order to characterize the multifaceted regulation of neo-angiogenesis, the term "angiogenic switch" was coined by Folkman and Hanahan (1991). As in normal tissue regeneration, both pro- and anti-angiogenic factors influence the careful orchestration of blood vessel development. Cancers, including prostate cancer, corrupt the delicate balance of normal blood vessel formation, tipping it toward proangiogenic factors. Based on the chaotic, unregulated expression of a multitude of proangiogenic factors and suppression of anti-angiogenic factors, cancer-associated blood vessels are abnormal – they are fenestrated and leaky but with increased thickening of the vessel walls because of pericyte proliferation Ruoslahti (2002). In part, this abnormal angiogenesis also facilitates the maintenance of a hypoxic microenvironment that is preferred by cancer cells and sustains glycolytic metabolism, the so-called

Wartburg effect (Koppenol et al. 2011). Furthermore, abnormal angiogenesis assists the creation of an immune-suppressive microenvironment, limiting cancer immune surveillance by the host immune system (Kim et al. 2007). In the following, we will elaborate upon the most important elements of angiogenesis that are modified in prostate cancer and offer potential rationale for the absence of benefits from angiogenic inhibitors in prostate cancer patients, which is similar to patients with other cancers who also fail to benefit from anti-angiogenic therapies.

Proangiogenic Factors

Vascular Endothelial Growth Factors (VEGFs)

The VEGF pathway includes five different ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E) and three cognate receptors, both of which modulate angiogenesis as well as lymphangiogenesis (Ferrara 2002). The most prominent and well-studied pair is VEGF-A and VEGFR-1/VEGFR-2. Like increased VEGFR-1 expression, the heightened expression of VEGF-A is also associated with poorer outcomes for prostate cancer patients (Green et al. 2007). ARs are also capable of transactivating VEGF-A promoters (Eisermann et al. 2013), but may additionally lead to alternative splicing of VEGF-A, potentially increasing expression of more potent proangiogenic VEGF-A splice variants (Bates et al. 2002; Mavrou et al. 2015). ARs can also affect the half-life of VEGF-A transcripts; in the case of active AR signaling, the Wilms tumor suppressor gene (*WT-1A*) may enhance VEGF-A transcript stability (Cash et al. 2007).

In addition, AR-independent mechanisms also feature in the regulation of VEGF-A in prostate cancer. A single nucleotide polymorphism (SNP) in the VEGF-A gene has been linked with prostate cancer risk (Sfar et al. 2006). Specifically, this SNP causes a decrease in the usage of an alternative initiation codon (Lambrechts et al. 2003). MicroRNAs (miRNAs) can also regulate gene transcription, like miR-29b that can negatively regulate VEGF-A expression. In prostate cancer cell lines, miR-29b was significantly reduced, leading to elevated VEGF-A levels (Szczyrba et al. 2013). Other potential VEGF-modifying miRNAs that could be deregulated in prostate cancer are miR-145, miR-205, and miR-374b (Boll et al. 2013; Yue et al. 2012).

Finally, the role of VEGF-A is not limited to the development of primary, localized prostate cancer. VEGF-A promotes local tumor evasion, provides support to the development of niches for distant bone metastases formation (Kaplan et al. 2005), increases osteoblast activity and bone remodeling (Kitagawa et al. 2005), and diminishes immune surveillance by impairing the functions of cytotoxic T cells (Mellman et al. 2011), antitumoral M1 macrophages, and dendritic cells.

Together, VEGF-A-VEGFR-1/VEGFR-2 are crucial components of the angiogenic switch in prostate cancer.

Platelet-Derived Growth Factors (PDGFs)

PDGF signaling is composed of four ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) and two receptors (PDGFR-α and PDGFR-β) (Farooqi and Siddik 2015). Biochemically, ligands form homo- or heterodimers. PDGFR-β is upregulated in virtually all prostate cancers, and high PDGFR-β levels correspond to shorter prostate cancer recurrence-free periods (Paulsson et al. 2009). The PDGFR-β ligand, PDGF-B, is not overexpressed in most prostate cancers, though PDGF-D is frequently overexpressed and again correlates with prostate cancer aggressiveness (Ustach et al. 2010). Loss of PTEN is a very frequent event in prostate cancer and leads to upregulated PDGF-D expression (Christensen et al. 2014). PDGF-D is also important in the metastatic process – it promotes differentiation of osteoclasts by increasing NFAT-1, the master regulator of osteoclastogenesis (Huang et al. 2012b). PDGFR-A is of lesser importance in primary prostate cancer; however, it may be involved in the formation of bone metastases (Sulzbacher et al. 2009).

Fibroblast-Derived Growth Factors (FGFs)

FGF signaling features 22 ligands and four receptors and FGFRs are frequently mutated in cancers (Turner and Grose 2010). With this, recurrent FGFR3 mutations have been identified in prostate cancer (Hernandez et al. 2009), while several of the ligands, including FGF2, FGF6, and FGF10, are known to be overexpressed in prostate cancer (Giri et al. 1999; Ropiquet et al. 2000). Beyond mutations, the receptor, FGFR1-IIIc, has been demonstrated to be upregulated (Giri et al. 1999). In addition, negative regulators of FGF signaling, including SPRY1, SPRY2, and SEF, can be lost, resulting in increased FGF signalling (Fritzsche et al. 2006). It was suggested that most of the changes in FGF signaling are AR independent (Kwabi-Addo et al. 2004).

Angiopoietins (Angs)

Ang signaling includes four ligands and several angiopoietin-like ligands (ANGPTLs), the latter of which bind to two receptors (TIE-1 and TIE-2) (Fagiani and Christofori 2013). Angiopoietin-2 is highly expressed in primary prostate cancer as well as in liver, bone, and lymph node metastases, whereas angiopoietin-1 is minimally expressed (Lind et al. 2005). Inhibition of Ang-2 in preclinical models of prostate cancer has been seen to result in blockade of prostate cancer development and progression (Morrissey et al. 2010).

Anti-angiogenic Factors

There are multiple endogenous anti-angiogenic factors. Angiostatin, endostatin, osteopontin, vasostatin, thrombospondin, and prothrombin are among many other more or less potent molecules but, when acting in combination, are capable of blocking the angiogenic process rather effectively (Nyberg et al. 2005). Of note is that these molecules are not absent in primary or metastatic prostate cancer; however, the higher levels of proangiogenic factors override them, being anti-angiogenic. Potential enhancement of anti-angiogenic molecules in tumors could help in diminishing angiogenesis; however, tumor-specific overexpression remains a challenge for clinical feasibility.

Mechanisms of Resistance to Angiogenesis Inhibition

Multiple compounds targeting the neo-angiogenic process of prostate cancer have been developed, including inhibitors of ligands or receptors, but to date none of these have shown promise. Worthwhile noting is that, similar to other cancers, angiogenic inhibitors have limited efficacy, and, eventually, all patients progress. This apparent lack of efficacy is linked to two major processes – innate and adaptive resistance (Bergers and Hanahan 2008).

Adaptive Resistance

Despite the apparent lack of long-term efficacy of angiogenic inhibitors, these compounds actually do perform what they were designed for. VEGF-A neutralizing antibodies and VEGFR-2 blockers along with kinase inhibitors can potently inhibit signaling and consequently block angiogenesis. However, under therapeutic pressure, cancer cells will adapt. Shutting down angiogenesis creates tumors with increased hypoxia as well as necrosis (McIntyre and Harris 2015). Cancer cells are used to hypoxia and managed to activate several alternate pathways to alleviate the acute crisis of drops in blood flow and nutrient supply (Rapisarda and Melillo 2009).

When only one aspect of angiogenesis, for example, the VEGF pathway, is blocked, cancer cells are capable of upregulating other factors, including FGFs (Rapisarda and Melillo 2009), angiopoietins (Rigamonti et al. 2014), and ephrins (Pircher et al. 2014). Furthermore, in cases of increased hypoxia by inhibition of angiogenesis, cancer cells recruit bone marrow-derived cells that aid restoring angiogenesis (Asahara et al. 1997). Finally, pericyte coverage of blood vessels

that remains after potent angiogenic inhibition might also save tumor cells (Song et al. 2005).

A very severe consequence of potent angiogenic inhibition is that cancer cells can become more aggressive to escape nutrient deprivation, and this tragically could bring about increased metastatic potential, e.g., more metastases (Pennacchietti et al. 2003). It has been previously shown that during angiogenic therapy, cancer cells are capable of activating pro-metastatic pathways, including, c-MET, Axl, and TGF-β (Sennino et al. 2012).

Innate Resistance

In the case of innate resistance, cancers are straightforwardly insensitive to angiogenic inhibition. This could be primarily because of the absence, or relative deficiency, of the particular pathway that is being targeted. Analysis of tumor-associated biomarkers could better identify patients who might be insensitive to a given anti-angiogenic therapy.

Anti-angiogenic Therapies of Advanced Prostate Cancer

Bevacizumab

The first anti-angiogenic treatment that arrived in the clinic was bevacizumab. The humanized monoclonal antibody targets VEGF-A and has been registered in a number of countries for the treatment of colorectal, lung, and renal cell cancers. Other indications with approval in several countries are breast cancer, glioblastoma, ovarian carcinoma, and carcinoma of the cervix. It has been among the most successful anticancer drugs and its global sales were worth about seven billion US dollars in 2015.

In early studies, bevacizumab did not exhibit single-agent activity in prostate cancer. There were, however, promising results in combination with docetaxel. Di Lorenzo reported a phase II study with 20 heavily pretreated patients, and 11 (55%) showed major PSA responses and 3 had objective responses (Di Lorenzo et al. 2008). One phase II study featuring a combination of bevacizumab, docetaxel, and estramustine included 79 patients with docetaxel-naive CRPC. A 50% PSA decline was observed in 58 patients (75%) and 23 of 39 patients with measurable disease had a partial response (59%) (Picus et al. 2011). While these response rates were encouraging, the combination had significant toxicity with a high rate of neutropenia and thromboembolic events.

CALGB90401 was a large, phase III trial with 1.050 men randomized to receive docetaxel either with bevacizumab at 15 mg/kg (DP-B) or placebo (DP). The primary endpoint was overall survival which was not significantly different among the two arms – 22.6 months for DP-B compared with 21.5 months for patients treated with DP (HR, 0.91; 95% CI, 0.78–1.05; stratified log-rank $p = 0.181$). The median PFS time was superior in the investigational arm (9.9 vs. 7.5 months, $p < 0.001$). Grade 3 or greater treatment-related toxicity was more common with DP-B (75.4% v 56.2%; $P <= 0.001$), as was the number of treatment-related deaths (4.0% v 1.2%; $P = 0.005$) (Kelly et al. 2012). The difference in cardiovascular events was particularly important regarding the increased risk of arterial thromboembolism (Patel et al. 2015).

Bevacizumab has therefore not been registered for the treatment of prostate cancer. Interestingly, with similar results in terms of progression-free survival (PFS) benefit, the addition of bevacizumab to chemotherapy has led to different conclusions for different types of tumors. For example, the AVADO trial compared docetaxel alone or with bevacizumab at two different doses for the treatment of first-line therapy for human epidermal growth factor receptor 2-negative, locally advanced, or metastatic breast cancer (Miles et al. 2010). There was no difference in overall survival between the three arms, and only a minimal PFS benefit was detected in one of them, being the combination of docetaxel with bevacizumab at 15 mg/kg, but not with 7.5 mg/kg, demonstrating superior median PFS to placebo plus docetaxel in an unstratified analysis (placebo mPFS, 8.2 months; 7.5 mg/kgmPFS,

9.0 months [HR, 0.86; $P = 0.12$]; 15 mg/kg mPFS, 10.1 months [HR, 0.77; $P = 0.006$]). Despite similar median PFS benefit, this trial was considered positive. This is an example how similar data can yield varied conclusions and different solid tumor types (Ocana et al. 2011).

Aflibercept

Aflibercept (also known as VEGF-Trap) is a recombinant fusion protein consisting of the extracellular domains of the human VEGF receptor 2 (VEGFR-2) fused to the Fc portion of human immunoglobulin G1. Aflibercept has high binding affinity to the isoform, VEGF-A, and also binds VEGF-B, PDGF-1, and PDGF-2, thereby inhibiting angiogenesis (Chu 2009). Aflibercept has been assessed alone and with other chemotherapies, including docetaxel, in preclinical models and exhibited activity in a DU145 prostate cancer cell line xenograft model. Aflibercept has also been assessed in phase I and III clinical trials with docetaxel, though no phase II trial of this combination has been conducted for men with metastatic CRPC (mCRPC) (Isambert et al. 2012).

The Venice trial was a phase III multicenter randomized double-blind placebo-controlled trial for patients with mCRPC (Tannock et al. 2013). Patients were randomized to docetaxel 75 mg/kg and aflibercept (6 mg/kg) or placebo every 3 weeks. 1224 men were randomly allocated to treatment. Median overall survival (the primary endpoint) was 22.1 months (95% CI 20.3–24.1) in the aflibercept group and 21.2 months (19.6–23.8) in the placebo group (stratified hazard ratio 0.94, 95.6% CI 0.82–1.08; $p = 0.38$). A higher incidence of grades 3–4 gastrointestinal side effects, hemorrhagic events, hypertension, fatigue, infections, and treatment-related fatal adverse events (21 [3.4%] vs. 9 [1.5%]) in the aflibercept group was recorded. Therefore, aflibercept has not been approved for the treatment of metastatic prostate cancer. In contrast, aflibercept was tested as a second-line treatment for metastatic colorectal cancer (in combination with 5-FU and irinotecan). Medium improvement of overall survival was 6 weeks, and this led to the approval of aflibercept in certain countries for the treatment of colon cancer (Van Cutsem et al. 2012).

Tyrosine Kinase Inhibitors (TKIs): Sunitinib, Sorafenib, and Cabozantinib

TKIs targeting both the VEGF and PDGF pathways revolutionized the treatment of metastatic kidney cancer in early 2000. Much efforts have also been placed into the development of this class of drugs for the treatment of metastatic prostate cancer.

Sunitinib blocks the VEGFR-2 and PDGF receptors, FLT3, and c-kit. A phase II trial that enrolled 36 patients who were progressing after docetaxel found that 4 patients (12.1%) had a $\geq$ 50% PSA decline and 7 (21.2%) had a $\leq$ 30% PSA decline (Sonpavde et al. 2010).

Another phase III trial featured 873 men with progressive mCRPC after docetaxel-based chemotherapy; patients were randomly assigned in a 2:1 ratio to receive sunitinib 37.5 mg/d continuously or placebo. Median OS was 13.1 months and 11.8 months for sunitinib and placebo, respectively (HR, 0.914; 95% CI, 0.762–1.097; $P = 0.168$). PFS was statistically significantly improved in the sunitinib arm (median 5.6 v 4.1 months; HR, 0.725; 95% CI, 0.591–0.890; $P < 0.001$). This difference was not deemed to be a clinically significant advantage, however, when considering the toxic side effects, such as fatigue (Michaelson et al. 2014).

Sorafenib has been studied in a phase II combination trial with bicalutamide. Eligible patients had rising PSA and minimal symptoms and were chemotherapy-naive. PSA declines of $\geq$50% took place in 12 (32%) of the 38 assessable patients, including 7 of 27 patients (26%) with prior antiandrogen use (Beardsley et al. 2012). While these results were encouraging, no large follow-up study has ever been reported.

Cabozantinib is a second-generation TKI that inhibits VEGFR-2 as well as MET, hence potentially preventing the development of resistance mechanisms. Cabozantinib also has important effects on the tumor microenvironment,

particularity against bone metastasis impacting the balance of osteoclast/osteoblast differentiation. Cabozantinib was tested in two phase II trials with 171 and 144 patients each.

In one randomized, phase II discontinuation study, 171 men were treated with cabozantinib – 72% of patients had regression in soft tissue lesions, whereas 68% of evaluable patients showed improvement upon bone scanning, including complete resolution in 12% (Smith et al. 2013). In addition, a second trial demonstrated significant improvements in clinically relevant parameters, like pain relief (57% of patients) and reduction or discontinuation of narcotic analgesics (55% of patients), along with improvements in measurable soft tissue disease, circulating tumor cells, and bone biomarkers (Smith et al. 2014).

Given these very encouraging results, cabozantinib was evaluated in two, randomized controlled, phase III trials. In particular, the so-called "Comet 1" and "Comet 2" studies investigated patients with mCRPC before or after treatment with docetaxel.

The Comet 1 trial randomly assigned 1.028 patients in a 1:1 ratio to either cabozantinib or prednisone. Median OS was 11.0 months with cabozantinib and 9.8 months with prednisone (HR, 0.90; 95% CI, 0.76–1.06; $P = 0.213$). Radiological PFS was improved in the cabozantinib group (median, 5.6 v 2.8 months; HR, 0.48; 95% CI, 0.40–0.57; stratified log-rank $P < 0.001$). Cabozantinib was associated with improvements in circulating tumor cell (CTC) conversion, bone biomarkers, and post-random assignment incidence of serial skeletal events (SSEs) but not in PSA outcomes (Smith et al. 2016).

Considering that Comet 1 failed the primary endpoint (OS), the Comet 2 study (docetaxel-naive CRPC patients) was abandoned.

On the contrary, cabozantinib has yielded positive results with other tumor types. For example, cabozantinib has been approved for the treatment of medullary thyroid cancer as well as for second-line treatment of metastatic clear cell carcinoma of the kidney (Choueiri et al. 2016; Elisei et al. 2013).

Tasquinimod

Tasquinimod is a quinoline-3-carboxamide derivative with anti-angiogenic activity. The inhibition of anti-angiogenesis has been demonstrated in a variety of assays, including the in vitro endothelial capillary tube formation assay. Human prostate cancer xenograft models also exhibited diminished tumor growth by at least 50% compared to control-treated animals (Dalrymple et al. 2007).

Tasquinimod can also inhibit regulatory myeloid-derived suppressor cells (MDSCs), possibly resulting in a decrease in the immune-suppressive tumor microenvironment. The agent has therefore a dual mechanism of action – immune regulation and anti-angiogenic effects (Olsson et al. 2015).

A phase I trial for men with CRPC established the maximum tolerated dose of tasquinimod at 0.5 mg per day, but when a stepwise intra-patient dose escalation was employed, 1.0 mg per day was also well-tolerated. Several disease stabilizations were seen (Bratt et al. 2009).

One randomized phase II trial with 201 male participants (134 assigned to tasquinimod, 67 to placebo) exhibited a 6 -month PFS rate (the primary endpoint) for tasquinimod and placebo groups at 69% and 37%, respectively ($P < 0.001$), and PFS was 7.6 versus 3.3 months ($P = 0.0042$) (Pili et al. 2011). Based on these results, two randomized phase III studies were performed.

1245 men with chemotherapy-naive mCRPC and evidence of bone metastases were assigned (2:1) to receive tasquinimod once per day or placebo until progression or toxicity. The patient population was typical with a median age of 71 years, Karnofsky performance status $\geq$90% close to 80%, and median PSA levels around 50 ng/l.

Estimated median mPFS by central review was 7.0 months (95% CI 5.8–8.2) with tasquinimod and 4.4 months (95% CI 3.5–5.5) with placebo (HR 0.639 [95% CI 0.544–0.751]; $P < 0.001$). With a median follow-up of 30.0 months for tasquinimod and 30.7 months for placebo, the median OS was 21.3 months (95% CI 19.5–23.0) with tasquinimod but 24.0 months (95% CI 21.4–26.9) with placebo (HR 1.097 [95% CI 0.938–1.282]; $p = 0.247$). The authors

concluded that in chemotherapy-naive men with mCRPC, single-agent tasquinimod statistically significantly improved mPFS versus placebo. With this, no OS benefit was observed with tasquinimod (Carducci et al. 2015) However, a randomized placebo-controlled maintenance study after docetaxel did indicate there was a mPFS benefit for tasquinimod; OS data is not yet mature (Fizazi et al. 2016).

Taking into account these relatively disappointing results, further development of tasquinimod has been put on hold.

Thalidomide

The anti-angiogenic effect of thalidomide was accidentally discovered more than 20 years ago. The drug was initially marketed in 1957 to relieve morning sickness in pregnant women. Subsequently, it was linked to over 10.000 cases of phocomelia and other deformations, such as that of the heart or internal organs, and its use is widely thought to be one of the dark chapters in modern medicine (Vargesson 2015). In 1994, it was demonstrated that thalidomide inhibits basic lymphoblast growth factors (BLGF) that induce angiogenesis (D'Amato et al. 1994). Since then, thalidomide has been evaluated as an anticancer drug with anti-angiogenic properties.

Thalidomide and eight thalidomide analogues have been tested in human prostate cancer xenograft models (Ng et al. 2004). Clinical development has included a randomized phase II study employing combination with docetaxel. Seventy-five patients with chemotherapy-naive metastatic CRPC were randomly assigned to receive three weekly docetaxel doses with or without daily thalidomide at 200 mg orally ($n = 50$). The proportion of patients with a greater than 50% decrease in PSA was higher in the docetaxel/thalidomide group (53% in the combined group, 37% in the docetaxel-alone arm). The median PFS in the docetaxel group was 3.7 months, while it was 5.9 months in the combined group ($P = 0.32$). At 18 months, OS in the docetaxel group was 42.9% and 68.2% in the combined group (Dahut et al. 2004).

Similar activity has been described by Figg and colleagues. Fifty-nine patients were treated with the combination, and responses increased to 53% for those receiving the combination treatment versus 36% with docetaxel alone. A greater number of thromboembolic event rates with the combination were documented in both trials (Figg et al. 2001; Chen et al. 2014).

The combination of docetaxel, thalidomide, and bevacizumab was tested in a single-arm, phase II trial that enrolled 60 patients. Eighty-eight percent of patients experienced a drop in PSA of at least 50%. The time to progression (TTP) was 18.3 months and the OS was 28 months. This was double the survival of historical controls based on the Halabi nomogram (Ning et al. 2010).

Despite these motivating findings, no phase III trials have been initiated. The additional toxicity of thalidomide and the increased rate of significant toxicities (grades 3–4) of neutropenia, additional thrombosis and vascular events, neuropathy, constipation, and fatigue have hampered continued development.

Lenalidomide

Lenalidomide is an analogue of thalidomide, selected for its better tolerance and side effect profile. It is thought to be both an immune modulator and an anti-angiogenic compound. Lenalidomide was studied in preclinical models, including prostate cancer cell lines, and demonstrated single-agent activity as well as synergism with docetaxel (Henry 2012).

Phase I and phase II clinical trials have been carried out in patients with solid tumors. With this, it has been seen that men with mCRPC had a 25% response rate (Sanborn et al. 2009). Responses reflected by PSA declines were observed when lenalidomide was combined with docetaxel in 47% of patients that had not received docetaxel prior and in 50% of patients that did.

In a phase II, single-arm study with lenalidomide before chemotherapy in men with mCRPC, 32 patients were enrolled; stable disease was observed for 20 patients suggesting a clinical

benefit rate of 63%. The median time to radiographic progression was 4 months (2–16 months); the median OS was 20 months. Of 27 PSA-evaluable patients, 13 (48%) had a reduction in PSA levels; 3 (11%) had >50% PSA decrease; the median time to PSA progression was 3 months (2–9 months) (Nabhan et al. 2014).

In considering these preliminary but promising results, a phase III study was undertaken (Mainsail). Patients were administered 75 mg of docetaxel and 5 mg prednisone twice daily with either 25 mg lenalidomide or placebo daily. The study included overall 1.059 patients. Unfortunately, the study was ceased early because of futility. The OS was 17.7 months in the lenalidomide group and had not been reached in the placebo group. Additionally, in the investigation arm, there were more grades 3–4 side effects, such as neutropenia, febrile neutropenia, diarrhea, pneumonia, dyspnea, asthenia, and pulmonary embolism. The authors ultimately felt that further research of the combination was not warranted (Petrylak et al. 2015).

Conclusion

Overall, prostate cancer can utilize multiple pro-angiogenic pathways, making it a promising target for anti-angiogenic therapy. Unfortunately, so far, all attempts to introduce anti-angiogenic drugs into the treatment algorithm for prostate cancer

Table 1 Results of phase III trials with anti-angiogenic agents in men with prostate cancer

Agent	n/Population	Treatment	PFS	OS
Bevacizumab (Kelly et al. 2012)	1050, chemotherapy-naive CRPC	Docetaxel (D) 75 mg/mg plus prednisone (P) 10 mg with either bevacizumab (B) 15 mg/kg IV every 3 weeks (DP + B) or placebo	DP + B arm 9.9 v DP + P 7.5 months ($P < 0.001$)	22.6 months compared to 21.5 months for placebo (HR, 0.91; 95% CI, 0.78–1.05; $P = 0.181$)
Sunitinib (Michaelson et al. 2014)	873, progressive mCRPC after docetaxel	Randomization 2:1 to receive sunitinib 37.5 mg/d continuously or placebo	Sunitinib median 5.6 v 4.1 months with placebo; HR, 0.725; 95% CI, 0.591–0.890; stratified log-rank test, $P < 0.001$)	OS was 13.1 months and 11.8 months for sunitinib and placebo, respectively (HR, 0.914; 95% CI, 0.762–1.097; $P = 0.168$)
Tasquinimod (Carducci et al. 2015)	1245, chemotherapy naive CRPC	Tasquinimod (TASQ) 0.25–1 mg v placebo	rPFS by central review was 7.0 months (95% CI 5.8–8.2) with TASQ and 4.4 months (95% CI 3.5–5.5) with placebo (HR 0.639 [95% CI 0.544–0.751]; $p < 0.001$)	Median OS was 21.3 months (95% CI 19.5–23.0) with TASQ and 24.0 months (95% CI 21.4–26.9) with placebo (HR 1.097 [95% CI 0.938–1.282]; $p = 0.247$)
Cabozantinib (Smith et al. 2016)	1028, progressive mCRPC after D and abiraterone and/or enzalutamide	Cabozantinib 60 mg/d v P matched placebo	rPFS was 5.6 months with cabozantinib and 2.8 months with P (HR, 0.48; 95% CI, 0.40–0.57; $P < 0.001$)	11.0 months with cabozantinib and 9.8 months with P (HR, 0.90; 95% CI, 0.76–1.06; $P = 0.213$)
Lenalidomide (Petrylak et al. 2015)	1059, chemotherapy-naive CRPC	D (75 mg/mg) on day 1 and P (5 mg twice daily) on days 1–21 and either lenalidomide (25 mg) or placebo	10.4 months (IQR 6–14) in the lenalidomide group and 10·6 months (IQR 8–16) in the placebo group	17.7 months (95% CI 14.8–18.8) in the lenalidomide group and not reached in the placebo group (HR 1.53, 95% CI 1.17–2.00, $p = 0.0017$)

have failed. Thousands of men have taken part in clinical trials, and in contrast with other types of cancers, few approvals have been granted. While most agents have elicited a PFS benefit (see Table 1), this did not translate into an OS benefit. In actual fact, lenalidomide lessened survival significantly compared to placebo. Several explanations have been posed to explain these failures.

First, prostate cancer manages to rapidly activate multiple adaptive resistance mechanisms, leading to failure of these therapies. A better understanding and description of patients failing angiogenic drug administration could result in personalization of the therapy regimens. In clinical studies, to date, no particular patient population was enriched based on any biomarkers, and deciphering intra-patient, population heterogeneity is currently not considered for the treatment of CRPC (Dayyani et al. 2011).

Another issue in prostate cancer is the lack of intermediate or surrogate endpoints. While PSA, radiological responses, and CTCs decline, and others have been studied to serve as surrogates for OS, none have been shown to possess significant power to predict OS benefit and hence are not recognized by regulatory agencies (Scher et al. 2011).

Another problem is the side effect profile of anti-angiogenic drugs. The increase of thromboembolic events is a constant problem and limits their use. Fatigue and increased neutropenia rates when in combination with other chemotherapeutic agents are particularly relevant for the elderly and frail prostate cancer patient population.

Currently, the development of anti-angiogenic drugs alone or in combination with existing chemotherapy (docetaxel) is restricted by the many negative clinical trial findings. Further research, however, should be considered in combination with immunological agents in select patient populations driven by relevant biomarkers.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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The Value of Anti-angiogenics in Bladder Cancer Therapy

Gerald Bastian Schulz and Alexander Karl

Contents

Introduction 594
Angiogenesis in Bladder Cancer: Microvessel Density and Other Early Approaches 595
Angiogenic Pathways in Bladder Cancer 596
Targeting Angiogenesis in Bladder Cancer Clinical Trials 599
Bevacizumab 599
Aflibercept 599
Sunitinib 600
Everolimus 600
Sorafenib 600
Pazopanib 600
Cabozantinib 600
Ramucirumab/Icrucumab 601
Vandetanib 601
Nintedanib/Regorafenib 601
Conclusion 601
Cross-References 602
References 602

Abstract

The therapy of metastatic bladder cancer (BC) mainly relies on platinum-based chemotherapy with very limited options like vinflunine in the second line. Therefore, there has not been much change in the median survival of this patient cohort within the last decades. Angiogenesis is a well-proven patho-mechanism in

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BC. Different angiogenic pathways have been elucidated within the last 20 years, among them vascular endothelial growth factor (VEGF), angiopoietin/Tie, matrix metalloproteinases (MMPs), fibroblast growth factor (FGF), thrombospondin 1 (TSP1), and hypoxia-inducible factor (HIF) as the most prominent ones. In order to be able to understand the mechanisms of novel compounds, it is helpful to have a basic knowledge of the relevance of these signaling pathways in the field of BC. Therefore, we not only sum up the majority of clinical studies on anti-angiogenics in BC but also explain the most important pathways responsible for bladder tumor angiogenesis in this chapter.

Many established anti-angiogenics like bevacizumab, sunitinib, sorafenib, aflibercept, pazopanib, and vandetanib have been tested in clinical trials for BC. Furthermore, the pipelines are filled with clinical trials on novel anti-angiogenic drugs like ramucirumab, icrucumab, regorafenib, nintedanib, and many more. However, no compound has yet proven significant single-agent efficacy. Therefore, up to now, no anti-angiogenic drug has been approved for BC therapy.

Consequently, future clinical trials will not only have to test for new anti-angiogenics, but also for different treatment algorithms as well as combination therapies. Although most of the studies showed disappointing overall results, subcohorts had a significant benefit. Therefore, novel approaches should increasingly focus on identifying patient subgroups with the greatest susceptibility to the respective anti-angiogenic therapy.

Keywords

Bladder cancer · Urothelial carcinoma · Sorafenib · Sunitinib · Ramucirumab · Pazopanib · Aflibercept · Bevacizumab · VEGF · FGF · Angiopoietin

Introduction

Bladder cancer (BC) is the ninth most common cancer entity and accounts for approximately 150,000 deaths each year worldwide. The incidence is about four times higher in the male population, which makes BC the fourth most common malignancy in men. In Europe and North America, about 90% of BC are urothelial carcinoma (UC), followed by squamous cell carcinoma (SCC). By far the most relevant clinical risk factor is smoking followed by occupational exposure to different toxins. About 75% of patients with BC are initially diagnosed with non-muscle-invasive BC (NMIBC) (pTa, CIS, pT1). The most common symptom finally leading to the diagnosis of BC is macrohematuria.

NMIBC is treated endourologically with transurethral resections of the bladder (TUR-B) as well as intravesical chemotherapy. Patients with high-grade NMIBC are eligible for intravesical immunotherapy with BCG (bacillus Calmette-Guérin) for up to 3 years. Follow-up of NMIBC in order to prevent both progression to MIBC and recurrence is based on regular urethrocystoscopy and urine cytology. This makes BC one of the most expensive cancer entities. Although much effort has been invested into novel diagnostic and prognostic markers, none has been implemented into clinical practice yet.

Gold standard for muscle-invasive bladder cancer (MIBC) is still radical cystectomy (RC). Available data on neoadjuvant chemotherapy is partially contradictory with absolute 5-year OS improvement between 5% and 8% (Babjuk et al. 2016). Adjuvant therapy algorithms are based on cisplatin in the first line, in combination with gemcitabine (GC), paclitaxel (PCG) or methotrexate, vinblastine, and Adriamycin (MVAC). As second-line treatment, only vinflunine is established at the moment. Whereas 5-year survival in NMIBC ranges around 90%, this decreases to $<$50% in MIBC. In metastatic disease median survival is around 15 months only. Interestingly, the huge difference in the aggressiveness between low-grade NMIBC and high-grade MIBC is paralleled on the molecular level by two distinct pathways. While in papillary low-grade tumors activating FGF3 mutations are frequently found, TP53 mutations are more common in carcinoma in situ (CIS) and MIBC (Knowles and Hurst 2015).

Compared to other cancer entities, there has been only very limited therapeutic progress in the field of BC. In 2016, the PD-1/PD-L1 inhibitor atezolizumab has been approved by the FDA for locally advanced or metastatic BC. As the last major clinical breakthrough, BCG intravesical immunotherapy has been implemented nearly 40 years ago; every opportunity that might ultimately lead to the improvement of BC therapy has to be embraced.

The role of tumor angiogenesis as a major driver for BC pathogenesis is broadly accepted. In the first part of this chapter, we want to elucidate the role of different angiogenic pathways in BC. Besides the VEGF pathway, fibroblast growth factor, angiopoietins, matrix metalloproteinases, as well as hypoxia-inducible factors are central stakeholders. In the following main part, both completed clinical trials and open studies on anti-angiogenic therapies will be presented in detail.

Angiogenesis in Bladder Cancer: Microvessel Density and Other Early Approaches

Angiogenesis is a central mechanism in the pathogenesis of BC and therefore also a highly attractive therapeutic target (Bochner et al. 1995). For more than 20 years, studies are being published on angiogenesis in BC. At the beginning, similar to other cancer entities, anatomical angiogenesis markers like microvessel density (MVD) were correlated with different clinical parameters. In the following years, as angiogenesis became one of the main focuses in cancer research, VEGF and other prominent pathways were investigated in the context of BC.

The most direct way to evaluate angiogenesis is the histopathologic assessment of endothelial cells. Within the last years, different approaches like the MVD, microvessel count (MVC), and the vessel surface area (VSA) have been used for this purpose.

An increase in MVD was correlated with advanced BC stage and grade and worse prognosis (Canoglu et al. 2004; Chaudhary et al. 1999; Sankhwar et al. 2015). Compared to NMIBC, MVD is elevated in MIBC (Shirotake et al. 2011). A study of 61 patients with NMIBC and MIBC significantly associated with high MVD with reduced survival. Furthermore, high MVD in NMIBC was correlated with progression to MIBC (Goddard et al. 2003). In MIBC, MVD was shown to be a prognostic marker for disease recurrence after radical cystectomy (RC) (Inoue et al. 2000a). On the other side, elevated VSA could be correlated with longer disease-free survival rates in the same cohort (Bertz et al. 2014). There are also studies showing an increased MVD in organ-confined tumors compared to T3–4 BC as well as nodal negative compared to lymph node-positive BC (Herrmann et al. 2007). Intravesical immunotherapy with BCG in high-grade NMIBC is able to prevent progression and recurrence. However, as this instillation therapy comes with the risk of significant side effects, prognostic markers for identifying possible therapy responders are needed. MVD has been shown to be an independent prognostic marker for recurrence after BCG-therapy (Ajili et al. 2012).

These results clearly illustrate the challenge of several coexisting scores for assessing clinical relevant angiogenesis. On the one hand, the partially contradicting data on tumor angiogenesis might be based on different quantitative markers for angiogenesis. On the other hand, several studies did not evaluate the MVD blinded, which might lead to bias. Therefore, simply correlating clinical data with just “more” or “less” angiogenesis might not be sufficient in future. Most probably the focus might shift on describing different aspects of angiogenesis and tumor microvessels like architecture, vessel count, and diameter as well as interplay with perivascular components like smooth muscle cells, extracellular matrix, and pericytes. To sum up, one can say that MVD as the most reliable marker for angiogenesis is associated with advanced disease and dismal prognosis.

Compared to angiogenesis, lymphangiogenesis is much less investigated in BC. However, lymphovascular invasion is a scientifically proven prognostic factor in BC and might contribute to metastatic spread. Indeed, VEGF-C, well known

for its role in controlling lymphangiogenesis, has been shown to be an independent prognosticator for adverse oncologic outcome. Furthermore, high VEGF-C expression in histopathological BC specimen could be correlated with stage, MVD, and lymph node metastasis (Suzuki et al. 2005). As most patients with BC die of metastatic disease, the inhibition of lymphangiogenesis might be a novel therapeutic target (Benoit et al. 2015; Yang et al. 2011).

Angiogenic Pathways in Bladder Cancer

VEGF/VEGFR was the historically first pathway associated with angiogenesis and also the first used as a target for anti-angiogenics. In NMIBC, elevated serum levels of VEGF were significantly associated with a decrease in cancer-specific survival (CSS). Moreover, serum VEGF levels at the top quintiles were shown to be an independent prognostic marker for overall survival (OS) and CSS (Puntoni et al. 2016).

In BC patients, urine levels of VEGF are not only significantly elevated compared to control groups, but are also positively correlated with stage. Tissue VEGF levels were correlated with urine VEGF levels in this study (Sankhwar et al. 2015).

In NMIBC, both tissue and serum protein levels of VEGF are upregulated compared to controls (Kozakowska et al. 2016; Sankhwar et al. 2015). RNA and protein expression of VEGF and its receptor VEGFR1 are elevated in BC tissue compared to control groups. With advanced stage and grade as well as in MIBC compared to NMIBC, VEGF expression in human histopathological specimen is significantly higher. However, recurrence-free survival as a clinical endpoint could not be significantly associated with the expression of these angiogenic molecules in this study (Yang et al. 2004). The role of the VEGF pathway in BC becomes even more complicated, as VEGF is upregulated in NMIBC compared to MIBC, whereas for VEGFR the opposite can be seen, as VEGFR is upregulated in MIBC compared to NMIBC (Kopparapu et al. 2013). In a cohort of 72 patients with UC, VEGFR2 expression in histopathological specimen could be correlated with stage and invasiveness, confirming the conclusions from abovementioned studies (Xia et al. 2006).

In summary, VEGF signaling is increased in BC and was correlated with clinical outcome in urine, serum, and histopathological specimen.

Endocan (ESM1), a protein preferentially expressed in endothelial cells, is overexpressed in different cancer types like lung, renal cell, and hepatocellular cancer. By binding to its receptor VEGFR2, VEGF regulates the expression of endocan in endothelial cells. Compared to benign bladder tissue, endocan levels are increased in BC microvessels and could be correlated with both staging and recurrence-free survival in BC. Therefore, external validation is required to evaluate endocan as a prognostic biomarker (Roudnicky et al. 2013; Yang et al. 2015). Moreover, serum and urine endocan levels are increased in BC compared to control groups. However, diagnostic sensitivity for BC only ranges between 50% and 62%. The specificity of this potential diagnostic marker is reduced, as endocan expression in serum and urine is increased in patient with urinary tract infections (Laloglu et al. 2016).

One of the first proven drivers for angiogenesis in both physiological and pathological conditions is hypoxia. Among the most prominent components of this signaling pathway are the hypoxia-inducible factors (HIF). These transcription factors stand upstream of the VEGF pathway, by which HIF is able to activate neo-angiogenesis. In NMIBC, both HIF-1α and HIF-1β are elevated on RNA level in BC tissue compared to control groups. Another study investigated HIF-1α in urine samples, demonstrating a significant gain in BC sensitivity when HIF-1α is combined with urine cytology. However, there was no correlation between HIF-1α and MVD in this study (Badr et al. 2013). HIF-1α might also be of use as a diagnostic marker, as the expression in human BC tissue has been correlated not only with VEGF and MVD, underlining the significance of this pathway for angiogenesis in BC, but also with stage and grade (Deniz et al. 2010). The

significant association between HIF-1α and MVD and VEGF was shown in a study including 99 patients with UC. Additionally, both HIF-1α and MVD proved to be independent factors for disease-free survival (DFS) (Chai et al. 2008). Immunohistochemical expression of HIF-1α was higher in both MIBC and CIS (Ioachim et al. 2006). For upper tract urothelial carcinoma (UTUC), HIF-1α expression was also found to be a prognostic factor for oncologic outcome (Ke et al. 2008). Although HIF might not be used as a target for anti-angiogenic therapy in the near future, this transcription factor might help us to identify patients susceptible to novel anti-angiogenics.

Heme oxygenase is an enzyme important for the conversion of heme to biliverdin. However, several studies indicated an additional role of heme oxygenase 1 (HO-1) in tumor angiogenesis, shown for glioma, prostate cancer, and pancreatic cancer. Interestingly, heme HO-1 has also been shown to be elevated in NMIBC (Kozakowska et al. 2016). Increased HO-1 protein levels are correlated with an increase in MVD in human BC specimen. As inhibition of HO-1 with zinc protoporphyrin (ZnPP) inhibits angiogenesis in vivo in BC, ZnPP might be of clinical relevance (Miyake et al. 2011). This is supported by other studies on this component, which could show that ZnPP not only decreases angiogenesis but far more important also tumor growth (Cheng et al. 2016).

Another mediator of angiogenesis in BC seems to be the macrophage migration inhibitory factor (MIF). MIF is a pro-inflammatory cytokine promoting both hypoxia-induced and non-hypoxic neo-angiogenesis. MIF mediates HIF-induced VEGF expression and activates tumor-associated macrophages (TAM) that are known to play a central role in angiogenesis (Chesney and Mitchell 2015). In a murine BC model, inhibition of MIF not only reduced the stage of BC but also angiogenesis, accentuating the relevance of this link in BC. The relevance of MIF for neo-angiogenesis was confirmed in different cancer entities like melanoma and breast, ovarian, gastric, hepatocellular, and lung cancer (Choudhary et al. 2013). As a phase I study evaluating an anti-MIF antibody is under way (NCT01765790), further studies will have to investigate the role of MIF in human BC.

The angiopoietin-tie (Ang-Tie) signaling system plays a central role for the equilibrium between neo-angiogenesis and vascular quiescence. The ligand angiopoietin 1 (Ang1) acts as an agonist for the mainly vascular-based receptor tyrosine kinase (RTK) Tie2 and mediates stabilization of the vasculature. In this simplified overview of this pathway, the antagonist Ang2 promotes neo-angiogenesis. However, the role of the Ang-Tie signaling system in BC angiogenesis is unclear so far. In a study comparing serum levels of different angiogenic molecules, Ang1 was significantly higher in patients with BC compared to control groups. The same study also found the soluble receptor Tie2 to be correlated with oncologic outcome (Szarvas et al. 2009). In NMIBC human tissue, Ang1 expression was shown to be significantly lower compared to the normal urothelium on both RNA and protein level, whereas VEGF expression was increased. Interestingly, high Ang2 expression was correlated with tumor recurrence in a multivariate analysis in this study, which could be verified by another study (Oka et al. 2005; Szarvas et al. 2008). These data fit to our current understanding of this pathway, where Ang1 inhibits angiogenesis and Ang2 acts as its antagonist. In a study comparing MIBC with NMIBC, both VEGF and Ang2, markers for increased angiogenesis, were elevated in NMIBC, whereas Ang1, the Tie2 ligand responsible for vessel stabilization and maturation, was elevated in MIBC. According to these results, there might be an angiogenic switch when BC becomes muscle invasive. Therefore, NMIBC might be characterized by a highly active vessel remodeling and angiogenesis, whereas in muscle-invasive stages, mature vessels are able to supply the tumor with a sufficient blood supply (Quentin et al. 2004). A quite new therapeutic approach which can be derived from abovementioned studies might be targeting vessel maturation instead of just inhibiting neo-angiogenesis. In conclusion, it is quite difficult to evaluate the data on the Ang-Tie system in BC, as this signaling pathway can promote

angiogenesis via Ang2 signaling as well as mediate vessel quiescence via Ang1, and yet it is completely unclear which is more detrimental. Clinical studies on angiopoietin inhibitors like the peptibody trebananib (AMG 386) are performed for different solid tumors. However, according to our knowledge, there is no clinical study with the focus on BC for angiopoietin modulators at the moment.

Basic fibroblast growth factor (bFGF) is a well-known mediator of angiogenesis (Przybylski 2009). When UC cell clones with high bFGF expression were transplanted into mice, these clones were of significantly higher malignancy compared to controls (Chikazawa et al. 2008). In a clinical study, bFGF and VEGF in surgical specimen were found to be independent prognosticators for adverse oncologic outcome after neoadjuvant chemotherapy (Inoue et al. 2000a). Furthermore, a significant association between urinary bFGF and BC stage has been proven (Gravas et al. 2004; Zaravinos et al. 2012). Dovitinib, a tyrosine kinase inhibitor of FGFR3 and VEGFR2, is tested in a phase II study in a cohort of BCG-refractory patients (NCT01732107). Another phase II study investigates JNJ-42756493, a pan-FGFR inhibitor in a cohort of metastatic UC (NCT02365597).

During the process of neo-angiogenesis, the perivascular space including basement membrane and extracellular matrix has to be remodeled. This complex and yet poorly investigated process is a predisposition for migrating endothelial cells, which are guided by angiogenic factors retained and subsequently released by the ECM. Important stakeholders controlling this remodeling machinery are matrix metalloproteinases (MMPs) and the respective inhibitors, tissue inhibitors of matrix metalloproteinases (TIMP). In peripheral blood leukocytes, elevated MMP9 was significantly associated with increased BC grade (Wieczorek et al. 2015). Interestingly, MMP9 expression in 131 patients with UC could be correlated with both stage and grade, with higher expression levels seen in advanced stage and high-grade tumors. This underlines the biological significance of MMP9 in BC angiogenesis (Donmez et al. 2009). In another study comparing MMP9 serum levels of BC patients with a healthy control group, MMP9 levels were not only significantly higher in the BC cohort, but also correlated with stage and grade (Guan et al. 2003). Additionally to MMP9, MMP1 seems to play a similar role in BC, as increased urine concentrations of MMP1 are seen in BC patients and high MMP1 levels were positively correlated with BC stage and grade (Durkan et al. 2001).

Among others, MMP9 expression is induced by heparin-binding epidermal growth factor-like growth factor (HB-EGF). Cancer cells expressing HB-EGF induce tumors with a higher vascularization (Ongusaha et al. 2004). As there are studies suggesting HB-EGF to act as a downstream target of VEGF, it might be worth evaluating HB-EGF as a novel target for anti-angiogenic therapy (Arkonac et al. 1998).

Interleukin-8 (Il-8) mediates tumor angiogenesis through tumor-infiltrating macrophages (Qazi et al. 2011). In BC, Il-8 inhibition reduces the expression of MMP9, which plays a relevant role in matrix remodeling. In a mouse model, inhibition of Il-8 by an antibody was able to reduce both tumor growth and MVD (Inoue et al. 2000b; Mian et al. 2003). The relevance of Il-8 in BC pathogenesis is underlined by a study showing Il-8 to be significantly elevated in the urine of BC patients compared to control groups as well as in MIBC compared to NMIBC (Sheryka et al. 2003). Summarizing, in the short and medium term, clinical relevance of MMPs and Il-8 might be mainly derived from their use as biomarkers and less likely from their therapeutic approaches.

In a murine BC model, the transcription factor Krüppel-like factor 5 (KLF5) has been shown to regulate VEGF. In the same study, the expression of KLF5 and VEGF correlated in human BC tissue, which makes KLF5 a possible novel target for anti-angiogenic therapy (Gao et al. 2015).

BLCA1 is a nuclear matrix protein used for BC detection. In human BC tissue, elevated BLCA1 expression has been correlated not only with clinical parameter like advanced stage but also angiogenic markers including VEGF, MMP9, and MVD (Feng et al. 2015). However, further studies are needed in order to decipher the relevance of KLF5 and BLCA1 for BC angiogenesis.

Angiotensin receptors are not only expressed in several tumors but might also play a significant role in angiogenesis (Miyajima et al. 2009). In BC, angiotensin II type 1 receptor (AT1R) expression in MIBC and high-grade NMIBC was higher compared to NMIBC and low-grade NMIBC. What is more, AT1R was significantly correlated to MVD, which makes AT1R a possible new target for anti-angiogenic therapy in BC (Shirotake et al. 2011).

Thrombospondin is a well-known inhibitor of angiogenesis (Lawler and Lawler 2012). In patients undergoing RC for BC, thrombospondin is not only downregulated but furthermore could also be correlated to oncologic outcome in a multivariate analysis (Shariat et al. 2010). Both advanced stage and grade are associated with decreased expression of thrombospondin. Interestingly, in the same study, VEGF and MMP9 were found to be alternated in inverse correlation to thrombospondin, underlining the significance of this pathway (Donmez et al. 2009). In NMIBC, thrombospondin was shown to be an independent predictor for progression to invasive disease (Goddard et al. 2002). Androgens act as thrombospondin inhibitors, which makes them indirect mediators of angiogenesis. In murine in vivo models for BC, castration inhibited tumor growth significantly. However, the relevance for antiandrogen therapy for BC is completely unclear (Johnson et al. 2008). Thrombospondin has been used as an anti-angiogenic agent in different phase I clinical studies against advanced tumors of different cancer entities, both as a single agent and within a combination therapy with bevacizumab and cisplatin/gemcitabine (Gietema et al. 2006; Gordon et al. 2008; Uronis et al. 2013). However, to our knowledge there are no open clinical trials for BC with thrombospondin-mimetic agents at the moment.

Endostatin, like thrombospondin, belongs to the group of endogenous angiogenesis inhibitors. Although the role of endostatin in BC has been investigated, the clinical relevance as a biomarker or even therapeutic target is largely unclear (Du and Hou 2003; Szarvas et al. 2012).

The role of microRNAs in BC, like in most of the other cancer entities, remains quite unclear although several groups have been working on this topic (Guancial et al. 2014). Murine in vivo experiments established microRNA-34a as both metastasis suppressive and anti-angiogenic. Moreover, microRNA-34a levels were also decreased in bladder cancer tissue (Yu et al. 2014).

Targeting Angiogenesis in Bladder Cancer Clinical Trials

Different clinical trials targeting BC angiogenesis have been performed. Therefore, anti-angiogenics have been used both as single agents and in combination with standard chemotherapy. However, only very few phase III trials were completed or are ongoing (Mazzola and Chin 2015; Pinto et al. 2010; Sonpavde and Bellmunt 2016). The following section gives an overview on the majority of clinical studies performed on anti-angiogenics for BC.

Bevacizumab

Bevacizumab most likely is the most prominent anti-angiogenic, interfering with the VEGF pathway. In a phase II study testing bevacizumab in addition to gemcitabine/cisplatin (GC) in a first-line chemotherapy setting for metastatic or unresectable UC, overall response rate was 72%, median progression-free survival (PFS) 8.2 months, and median overall survival (OS) 19.1 months (Hahn et al. 2011). An ongoing phase III trial compares GC chemotherapy for metastatic or unresectable BC with GC plus bevacizumab (NCT00942331).

Aflibercept

Another approach to target the VEGF system is aflibercept, a fusion protein able to neutralize different VEGF isoforms. In a phase II study, aflibercept was administered to a cohort of BC patients previously treated with a platinum-based chemotherapy. Unfortunately, there has not been a significant improvement of oncologic outcome (Twardowski et al. 2010).

Sunitinib

Sunitinib is a receptor tyrosine kinase (RTK) inhibitor targeted against different receptors, among them both VEGFR and PDGFR. In a multicentric, double-blinded phase II study, sunitinib versus placebo were investigated as a maintenance therapy with 6-month progression rate as the primary endpoint. Fifty-four patients were included after four to six cycles of chemotherapy with locally recurrent or metastatic BC. Although serum VEGFR2 levels were reduced under sunitinib maintenance, the 6-month progression rate was not reduced by the anti-angiogenic agent (Grivas et al. 2014). In addition, a phase II study tested two different schedules of sunitinib in a cohort of metastatic BC with previous chemotherapy. Although oncologic response was seen in some patients with tumor regression lasting between 0.6 and 23.4 months, overall results were disappointing, and the threshold of at least 20% of activity defined by RECIST was not achieved (Gallagher et al. 2010). Another phase II clinical trial tested the combination of gemcitabine, cisplatin, and sunitinib for BC in both the adjuvant and neoadjuvant setting. However, the trial was closed due to excess toxicity of the combination therapy (Galsky et al. 2013).

Everolimus

A phase II study including 37 patients tested everolimus in a patient cohort of UC after failure of a platinum-based chemotherapy with disease control rate (CCR) as primary endpoint. Everolimus was well tolerated and DCR was 27% after 8 weeks. Importantly, efficacy was enhanced in a patient subcohort with higher initial angiopoietin-1 levels, which might be of utmost importance when selecting suitable patients for anti-angiogenic therapy in BC (Seront et al. 2012).

Sorafenib

A multicenter phase II study evaluated sorafenib in addition to conventional chemotherapy with GC compared to GC alone as first-line adjuvant chemotherapy. Primary endpoint was PFS in this study, including 89 patients. In the final analysis, no significant difference between the two arms in respect to ORR, median PFS, and OS was observed (Krege 2014). Sorafenib was also tested as first-line chemotherapy in metastatic UC in a phase II study. However, sorafenib did not show any significant improvements (Sridhar et al. 2011).

Pazopanib

Pazopanib is a multi-targeting RTK inhibitor with anti-angiogenic activity by targeting FGFR, PDGFR, and VEGFR. In a phase II study, pazopanib was tested in 19 patients with metastatic UC after previous chemotherapy. However, results were disappointing and the study was closed after the interim analysis (Pili et al. 2013). Another randomized phase II study compared pazopanib with paclitaxel in 131 patients with advanced BC and previous platinum-based chemotherapy. Primary endpoint was OS. The study was closed as futility criteria were fulfilled. In this trial, pazopanib did not prove to be superior to paclitaxel as second-line therapy (Powles 2016). Pazopanib was also tested in a single-arm phase II study (NCT01031875) in a cohort of platinum-based chemotherapy pretreated patients with metastatic UC. An objective response rate of 17.1% was reported in this study (Necchi et al. 2012). Interestingly, IL-8 levels were identified as a prognostic marker for oncologic response to pazopanib (Necchi et al. 2014).

Cabozantinib

Cabozantinib is a small molecular inhibitor targeting the VEGF and MET pathways. A phase II study for advanced/metastatic BC is under way, and study completion is estimated for August 2018 (NCT01688999). Interestingly, levels of regulatory T cells (Treg) in the peripheral blood before the administration of cabozantinib correlate with partial response (Apolo 2014).

Ramucirumab/Icrucumab

In a randomized, controlled phase II study (NCT01282463), enrolling 140 patients after platinum-based chemotherapy for advanced UC docetaxel was compared to the combination of docetaxel/ramucirumab and docetaxel/icrucumab (Petrylak 2016). PFS was used as the primary endpoint. Ramucirumab is a monoclonal antibody directed against VEGFR-2, inhibiting binding of all VEGF ligands. Icrucumab inhibits ligand interaction and subsequent phosphorylation of VEGFR-1. In this promising trial, combination of docetaxel with ramucirumab prolonged PFS compared to chemotherapy with docetaxel alone (5.4 months vs. 2.8 months). On account of these data, a randomized, double-blind, placebo-controlled phase III trial comparing docetaxel plus ramucirumab versus docetaxel plus placebo is currently recruiting patients (NCT02426125). Primary endpoint is PFS, and final data collection date for primary outcome measure is estimated on April 2017.

A completely new and innovative approach is the combined inhibition of angiogenesis with immune-checkpoint inhibitors like the PD-1 inhibitor pembrolizumab, which is being tested in a phase I study (NCT02443324).

Vandetanib

Vandetanib is a receptor tyrosine kinase inhibitor targeted against VEGFR and EGFR. In a phase II trial (NCT00880334), 142 patients with advanced UC after platinum-based chemotherapy were assigned into a docetaxel only or docetaxel plus vandetanib arm. PFS, ORR, and OS were not improved by the addition of vandetanib (Choueiri et al. 2012).

Nintedanib/Regorafenib

The importance of FGF and VEGF in BC angiogenesis was described above. Nintedanib is a small molecule tyrosine kinase inhibitor targeting PDGFR, FGFR, and VEGFR. Besides different other cancer entities, nintedanib is tested for advanced UC in a phase II trial (NCT02278978). Another pathway relevant for BC angiogenesis is the Ang-Tie system. Regorafenib is a multiple tyrosine kinase inhibitor targeted against TIE2, VEGFR, PDGFR, and FGFR, and a phase II trial for BC is under way (NCT02459119).

Conclusion

Treatment options and also prognosis for patients with advanced BC are still very limited up to now. As BC is the ninth most common malignancy, improvements are eagerly awaited. However, especially when compared to many other cancer entities, there has not been much innovation during the last decades.

Like in many other oncologic entities, anti-angiogenic therapies did not bring the expected and urgently needed improvements compared to standard care. Neither bevacizumab, sorafenib, sunitinib nor pazopanib significantly improved oncologic outcome in advanced or metastatic BC. To be clear, at the moment there is no anti-angiogenic therapy approved for BC. Still, there is good reason to be optimistic for anti-angiogenics in the midterm, with good results in a phase II study for ramucirumab and an ongoing phase III trial testing bevacizumab in addition to gemcitabine/cisplatin in the adjuvant setting. This optimism is supported by the evidence generated by a whole bunch of publications showing that angiogenesis plays a significant part in BC pathogenesis. Besides VEGF, Ang-Tie, FGF, TSP1, MMP, and HIF, there are still many more angiogenic signaling pathways unexplored in BC. Furthermore, several completely new compounds are tested in clinical studies, and results are eagerly awaited.

So where will we head in the future? First, as it becomes more and more evident that there will be no single anti-angiogenic for all patients with advanced BC, we will have to focus on biomarkers to identify subcohorts which are susceptible to the respective compound. Secondly, more consideration has to be given to combination therapies as well as different treatment sequences.

Lastly, profound basic as well as translational research will have to be conducted to both identify new targets and understand mechanism of resistance to angiogenic treatments.

Cross-References

- Anti-angiogenic Cancer Therapy: Development of Resistance
- Anti-angiogenic Targets: Angiopoietin and Angiopoietin Receptors
- Anti-angiogenics and Radiation Therapy
- Biomarkers for Anti-angiogenic Therapy
- Combination of Anti-angiogenics and Other Targeted Therapies
- Cytotoxics and Anti-angiogenics: Metronomic Therapies
- Imaging Tumor Angiogenesis
- Mechanisms of Anti-angiogenic Therapy
- Mechanisms of Tumor Angiogenesis
- Pathology of Tumor Angiogenesis
- The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Part XVII

Anti-angiogenics in Brain Tumors

The Value of Anti-angiogenics in Primary Brain Tumor Therapy

E. Schorb and C. F. Waller

Contents

Introduction 610
Glioblastoma Multiforme 610
Diffuse Astrocytic and Oligodendroglial Tumors 611
Meningioma 611
Medulloblastoma 611
Other Types of Brain Tumors 612

Angiogenesis and Glioblastoma 612

Angiogenesis and Astrocytic and Oligodendroglial Tumors 612

Angiogenesis and Meningioma 613

Angiogenesis and Medulloblastoma 613

Anti-angiogenic Agents 613

Mechanisms of Resistance 614

Clinical Trials 615

Newly Diagnosed Glioblastoma 615

Recurrent Glioblastoma 617
Bevacizumab beyond Progression 617

Meningioma 618

Clinical Implications of Bevacizumab Therapy in Glioblastoma 618

Summary 620

Conclusion 620

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Cross-References 620
References 621

Abstract

Glioblastoma Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor in adults. The current standard treatment is tumor resection followed by adjuvant radiotherapy and concomitant chemotherapy with temozolomide. Even with this intensive and multimodal approach, the prognosis remains poor with a median survival of 14–16 months. Glioblastomas are strongly vascularized tumors that frequently contain pathological and dysfunctioning blood vessels. Thus, angiogenesis inhibitors have been investigated increasingly and become a promising treatment approach. The most common anti-angiogenic agent bevacizumab was approved by the United States Food and Drug Administration as monotherapy in relapsed glioblastoma patients in 2009. However, despite promising preclinical data and early clinical trials, anti-angiogenic therapy has failed to show a survival benefit in randomized controlled trials.

Anaplastic Oligodendroglial Tumors Similar to GBM therapy, the treatment of recurrent anaplastic oligodendroglial tumors is also challenging with only partially effective therapeutic options and modalities. In comparison to glioblastomas, only very limited data exist regarding the activity of anti-angiogenic compounds in recurrent anaplastic gliomas.

Meningioma Meningioma is another common central nervous system tumor in adults with mostly benign, localized, and noninvasive character. However, some meningiomas tend to be more aggressive with limited therapeutic options in case of recurrence or progression. Preclinical studies are scarce; however, data from few prospective clinical trials suggest a possible role for anti-angiogenic treatment.

The aim of this chapter is to review preclinical and clinical trial data of anti-angiogenic therapy in the above mentioned as well as in further less common brain tumors.

Keywords

Glioblastoma multiforme · Oligodendroglial tumors · Meningioma · Medulloblastoma · Monoclonal antibody · Bevacizumab · Tyrosine-kinase inhibitors · Resistance · Survival · Clinical trials

Introduction

Glioblastoma Multiforme

Glioblastoma multiforme (GBM) accounts for approximately 60–70% of all gliomas (Wen and Kesari 2008) and 15% of all primary brain tumors (Ostrom et al. 2014) and is the most common primary malignant brain tumor in adults. The current standard treatment for GBM patients <70 years is maximal tumor resection followed by adjuvant radiotherapy and chemotherapy with temozolomide (Weller et al. 2014). With a median progression free survival of 6.9 months and a 37% relative reduction in the risk of death for patients treated with radiotherapy and temozolomid, as compared with those who received radiotherapy alone, this multimodal treatment approach has demonstrated efficacy. However, with a median survival of 14–18 months and 5-year survival rates of less than 5% the prognosis for GBM patients remains very poor (Stupp et al. 2005; Wen and Kesari 2008; Ostrom et al. 2014). This disappointing survival data may be partly explained by the capacity of GBM cells to spread and invade into surrounding brain parenchyma and of the development of resistance of GBM cells during therapy. New treatment approaches have been developed during the past years but progress in finding more effective therapies in this indication has been disappointing, and survival rates for patients with newly diagnosed

GBM have hardly improved. Despite efforts toward the molecular characterization of GBM, only a few molecular features such as MGMT promotor methylation and IDH1/2 mutation have been shown to be of clinical importance. Apart from molecular characteristics, other GMB-specific targets like tumor infiltration and migration, angiogenesis, vasculogenesis, hypoxia, and gliosis may be of importance in the development of targeted therapeutic options. GBM is histologically characterized by microvascular proliferation with a high expression of proangiogenic factors. Thus, anti-angiogenic agents have been investigated in this indication in the hope of expanding treatment options.

Diffuse Astrocytic and Oligodendroglial Tumors

The treatment of recurrent diffuse astrocytic and oligodendroglial tumors is challenging, as only partially effective therapeutic modalities are available. These therapies include chemotherapy, radioactive implants, stereotactic irradiation, targeted therapy, and reoperation (Roth et al. 2013). Chemotherapy is of modest efficacy, primarily due to limited response duration (Stewart 2002). In an analysis of 8 phase 2 studies of chemotherapy for recurrent high grade glioma, Wong reported response rates in recurrent anaplastic astrocytomas of 14% with a progression-free survival (PFS) of 31% at 6 months (Wong et al. 1999). In comparison to GBM, only a small data set exists for activity of bevacizumab in recurrent anaplastic gliomas (Stark-Vance 2005; Pope et al. 2006; Vredenburgh et al. 2007a, b; Chen et al. 2007; Norden et al. 2008).

Meningioma

Meningiomas are common primary tumors of the central nervous system (CBTRUS 2010). The majority of meningiomas are of benign character (World Health Organization[WHO] grade I), but nearly 20% are atypical (WHO grade II) and 1–2% are anaplastic (WHO grade III) (Mawrin and Perry 2010). Standard therapy for symptomatic or growing benign meningiomas is maximum safe resection. In case of atypical or anaplastic lesions, progressive grade I lesions or inoperability of the tumor radiation therapy is usually added (Alexiou et al. 2010; Wen et al. 2010). Notwithstanding, survival outcome for patients with progressive grade II and III meningiomas remains poor. Reported 5-year survival rates range from 28 to 61% (Hanft et al. 2010). Hydroxyurea has demonstrated modest antitumor activity in some series but overall efficacy of several chemotherapy agents remains disappointing (Schrell et al. 1997; Mason et al. 2002; Rosenthal et al. 2002; Loven et al. 2004; Newton et al. 2004; Newton 2007). Several putative therapeutic targets have been shown to be expressed in a large majority of meningiomas including receptors for progesterone, growth hormone, and vascular endothelial growth factor (VEGF) (Baxter et al. 2014). Targeted therapies that inhibit specific activators of growth factor signaling pathways have also been tested in clinical trials without proven efficacy to date among nonenriched progressive meningioma patients (Wen et al. 2009; Norden et al. 2010). Thus, effective treatment for patients with meningiomas that recur or progress following resection and radiotherapy still remains a major challenge.

Medulloblastoma

Medulloblastomas account for 12% of childhood brain tumors. In adults, medulloblastomas are rare (Ostrom et al. 2014). During the past years four molecular subgroups of medulloblastoma have been described – Wnt signaling pathway (WNT, 10% of medulloblastomas), sonic hedgehog signaling pathway (SHH, 30% of medulloblastomas), group 3 (15% of medulloblastomas), and group 4 (45% of medulloblastomas) – and have led to a fundamental change in medulloblastoma classification. Each of these subgroups is characterized by unique molecular profile and clinical outcome (Kool et al. 2012). Of the 4 subgroups of

medulloblastoma, group 3 has the worst prognosis. Current therapies for medulloblastoma mainly consist of surgery, radiotherapy, and cytotoxic treatment but mortality remains significant and many survivors suffer from severe treatment-related effects of radiation and cytotoxic chemotherapy. Recurrence of medulloblastoma still has a dismal prognosis with no significant salvage rate (Packer and Vezina 2008).

Other Types of Brain Tumors

Anti-angiogenic therapy has also been investigated in several other nonglial brain tumors, such as vestibular schwannomas, ependymomas, and miscellaneous histotypes. However, to date, there is only a very limited number of preclinical and clinical data in these orphan diseases.

Angiogenesis and Glioblastoma

Angiogenesis is an important therapeutic target in the treatment of GBM as it is one of the most vascularized types of cancer (Brem et al. 1972). Thus, many preclinical studies use GBM as a tumor model of angiogenesis (Jain et al. 2007). VEGF is an important regulator of angiogenesis that is described to be highly expressed within brain tumors (Salmaggi et al. 2003). Glioblastomas are histologically characterized by microvascular proliferation and express high levels of VEGF (Kaur et al. 2005). Glioblastoma vasculature is functionally and structurally abnormal, which leads to hypoxic regions. Hypoxia and angiogenesis are known to be related to tumor growth and invasion; hypoxia results in upregulation of VEGF (Du et al. 2008) and promotes cancer cell invasion, genetic instability, altered metabolism, and creation of an immunosuppressive environment (Jain 2014). The grade of VEGF expression is correlated with the grade of malignancy and prognosis in GBM. In comparison to grade II glioma, the expression of VEGF in glioblastoma is approximately 10 times higher (Schmidt et al. 1999). In addition to VEGF, other proangiogenic factors have been described to be upregulated in glioblastomas, e.g., hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), angiopoietins, and interleukin-8 (Shih and Holland 2006; Reiss et al. 2005; Brat et al. 2005; Schmidt et al. 1999). Moreover, angiogenesis is also constitutively activated through nonhypoxia dependent pathways such as Ras/mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) (Jayson et al. 2016). Given the number of pathways involved in angiogenesis, there are multiple opportunities for therapeutic targeting.

The angiogenetic agent most investigated in GBM is bevacizumab, a humanized anti-VEGF antibody, which is associated with the improvement of progression-free survival and performance status without a survival benefit in patients with glioblastoma. The mechanisms of action of Bevacizumab do not only include the inhibition of tumor angiogenesis, but also indirect effects such as the depletion of niches for glioma stem cells and stimulation of antitumor immunity. Several mechanisms have been reported to mediate adaptation and resistance to bevacizumab, including the activation of alternative pro-angiogenic pathways, but the resistance mechanisms have not been fully explained to date.

Angiogenesis and Astrocytic and Oligodendroglial Tumors

On the basis of the evidence from glioblastoma patients, anti-angiogenic compounds, such as VEGF inhibitor bevacizumab, have also been tested in anaplastic astrocytic or oligodendroglial tumor patients. Several smaller studies using bevacizumab alone or in combination with other agents in patients with recurrent astrocytic or oligodendroglial tumors suggest that bevacizumab also is active in these tumors (Chamberlain et al. 2009a, b; Taillibert et al. 2009; Kreisl et al. 2011; Seystahl et al. 2013). However, the definite role of bevacizumab and other anti-angiogenic compounds still needs to be defined. The randomized TAVAREC trial (NCT01164189), which is assessing the significance of bevacizumab in recurrent grade II and grade III gliomas, has recently completed recruitment.

Angiogenesis and Meningioma

VEGF-mediated angiogenesis is also supported by growing evidence in some meningiomas, especially in higher grade subtypes (Pistolesi et al. 2004; Rogers et al. 2010). The rationale for therapeutic targeting of angiogenesis for recurrent/progressive meningioma patients is based on the knowledge that levels of VEGF, VEGF-receptor, and microvessel density – similar to GBM – increase with meningioma grade and may be of prognostic significance (Provias et al. 1997; Pistolesi et al. 2004; Barresi and Tuccari 2009). Pistolesi et al. performed polymerase chain reaction and immunohistochemical staining (IHC) on 40 samples of intracranial meningioma and demonstrated that the level of microvessel density and VEGF expression was significantly associated with grade II and III meningiomas (Pistolesi et al. 2004). Furthermore, levels of VEGF and VEGF-R have also been positively correlated with the extent of peritumoral brain edema in meningioma patients (Provias et al. 1997; Otsuka et al. 2004; Yoshioka et al. 1999; Ragel and Jensen 2010; Goldman et al. 1997; Kalkanis et al. 1996).

Angiogenesis and Medulloblastoma

To date, only little is known about the role of angiogenesis in the pathogenesis of medulloblastoma. Recently, Thompson et al. demonstrated that angiogenesis is a key factor in group 3 medulloblastoma tumor pathogenesis and survival (Thompson et al. 2017) and therefore may be an important therapeutic target.

Anti-angiogenic Agents

The importance of blood supply for tumor growth and metastasis has become an important target in the treatment of many solid tumors during the last years.

Early trials on anti-angiogenic agents in glioblastoma with thalidomide – a weak angiogenesis inhibitor, lenalidomide – an analog of thalidomide, carboxyamidotriazole, and the copper-chelating drug penicillamine failed to show a clear benefit in glioblastoma. Lenalidomide showed modest activity when used as a single agent or in combination with carmustine for recurrent glioblastoma. In patients with newly diagnosed GBM, there was no benefit observed when it was combined with the DNA alkylating agent temozolomide and radiation therapy (Fine et al. 2000; Marx et al. 2001; Chang et al. 2004; Fine et al. 2003; Kesari et al. 2008). Lenalidomide, carboxyamidrotriazole, and penicillamine also did not show a clinical benefit compared to standard treatment (Drappatz et al. 2009; Fine et al. 2007; Mikkelsen et al. 2007; Brem et al. 2005).

Recently, clinical trials have focused on more potent inhibitors of angiogenesis, particularly the recombinant, humanized monoclonal antibody bevacizumab, which inhibits the interaction between VEGF-A and VEGFR1/2 and neutrophils (Gatson et al. 2012) and binds VEGF-A with high affinity and specificity. The initial proposed mechanism of action is through decreased tumor perfusion, thereby depriving the tumor of nutrients and oxygen (Folkman 1971). More recent studies have suggested that in the initial stages of treatment and at low doses, anti-angiogenic agents such as bevacizumab can normalize tumor blood vessels and thereby improve vessel function and reduce tumor-associated edema (Jain 2014).

Bevacizumab was shown to inhibit angiogenesis and tumor growth in preclinical models of glioblastoma by several groups (Kim et al. 1993; Rubenstein et al. 2000; Jahnke et al. 2009; Lee et al. 2000). In a proportion of glioblastoma patients, an increased tumor perfusion was described after bevacizumab therapy which may sensitize the tumor to radiation and chemotherapy (Winkler et al. 2004; Batchelor et al. 2007). Bevacizumab is associated with the improvement of progression-free survival and clinical performance status in patients with glioblastoma and was approved by the United States Food and Drug Administration (FDA) in 2009. However, the performed randomized trials uniformly suggest that these favorable clinical effects of

bevacizumab do not translate into an overall survival benefit.

Numerous receptor tyrosine kinase inhibitors of VEGF and other proangiogenic pathways including aflibercept, cediranib, nintedanib, pazopanib, sorafenib, sunitinib, vandetanib, and enzastaurin have also been tested in clinical trials (Batchelor et al. 2010, 2013; Iwamoto et al. 2010; Wick et al. 2010a; De Groot et al. 2011; Reardon et al. 2011b, 2013; Kreisl et al. 2012, 2013; Muhic et al. 2013; Odia et al. 2016). However, with the exception of cediranib and enzastaurin, an oral serine/threonine kinase inhibitor, none have progressed beyond phase 2 clinical trials.

Cediranib is an oral inhibitor of VEGF receptor tyrosine kinases. However, a phase 3 trial in recurrent glioblastoma patients treated with cediranib monotherapy or cediranib with lomustine, an alkylating nitrosourea, versus lomustine alone did not demonstrate difference in progression-free survival (PFS) or overall survival (OS) (Batchelor et al. 2010). Enzastaurin is an oral serine/threonine kinase inhibitor, which targets the protein kinase C and PI3K/AKT pathways. A randomized phase II trial which investigated treatment of enzastaurin in combination with bevacizumab in recurrent glioblastoma patients showed promising results. Median OS was 2.0 months and median PFS 2.0 months with an objective response rate of 22% (Odia et al. 2016), whereas an international randomized phase 3 trial of enzastaurin in recurrent glioblastoma revealed that enzastaurin, with an objective response rate of only 2.9%, was not superior to lomustine for GBM at first recurrence (Wick et al. 2010a). Authors hypothesized that combined enzastaurin and bevacizumab, with their different mechanisms of VEGF inhibition, would lead to additive anti-angiogenic effects and improved antiglioma efficacy in GBM.

Mechanisms of Resistance

Despite radiographic responses and improvement of PFS, response to anti-angiogenic therapy is generally not durable due to the fact that alternative mechanisms of vessel recruitment are ultimately utilized. Local hypoxia may be a trigger of alternative proangiogenic factors (DeLay et al. 2012; Lu and Bergers 2013; Casanovas et al. 2005; Rigamonti et al. 2014; Huang et al. 2010). Preclinical studies reveal that dual targeting of VEGF and angiopoietin-2 may overcome the resistance to anti-VEGF monotherapy (Kloepper et al. 2016; Peterson et al. 2016; Park et al. 2016).

Another potential escape mechanism to anti-angiogenic therapy is vessel co-option, the process whereby tumors utilize native brain vessels to recruit blood supply (Jain 2014). The molecular mechanisms of vessel co-option are still poorly understood.

Furthermore, some tumor vessel subtypes have decreased sensitivity of pericytes and are therefore supposed to have inherent insensitivity to VEGF inhibition (Sitohy et al. 2011; Benjamin et al. 1999).

There is also preclinical data that anti-angiogenic therapy induces transformation from a proneural to a more invasive mesenchymal phenotype, including upregulation and increased phosphorylation of the receptor tyrosine kinase c-Met (Piao et al. 2013; Lu et al. 2012; Jahangiri et al. 2013; Lucio-Eterovic et al. 2009). Glioblastoma mouse models revealed that inhibition and knockdown of c-Met inhibit tumor growth and prolong survival (Piao et al. 2013; Lu et al. 2012; Jahangiri et al. 2013). Despite strong preclinical evidence supporting a role for c-Met in bevacizumab resistance, the c-Met inhibitor XL184 which was tested in a phase 2 clinical trial proved particularly ineffective against glioblastomas that previously progressed during bevacizumab treatment (Wen et al. 2010). A phase 1 trial of another c-Met inhibitor INC280 is currently ongoing (NCT02386826).

Another important aspect is that treatment with bevacizumab is described to be associated with nonenhancing, diffuse, or distant recurrence (Norden et al. 2008). Given the potential disease progression via nonenhancing, infiltrative, or invasive disease with anti-angiogenic treatment, combination therapy with agents that target invasion may also be a promising future strategy to overcome resistance to anti-angiogenic monotherapy.

Clinical Trials

A multitude of anti-angiogenic agents have been evaluated for glioblastoma in newly diagnosed as well as in recurrent glioblastoma including tyrosine kinase inhibitors, monoclonal antibodies against VEGFR, and a soluble decoy receptor (Table 1). Unfortunately, many of them showed negative results. Barriers to successful development of therapeutic agents are manifold and often not completely understood, but partly may be explained by the unique characteristics of GBM and its host organ with challenging drug delivery. Furthermore, the tumor is pathologically characterized by abnormal blood vessels and areas of necrosis which could lead to heterogeneous drug distribution.

Newly Diagnosed Glioblastoma

Anti-angiogenic therapy with bevacizumab in combination with standard treatment with radiation therapy and chemotherapy with temozolomide was investigated in several early, single arm phase 2 studies. In comparison to historical controls, the addition of bevacizumab was associated with a significant improvement of PFS, but only a modest improvement of OS (Lai et al. 2011; Vredenburgh et al. 2011, 2012). Recently, two randomized, placebo-controlled phase 3 trials investigated the addition of bevacizumab to standard temozolomide plus radiation in patients with newly diagnosed glioblastoma: The AVAglio study compared patients randomized to either bevacizumab or placebo in combination with standard chemoradiation. PFS was significantly prolonged at 10.6 months in the bevacizumab group compared to 6.2 months in the standard therapy group (Chinot et al. 2014).

The RTOG 0825 study also compared bevacizumab versus placebo in combination with standard radiation and chemotherapy with temozolomide. There was also a trend toward an improvement of PFS in the bevacizumab group (10.7 months versus 7.3 months in the placebo group) without reaching statistical significance (Gilbert et al. 2014). But both studies failed to demonstrate a benefit in OS, possibly due to the high crossover rates of 30 to 50%, which might have obscured the true impact on OS as a large number of patients in the placebo arm were subsequently treated with bevacizumab at the time of disease progression. Secondary endpoints such as performance status, corticosteroid requirement, and quality of life measures were also assessed within the two randomized trials. The AVAglio trial showed a prolonged maintenance of performance status, decreased steroid use, and prolonged time to deterioration in prespecified cognitive domains in the bevacizumab arm, whereas the RTOG 0825 trial showed that bevacizumab led to worsened cognitive function. The cause of the differences remains unclear. Possible explanations include different radiographic response criteria, substantial dropout in the RTOG trial, and differences in statistical modeling.

Combination therapies with bevacizumab in newly diagnosed glioblastoma have also been assessed. The GLARIUS study, a randomized phase 2 trial in patients whose tumors expressed the DNA repair enzyme O6-methyl guanine DNA methyltransferase (MGMT), compared standard radiation and chemotherapy with temozolomide versus radiation with bevacizumab and irinotecan, an inhibitor of topoisomerase I. Loss of MGMT function through gene promoter methylation had been shown to result in increased sensitivity to therapy with temozolomide in glioblastoma (Hegi et al. 2005). The GLARIUS trial showed that PFS was significantly prolonged at 9.7 months in the bevacizumab plus irinotecan arm compared to 5.99 months in the standard chemoradiation arm. But similar to the AVAglio and RTOG 0825 trial, OS did not significantly differ with OS of 17.5 months in the control arm compared to 16.6 months in the experimental arm. Furthermore, there was no statistically significant difference in delaying the time to deterioration in prespecified dimensions of quality of life (Herrlinger et al. 2016). In another phase 2 trial, similar results were seen regarding the use of bevacizumab and everolimus, a mTor-inhibitor, as part of first-line combined modality therapy for glioblastoma (Hainsworth et al. 2012).

Table 1 Representative clinical trials of anti-angiogenic agents in glioblastoma

Study	Agent	Ref	Phase	Disease type	Patients (n)	ORR (%)	Median PFS (months)	Median OS (months)
GLARIUS	Bev + irinotecan/XRT vs. TMZ/XRT	Herrlinger et al. 2016	2	nGBM	170	n.r.	9.7 5.9	16.6 17.3
RTOG 0825	BEV + TMZ/XRT vs. TMZ/XRT	Gilbert et al. 2014	3	nGBM	637	n.r.	10.7 7.3	15.7 16.1
AVAGlio	BEV + TMZ/XRT vs. TMZ/XRT	Chinot et al. 2014	3	nGBM	921	n.r.	10.6 6.2	16.9 16.8
NCI	Bev	Kreisl et al. 2009	2	rGBM	48	35	4.0	7.8
NCT00369590	Aflibercept	de Grot et al. 2011	2	rGBM	42	18	3.0	9.8
NCT00350727	Pazopanib + lapatinib	Reardon et al. 2013	2	rGBM	41	5	~ 2.0	n.r.
NCT00459381	Pazopanib	Iwamoto et al. 2010		rGBM	35	5.9	3.0	8.8
NCT00597493	Sorafenib + TMZ	Reardon et al. 2011b	2	rGBM	32	3	1.6	10.4
NCT01462695	Sunitinib	Kreisl et al. 2013	2	rGBM	32	10	3.0	9.4
NCT 00272350	Vandetanib	Kreisl et al. 2012	2	rGBM	32	12.5	1.3	6.3
NCT01251484	Nintedanib	Muhic et al. 2013	2	rGBM	25	0	1.0	6.0
BELOB	BEV vs. Lomustine vs. BEV + lomustine	Taal et al. 2014	2	rGBM	153	38 5 39	3.0 1.0 4.0	8.0 8.0 12.0
BRAIN	BEV vs. BEV + irinotecan	Friedman et al. 2009	2	rGBM	167	28.2 37.8	4.2 5.6	9.2 8.7
Enzastaurin	Enzastaurin vs. Lomustine	Wick et al. 2010	3	rGBM	266	2.9 4.3	1.5 1.6	6.6 7.1
REGAL	Cediranib vs. Cediranib + lomustine vs. Lomustine + placebo	Batchelor et al. 2013	3	rGBM	325	15.3 17.2 8.9	3.3 4.5 2.9	8.0 9.4 9.8
EORTC 26101	BEV + lomustine vs. Lomustine	Wick et al. 2015	3	rGBM	437	n.r.	4.2 1.5	9.1 8.6

Abbreviations: *Ref* Reference, *ORR* overall response rate, *Bev* bevacizumab, *XRT* radiation therapy, *vs* versus, *TMZ* temozolomide, *nGBM* newly diagnosed glioblastoma, *rGBM* recurrent glioblastoma, *n.r.* not reported.

Recurrent Glioblastoma

Bevacizumab was approved by the FDA as monotherapy for recurrent GBM on the basis of two prospective, phase 2 studies. A single-arm study investigating 48 recurrent glioblastoma patients treated with bevacizumab showed an ORR of 35% and PFS at 6 months of 29% (Kreisl et al. 2009). The randomized phase 2 BRAIN study compared bevacizumab to bevacizumab plus irinotecan and revealed that bevacizumab, alone or in combination with irinotecan, was well tolerated and active in recurrent glioblastoma. The overall response rates (ORR) were 28.2% and 37.8% with PFS at 6 months of 42.6% and 50.3%, respectively (Friedman et al. 2009). However, one has to consider that the trial was not designed as a superiority trial and allowed for crossover from bevacizumab monotherapy to the combination approach, which may confound the results.

The European Medicines Agency declined approval of bevacizumab due to the lack of a nonbevacizumab control arm, the only modest improvement in OS, and challenges with radiographic response assessment (Wick et al. 2010b).

Additional studies have evaluated the use of bevacizumab in combination regimens. Within the randomized phase 2 BELOB trial, 148 patients with recurrent GBM were randomized to lomustine, bevacizumab, or both. Combination therapy resulted in a PFS at 6 months of 41% compared to 11% and 18% with OS at 9 months of 59% compared to 43% and 38% for lomustine and bevacizumab alone, respectively (Taal et al. 2014). This formed the basis for the phase 3 study EORTC 26101, which was conducted to compare lomustine versus lomustine plus bevacizumab. While the median PFS was increased from 1.5 to 4.2 months for combination therapy, there was no significant difference in OS for combination treatment versus lomustine alone (Wick et al. 2015).

Bevacizumab treatment was also evaluated in phase 2 trials investigating combination therapy with irinotecan, cetuximab, carboplatin, etoposide, fotemustine, sorafenib, temozolomide, erlotinib, panobinostat, and temsirolimus (Lee et al. 2015; Francesconi et al. 2010; Reardon et al. 2009, 2011a, 2012a; Ali et al. 2008; Bokstein et al. 2008; Kang et al. 2008; Zuniga et al. 2009; Nghiemphu et al. 2009; Desjardins et al. 2012; Sathornsumetee et al. 2010; Galanis et al. 2013; Drappatz et al. 2012; Lassen et al. 2013; Møller et al. 2012; Soffietti et al. 2014; Raizer et al. 2016; Field et al. 2015). Additionally, bevacizumab and re-irradiation were evaluated (Cuneo et al. 2013; Cabrera et al. 2012; Gutin et al. 2009). Unfortunately, none of these trials have demonstrated outcomes superior to historical controls treated with bevacizumab alone.

Bevacizumab beyond Progression

The continued inhibition of VEGF beyond progression has been investigated in different cancers. The continued use of bevacizumab after tumor progression was first used in metastatic colorectal cancer (BRiTE study; Grothey et al. 2008) and was strongly and independently associated with improved survival compared to no postprogression treatment or postprogression treatment without bevacizumab.

For recurrent GBM, a retrospective, pooled analysis of five phase II trials showed a modest survival benefit in patients treated with bevacizumab after tumor progression compared with postprogression treatment without bevacizumab (Reardon et al. 2012b) revealing continuation of bevacizumab is an independent prognostic factor of improved overall survival and might be a viable option for selected patients. Reardon et al. suggested that cessation of bevacizumab in case of tumor progression is more likely to lead to diffuse, distant, or multifocal patterns of tumor progression. On the contrary, Anderson et al. described bevacizumab discontinuation to be unrelated to disease progression (e.g., side effects), to rebound recurrence or worsening of PFS in patients who benefit from bevacizumab. In this investigation, patients who discontinued bevacizumab therapy additionally had an improved response to salvage therapy (Anderson et al. 2014).

The ongoing multicenter, double-blind, randomized phase IIIb TAMIGA trial which

compares lomustine plus either bevacizumab beyond progression or placebo following disease progression after first-line treatment with radiotherapy, temozolomide, and bevacizumab for newly diagnosed glioblastomas has recently completed recruitment in Europa and Canada (NCT01860638). Results will help us to determine the validity of bevacizumab treatment beyond progression in recurrent GBM.

Meningioma

Several clinical trials in patients with refractory meningioma have also suggested a possible role for anti-angiogenic therapy in this indication. Results from a phase 2 trial with sunitinib, an oral multitargeted tyrosine kinase inhibitor with anti-angiogenic activities, in grade II/III meningioma patients revealed a median PFS of 5.1 months (Kaley et al. 2015).

Vatalanib, a tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR), only showed modest activity in patients with refractory meningioma (Raizer et al. 2014).

Regarding bevacizumab treatment, two retrospective studies have been reported: the first one evaluated patients with atypical and anaplastic meningiomas treated with bevacizumab and revealed a median PFS of 26 weeks, with a PFS rate at 6 months of 43.8% (Nayak et al. 2012). In the second retrospective analysis, patients with recurrent meningioma treated with bevacizumab had an overall median PFS and a PFS rate at 6 months of 17.9 months and 85.7%, respectively. Results of a subgroup analysis show that patients with grade II/III meningioma had a slightly longer median PFS (15.8 months) than grade I patients (12.2 months) (Lou et al. 2012).

In a recently published retrospective analysis of serial cranial MRI in patients with WHO II and III recurrent meningioma treated with systemic therapy a detailed analysis of the MRI images was obtained before, during, and after treatment and the tumor volume, maximum tumor diameter, and volume of peritumoral edema were measured (Furtner et al. 2016). Using this information, growth rates of tumor diameter and tumor volume were calculated. While some growth inhibition with other systemic agents was revealed, the most pronounced decrease in growth rates was seen in patients treated with bevacizumab.

In a recently published small phase II trial, bevacizumab and everolimus were administered in patients with refractory, progressive intracranial meningioma. Treatment with bevacizumab and everolimus was well tolerated in most patients and resulted in prolonged disease stability (>12 months) in six patients (35%) without revealing any objective tumor responses (Shih et al. 2016).

Clinical Implications of Bevacizumab Therapy in Glioblastoma

Bevacizumab treatment is generally well tolerated in patients with glioblastoma. Treatment-related toxicities are comparable to those that have been described in other solid cancers. Reported rates of grade 3 or higher adverse events with bevacizumab in patients with recurrent glioblastoma range between 18% and 66% with lower rates of serious treatment-related adverse events when bevacizumab is used as a single agent (Friedman et al. 2009; Kreisl et al. 2009; Gilbert et al. 2014; Chamberlain et al. 2009a, b). The most common adverse events attributable to bevacizumab treatment in recurrent GBM include minor bleeding (i.e., epistaxis), hypertension, impaired wound healing, and proteinuria (Vredenburgh et al. 2007a, b; Friedman et al. 2009), which are similar to bevacizumab associated toxicities in other cancer types (Hurwitz et al. 2004; Sandler et al. 2006; Miller et al. 2007). The majority of these toxicities appear to be due to on-target, class-specific actions of angiogenic inhibition, and reflect disruption of VEGF in normal tissue. The rates of serious adverse events such as wound-healing complications, gastrointestinal perforation, reversible posterior leukoencephalopathy syndrome (RPLS), and cardiac failure in glioblastoma studies are low (each ≤2% incidence) (Vredenburgh et al. 2007a, b; Friedman et al. 2009; Norden et al. 2008; Gutin et al. 2009; Chamberlain 2008).

While the reported rate of grade 2 or higher bleeding events has been about 5.3%, life-threatening intracranial hemorrhages have occurred in only a small percentage (≤3%) of GBM patients treated with bevacizumab (Vredenburgh et al. 2007a, b; Friedman et al. 2009; Norden et al. 2008; Gutin et al. 2009; Chamberlain 2008). This incidence corresponds with the expected range for spontaneous events in patients with GBM (between 2% and 3%) (Wakai et al. 1982; Schrader et al. 2000).

Independently of the therapy applied, GBM patients have a significant risk of mainly venous thromboembolism. Semrad et al. described a 2-year cumulative incidence of symptomatic venous thromboembolism of 7.5% in patients with malignant glioma (Semrad et al. 2007). Consistently, relatively high rates of deep vein thrombosis and pulmonary embolism (ranging from 1.6%–12.5%) have also been reported in the studies evaluating bevacizumab-containing therapy in relapsed GBM (ranging from 1.6%–12.5%) (Vredenburgh et al. 2007a, b; Friedman et al. 2009; Norden et al. 2008; Gutin et al. 2009; Chamberlain 2008).

Thus, results from clinical trials suggest that despite small risks of severe and life-threatening complications, including intracranial hemorrhage and thromboembolic complications, bevacizumab-containing therapy is well tolerated with manageable, class-specific toxicities. The potential negative impact on health-related quality of life and neurocognitive functions during bevacizumab treatment is discussed controversely, but no controversity exists regarding stability of the above mentioned measures in the relapse/refractory setting (Friedman et al. 2009).

In several reported studies, survival rates after bevacizumab initiation were similar whether treatment was begun after the first or later recurrence (Piccioni et al. 2014; Friedman et al. 2009; Kreisl et al. 2009). As deferred use of bevacizumab does not seem to diminish efficacy in GBM patients, initiation of treatment can be considered at any point in the course of disease progression.

Due to bevacizumab's potential of cerebral edema, GBM patients receiving bevacizumab treatment have been successfully managed on reduced doses of dexamethasone (Kreisl et al. 2009). Lower dosages of concurrent corticosteroid usage are not only a positive side effect but are also recommended, because response rates to bevacizumab treatment have been shown to be lowered by concomitant steroid use (Friedman et al. 2009; Nagane et al. 2012).

Younger patients may be more optimal candidates for bevacizumab use as they tend to have more favorable survival outcomes as well as a lower risk of suffering adverse side effects (Nagane et al. 2012). Patients with glioblastoma who are receiving bevacizumab treatment should be monitored closely for treatment-related complications.

In patients suffering from bevacizumab resistance, neurosurgical interventions should be performed cautiously due to the risk of wound healing complications. Assistance from a plastic surgeon with closing the wounds is suggested by some authors (Golas et al. 2014).

Another challenge is radiologic evaluation during bevacizumab treatment. In excess of radiological evaluation by means of the RANO criteria, it is important to be aware of the multiple patterns of progression that can occur during bevacizumab treatment (local, distant, diffuse, or multifocal) (Pope et al. 2011). Furthermore, the decrease in the volume and intensity of contrast enhancement complicate the determination of objective responses.

Glioblastoma patients who progress during bevacizumab therapy show a median overall survival of 3.8 months (Magnuson et al. 2014). Apart from the risk of wound healing complications salvage surgical debulking is challenging because progression after bevacizumab is often non-enhancing and disseminated. Thus, surgical treatment may not always be a feasible option (Bloch et al. 2013; DeLay et al. 2012). To date, no direct comparison of different treatment schedules or dose–response studies has been conducted. Retrospective data suggest that treatment of patients with high-grade glioma with low doses of bevacizumab (5 mg/kg per week or 7.5 mg/kg every 3–4 weeks) may be superior to standard dosing (Lorgis et al. 2012; Levin et al. 2015), whereas meta-analysis was unable to demonstrate a dose–response difference between 5 and 10 or

15 mg/kg of bevacizumab with respect to response and survival rates (Wong et al. 2011).

Summary

Despite growing experience regarding anti-angiogenic therapy, treatment of high grade glioma patients remains a major challenge. Bevacizumab still plays a role in the treatment of recurrent glioblastoma, but given the lack of survival benefit the main objectives are quality of life aspects and potential saving of steroids. However, optimal dosing and timing of anti-angiogenic therapy have to be defined because the ideal regimen of bevacizumab in treating GBM patients remains unclear.

The combination of bevacizumab with radiotherapy, either in recurrent tumors (Gutin et al. 2009) or in newly diagnosed patients concomitantly with temozolomide (Lai et al. 2008; Narayana et al. 2008), has shown to be safe and feasible. However, the optimal chemotherapeutic agent for a combination therapy with bevacizumab still needs to be defined.

Anti-angiogenic therapy has also been tested in other brain tumors. In recurrent meningioma patients, prolonged disease stability without objective tumor responses has been demonstrated. Furthermore, the combination of different targeted therapies was shown to be well tolerated.

There is also some evidence for anti-angiogenic therapy in other nonglial brain tumors, such as vestibular schwannomas, ependymomas, medulloblastomas, and miscellaneous histotypes. Bevacizumab has shown to be effective in bilateral schwannomas in neurofibromatosis type II (Morris et al. 2017; Farschtschi et al. 2016). In other tumor subtypes, bevacizumab could be an option as salvage treatment after failure of standard therapy (Bonney et al. 2016; Piha-Paul et al. 2014).

Thus, beside optimization of treatment administration the use of combination strategies has to be defined. There are still many open questions regarding the optimal use of anti-angiogenic agents in order to improve response and duration of this approach and to convey a survival benefit. The best setting in which to use bevacizumab is still not well defined and has to be based on the individual clinical situation. In everyday practice, bevacizumab is commonly used in the presence of peritumoral edema or a significant mass effect or in order to limit corticosteroid use.

Additional research is still needed to explore mechanisms of resistance, combination strategies, and biomarkers to predict therapeutic response.

The future may be targeting several pathways or combining anti-angiogenic agents with other classes of drugs. Furthermore, additional studies are warranted to determine the optimal treatment for patients exhibiting progression of glioblastoma during bevacizumab treatment.

Conclusion

Angiogenic inhibition holds great promise for the treatment of brain tumors. Bevacizumab is the best characterized anti-angiogenic therapy and received FDA approval for the treatment of patients with recurrent GBM following prior temozolomid-based therapy. Overall, treatment with bevacizumab in multiple brain tumor studies seems to be well tolerated, but several important questions such as the best clinical setting to use the treatment, optimal combination partners, treatment duration in responding as well as in non-responding patients, and radiographic response criteria still need to be answered. These issues are currently addressed in ongoing clinical trials.

Cross-References

- ▶ Anti-angiogenic Cancer Therapy: Development of Resistance
- ▶ Combination of Anti-angiogenics and Other Targeted Therapies
- ▶ The Role of the VEGF Signaling Pathway in Tumor Angiogenesis

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Anti-angiogenics in Brain Metastases: Perspectives and Experiences

Frank Winkler

Contents

Introduction 628

Novel Concepts for Brain Metastases Prevention with Anti-angiogenics 629

Therapy of Brain Metastases by Anti-angiogenics 631
Palliative Benefits by Anti-angiogenics in Brain Metastases 632
Pragmatic Use of Anti-angiogenics in Heavily Pretreated Patients 633

Conclusions and Outlook 633

Cross-References 634

References 635

Abstract

Metastasis to the brain is an increasing complication of solid cancers, associated with high morbidity and mortality. Our understanding of key molecular and cellular determinants of early brain colonization and metastatic growth has significantly increased in the last decade, including crucial interactions with brain blood vessels. In lung adenocarcinoma, an early angiogenic switch appears mandatory for outgrowth beyond a micrometastatic state. This can explain preventive effects of anti-angiogenics against brain metastases formation in lung adenocarcinoma, which are suggested by mouse experiments and retrospective analysis of a clinical trial. Interestingly, there is no indication that those preventive effects of anti-angiogenics against the formation of macrometastases can be found outside the brain or in other tumor entities. In established brain macrometastases of various cancers, a growth pattern with strong angiogenic features can frequently be observed. Accordingly, an increasing amount of data speaks for a clinically meaningful activity of anti-angiogenics, particularly the anti-VEGF-A antibody bevacizumab, against brain macrometastases of patients. Moreover, the antiedema effects of this class of drugs, including their activity against radionecrosis, make anti-angiogenics useful agents in clinical practice with beneficial effects on neurological deficits and quality of life. This is even true for anti-angiogenics like bevacizumab given as single agents without combined chemotherapy. Taken

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together, there is accumulating evidence that anti-angiogenics might have a particularly meaningful role in the prevention and treatment of brain metastases. The lack of prospective randomized trials means that they have to be considered experimental therapies in this situation today. Thus, more robust clinical data are necessary to fully clarify the role of anti-angiogenics in brain metastases, a disease where better strategies for prevention and treatment are urgently needed.

Keywords
Brain metastases · Anti-angiogenics · Brain macrometastases · Prevention and treatment

Introduction

The major cause of death from cancer is due to metastases that are resistant to therapy. Brain metastases are particularly challenging targets for therapy and prevention (Preusser et al. 2012; Steeg et al. 2011). Up to 40% of patients with metastastic cancer develop symptomatic brain metastases during their clinical course of disease (Valiente et al. 2018). Lung cancer, breast cancer, and melanoma are the most frequent primary tumor sites, followed by colorectal and renal carcinoma (Davis et al. 2012). The incidence of brain metastases appears to be rising, partly due to better systemic control, prolonged life spans of cancer patients, and better radiological diagnostics. Thus, metastasis to the brain becomes an increasing problem for patients suffering from solid tumors who are otherwise responding well to local and systemic therapies (Valiente et al. 2018). Many therapeutics that work well on systemic metastases have much less, if any, activity in the brain, making it to a "sanctuary site" (Steeg et al. 2011). Finally, the organ that is colonized by circulating cancer cells (Fig. 1) is unlike any other in the body: the anatomical, physiological, and molecular microenvironment of the brain is special, and its importance for physical well-being and personality is obvious. All this makes it plausible to bring brain metastases more into focus of oncology today and to search for better strategies for therapy and prevention.

Local therapies like neurosurgical removal or radiotherapy remain the therapeutic backbone in patients with symptomatic brain metastases. However, systemic targeted and immunotherapies play an increasing role today. Considerably high response rates of brain metastases have been found for BRAF/MEK inhibitors in melanomas and EGFR or ALK inhibitors in lung adenocarcinomas which harbor the respective molecular alterations, but also for immune checkpoint inhibitors in brain metastases of melanoma and lung cancer (Valiente et al. 2018). Classical chemotherapeutics and also some targeted therapeutics cannot cross the blood-brain barrier in relevant concentrations, which prevents them from developing meaningful clinical activity against brain metastases (Osswald et al. 2016). Conceptually, anti-angiogenics do not have to fully cross the blood-brain barrier: their prime target, the endothelial cell, can easily be reached from the blood stream where they circulate. Therefore, anti-angiogenics have raised high hopes in neuro-oncology. While the results from phase III clinical trials of high-grade gliomas have not met those high expectations (Winkler et al. 2018), the situation in brain metastases might be different: their growth pattern is much less invasive and suggests a higher dependency on blood vessel interactions, compared to primary brain tumors (Kienast et al. 2010; Valiente et al. 2018; Winkler 2017) (Figs. 1 and 2). Extensive neo-angiogenesis is indeed a well characterized histological hallmark of many brain metastases, which supports the concept that in the specific context of brain metastatic disease, anti-angiogenic therapy might have a particularly high therapeutic impact (Berghoff et al. 2015; Kienast et al. 2010).

Therefore, a specific view on brain metastases is justified. This chapter summarizes the current translational and clinical knowledge about the role of anti-angiogenics in the treatment and potentially also prevention of brain metastases.

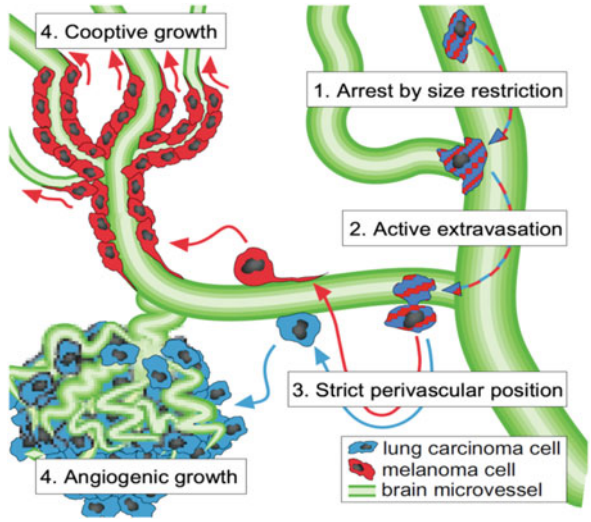

Fig. 1 Schematic illustration of the brain metastatic cascade. The mandatory steps that every cancer cell has to master to eventually grow to a brain macrometastasis include: (1) arrest in brain microvessels by size restriction, and additional specific interactions with endothelial cells; (2) an active extravasation process; (3) strict perputation of a perivascular niche position that is crucial for survival, and also for maintenance of dormancy of a cancer cell subpopulation in the brain; and (4) further growth by interaction with blood vessels, either by cooption of pre-existing brain vessels, or induction of angiogenesis. (From: Kienast et al. 2010)

Novel Concepts for Brain Metastases Prevention with Anti-angiogenics

BM are inevitably associated with a high morbidity and mortality (Davis et al. 2012), and they are a considerable burden for patients and caregivers. For the example of patients with stage III nsNSCLC, survival rates at 5 years are below 20%, mostly due to subsequent spreading of the tumor to the brain and other distant sites (DeSantis et al. 2014). Remarkably, despite metastasis being the leading cause of death in patients suffering from solid tumors, there exists little clinical evidence regarding metastasis prevention (Steeg 2012). For BM in small cell lung cancer, prophylactic cranial radiotherapy (pWBRT) has been shown to result in a decreased incidence of future brain metastases, and improved overall survival (Auperin et al. 1999); because of its considerably neurotoxicity, pWBRT has not prevailed in other entities. Strategies to prevent brain metastasis formation with a nonneurotoxic strategy hold the promise to translate into a significant benefit for cancer patients (Kienast and Winkler 2010; Steeg et al. 2011).

There exist several clinical and preclinical hints that chronic anti-angiogenic therapy with bevacizumab could prevent formation of metastasis in lung cancer. A subgroup analysis of the ECOG 4599 trial suggested that absence of a macroscopic residual tumor might improve the overall survival in favor for bevacizumab (Sandler et al. 2006). A similar trend was demonstrated in the second large phase III study in favor of bevacizumab efficacy in stage IIIB vs. stage IV (Reck et al. 2009). This suggests that the greatest benefit of bevacizumab should be expected for patients who do not have metastatic disease, i.e., a high tumor burden already. Own preclinical experiments using a novel mouse model where single metastasizing cancer cells were tracked by intravital microscopy have demonstrated that early angiogenesis was mandatory for successful macrometastasis formation in the mouse brain (Fig. 2). Consequently, prolonged anti-VEGF A treatment prevented this early angiogenic switch and forced small micrometastases (5–10 nsNSCLC cells only) into a state of chronic dormancy without any signs of further growth over many weeks

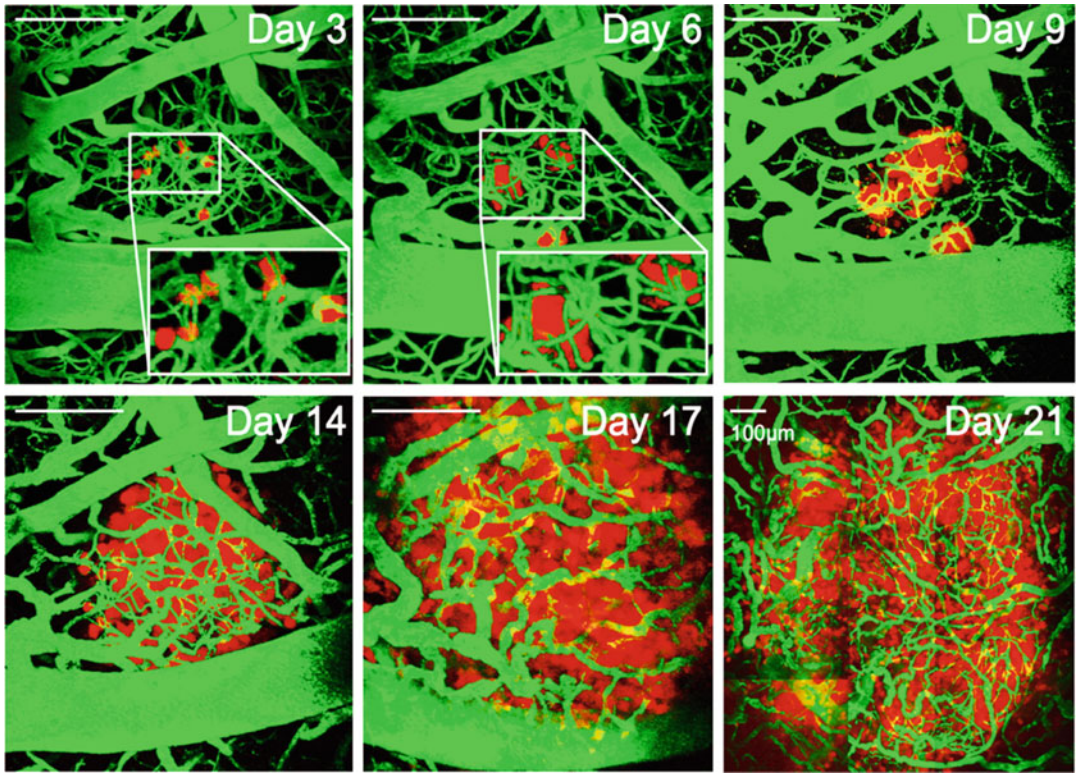

Fig. 2 Brain metastases formation of lung adenocarcinoma cells (red) in the mouse brain is characterized by early, marked angiogenesis (brain blood vessels: green). Extravasated cancer cells merge to a larger metastasis after brain colonization, inducing a strong angiogenic response from day 14 on. After that, exponential growth could be observed, paralleled by massive angiogenesis, which lead to a symptomatic brain metastasis at day 21. Scale bars, 100 µm. (From: Kienast et al. 2010)

(Kienast et al. 2010). In contrast, outgrowth of melanoma cells in the brain, which grew by co-option of pre-existing brain vessels, was not influenced by bevacizumab administration. To characterize the role of bevacizumab on BM prevention, we retrospectively analyzed three phase III clinical trials regarding the incidence of BM in nsNSCLC [AVAiL trial, (Reck et al. 2009, 2010)] and HER2 negative and positive mBC [AVADO and AVEREL trials, respectively; (Gianni et al. 2013; Miles et al. 2010)] patients, where bevacizumab was part of the standard treatment (Ilhan-Mutlu et al. 2016). Among the patients with nsNSCLC (AVAiL trial), BM as first site of recurrence was significantly lower in the bevacizumab arm when compared to the control chemotherapy arm (2.6% vs. 5.8%; $p = 0.01$), with a lower risk of BM development over time (HR 0.36, $p = 0.001$). No significant effect of bevacizumab regarding BM occurrence was observed in patients with HER2 negative and positive mBC. This suggested that bevacizumab might prevent or delay the formation of BM in nsNSCLC patients, whereas no effects were seen for BM prevention in mBC and for nsNSCLC metastasis outside the brain. In all three trials, preexisting BM were an exclusion criterion for study entry; this criterion lead to a bias for patients with a metastatic pattern preferentially involving sites other than the brain (i.e., a low number of total BM events over time), but allowed exploration of the effects of bevacizumab on the development of new BM during and after therapy.

To shed further light on the potential of anti-VEGF-A therapies for brain metastasis prevention, different doses of bevacizumab were

investigated in mouse nsNSCLC metastasis models. Using subclinical bevacizumab (5 mg/kg, twice weekly, intraperitoneal administration) in brain-seeking lung adenocarcinoma cells, a BM preventive effect could be achieved, which also translated into a survival benefit in these mice (Ilhan-Mutlu et al. 2016). No effects could be observed on the incidence of nonbrain metastases, confirming a brain-specific preventive effect. Together these data speak for the potential of anti-VEGF-A therapies to prevent metastatic outgrowth, which appears to be specific for the brain, and particularly relevant for nsNSCLC.

Remarkably, further support for the concept of chronic anti-angiogenesis in suppression of early tumor outgrowth comes from two recent studies: Both demonstrated that chronic impairment of angiogenesis is the reason why tumor growth is inhibited or prevented in two different mouse models of Down Syndrome (trisomy 21) (Baek et al. 2009; Reynolds et al. 2010). Chromosome 21 harbors 225 genes only, of which many are now identified as endogenous angiogenesis inhibitors, partly via the VEGF pathway – and a mild increase of 50% gene dose by the third chromosome appears sufficient to suppress the growth of solid tumors with high efficacy (Baek et al. 2009; Folkman 2007; Reynolds et al. 2010). Most importantly in this context, it is well known that the age-corrected mortality from solid tumors in individuals with Down Syndrome is very low, less than 10% than expected (Yang et al. 2002). Together with the preclinical data, this argues for an effective suppression of early tumor outgrowth by anti-angiogenesis in humans, which should be achieved with considerable low doses of anti-angiogenic agents.

Combined these data provide a strong biological and clinical rationale to test chronic anti-angiogenic therapy for its potential to prevent the occurrence of brain metastases in patients. A controlled, prospective clinical trial is warranted, with stage III nsNSCLC patients being today the most plausible patients that should be included in such a trial. These patients are at particularly high risk to develop brain metastases in the future and to suffer from morbidity and mortality caused by them.

Therapy of Brain Metastases by Anti-angiogenics

Anti-angiogenics are widely used in the clinic for several tumor entities responsible for brain metastases, including nonsmall cell lung, breast, colon, and renal cancer; however, all phase III approval studies have excluded brain metastases at study entry, making it more difficult to obtain sufficient information on the activity of anti-angiogenics on clinically relevant brain metastases today. The exclusion of brain metastases patients is typical for many trials in Oncology and hinders progress in brain metastases research. For anti-angiogenics, the initial concern that drugs like bevacizumab might increase the risk of CNS bleeding complications was another reason for this exclusion. However, this concern has been shown to be unjustified in larger analyses, where CNS hemorrhages or other relevant complications were not enriched in cancer patients suffering from brain metastases and receiving bevacizumab vs. those without brain metastases, or without bevacizumab treatment (Besse et al. 2010; Socinski et al. 2009). All in all, these studies showed that brain metastases do not significantly increase the risks associated with bevacizumab therapy and that the specific risk of CNS bleeding complications remains low in all groups.

Lung cancer: The BRAIN trial investigated 67 patients in a single arm design the combination of a platin-based chemotherapy plus bevacizumab in patients with newly diagnosed, asymptomatic BM from nonsquamous nonsmall cell lung cancer (NSCLC) (Besse et al. 2015). Here, both intracranial and extracranial response rates were considerably high: 61.2% and 64.2%, respectively, with a PFS of 6.7 months, and median OS of 16.0 months. Another cohort of second-line bevacizumab plus erlotinib which was terminated prematurely due to low enrolment ($n = 24$) showed a PFS of 6.3 months, a median OS of 12.0 months, and an ORR of only 12.5%. Other retrospective analyses and case studies confirmed that NSCLC patients with and without brain metastases showed similar PFS and OS when bevacizumab was part of the treatment regimen and that bevacizumab was tolerated similarly well

in patient with vs. without brain metastases (Bennouna et al. 2018; Stefanou et al. 2016). Finally, case reports and case series support a meaningful activity of bevacizumab in NSCLC brain metastases (Danciu et al. 2011; De Braganca et al. 2010; Su and Rau 2015). A case series of 18 patients with brain metastases from solid tumors who received bevacizumab ($n = 13$ NSCLC, $n = 4$ kidney) reported a 60% partial response rate, 40% disease stabilizations, PFS of 14 months, OS 15 months, and long-lasting clinical benefits and brain edema reductions. Together these studies and reports can be seen as supportive evidence for a meaningful clinical activity and safety of bevacizumab in NSCLC.

Breast cancer: In a single-arm phase II study that enrolled 35 patients with brain metastases refractory to whole-brain radiotherapy, bevacizumab was given 1 day before etoposide and cisplatin. 77.1% achieved a CNS-objective response, including 37.1% with a > 80% volumetric reduction of CNS lesions. CNS progression-free survival was 7.3 months, and OS was 10.5 months, which are favorable values for this heavily pretreated patient population (Lu et al. 2015). The rationale to give bevacizumab 1 day before the chemotherapeutic drugs was to achieve vascular normalization, improving the efficacy of cytotoxic therapies. Vascular normalization has been demonstrated before to occur in primary brain tumors (Batchelor et al. 2013; Winkler et al. 2004), and subsequently also in breast cancer brain metastases (Chen et al. 2016). Similarly to NSCLC, multiple additional case reports confirm a meaningful clinical activity, including favorable tumor response rates and improvements of symptom burden, in breast cancer brain metastases (Labidi et al. 2009; Yamamoto et al. 2012).

Colorectal cancer: After an early promising case report (Bhaskara and Eng 2008), a case series of five patients with colorectal brain metastases reported a partial response in one and stable disease in three patients, and favorable brain progression-free (14.8 months) and OS (26.2 months) survival times. Again, the safety profile was good.

Renal cancer: A case series of 16 patients suffering from newly diagnosed, untreated brain metastases from renal cell carcinoma treated with first-line sunitinib reported a median time to progression of 2.3 months, a median OS of 6.3 months, and an overall good tolerability of sunitinib (Chevreau et al. 2014). Similarly, the analysis of the expanded access program of sunitinib in renal cell carcinoma brain metastases patients ($n = 231$) revealed a median PFS of 5.6 months and a median OS of 9.2 months (Gore et al. 2011).

Palliative Benefits by Anti-angiogenics in Brain Metastases

In brain metastases, local treatment options have limitations: for radiotherapy, these are due to the risk of radionecrosis, particularly for too-extensive or recurrent radiotherapy, radiation-induced brain edema, and radiation-induced white matter leukodystrophy; for surgery, these are due to eloquent locations, too high numbers of metastatic foci, and compromised clinical state of the patient. Therefore, the treatment of patients with brain metastases, particularly recurrent and/or highly symptomatic state, is a major clinical challenge. Steroids are widely used to control clinical symptoms caused by perifocal edema. However, steroid treatment has potentially quality of life impairing side effects, such as symptoms related to iatrogenic Cushing syndrome which often appear after a treatment period of a few weeks. Mood changes, metabolic derailment, sleep disorders, and myopathy caused by the iatrogenic Cushing syndrome add to the symptoms of advanced cancer and can further impair the quality of life in these patients. Anyhow, the steroid effect on the tumor edema is frequently needed, as patients suffer from signs of increased cranial pressure including headache, nausea and vomiting, and/or focal neurological deficits otherwise. Therefore, treatment of (recurrent) symptomatic BM faces a dreadful vicious circle of irreplaceable steroid treatment and steroid side effects (Roth et al. 2010; Soffietti et al. 2017). Thus, effective systemic treatments are urgently needed that provide a radiation-independent growth reduction and a steroid-independent

symptom control. Anti-angiogenics, particularly those blocking the VEGF-A pathway, are of particularly high interest in this context: VEGF-A is a key factor of massively increased vascular permeability and subsequent brain edema in brain tumors (Dvorak 2002; Jain et al. 2007). Consequently, as experience from clinical trials and clinical practice tells, bevacizumab, and other VEGF-A blocking agents can relevantly reduce brain edema, thus saving steroids and improving neurological functioning and quality of life of brain tumor patients (Berghoff et al. 2014; Berghoff and Preusser 2018; Felsberg et al. 2017).

Pragmatic Use of Anti-angiogenics in Heavily Pretreated Patients

Frequently, an increase in contrast enhancement and/or edema in pretreated brain metastases can be found after radiosurgery, with no established and generally accepted radiological or even histological parameters to distinguish real tumor recurrence from radionecrosis. This is a common situation in clinical practice, regularly associated with uncertainties about best further treatment strategies, and high steroid use. Figure 3 gives an example of one typical case, where steroids failed to improve the severe neurological deficits, while bevacizumab lead to a relevant clinical improvement. The antitumor activity of anti-angiogenics like bevacizumab suggested by the reports cited above, in combination with its proven beneficial effects in radiation necrosis (Boothe et al. 2013; Delishaj et al. 2017; Levin et al. 2011), makes this class of drugs to a plausible choice for such heavily pretreated brain metastases. This is particularly the case if steroids fail to improve clinical symptoms, or if steroid dependency is chronically high, and side effects of steroids accumulate.

Conclusions and Outlook

Although we lack definitive data from controlled prospective clinical trials for a full assessment of the role of anti-angiogenics in brain metastases, there is compelling data that this class of drugs, particularly bevacizumab, has a meaningful clinical activity in this challenging oncological condition. Despite the notorious difficulty to decipher "true" antitumor effects of anti-angiogenics from their antiedema, and anticontrast enhancing effects (Mathews et al. 2008), the net clinical benefit for patients has been demonstrated multiple times in larger series and case reports – for all main tumor entities that give rise to brain metastases, with the exception of melanoma. Therefore, anti-angiogenics like bevacizumab can be seen as a useful addition to the therapeutic armamentarium for cancer patients suffering from brain metastases and that fail standard therapies, particularly for patients with high symptom burden (neurological deficits, signs of increased intracranial pressure), and for those dependent on relevant doses of steroids. In fact, current data even speak for a particularly high clinical activity of anti-angiogenics in metastasis to the brain compared to other sites in the body, even as single agents which is rarely found for bevacizumab outside the brain; more studies are needed to support this notion.

The dependency on relevant doses of steroids that is common in patients with symptomatic brain metastases is also clearly limiting the therapeutic benefits of immunotherapies, particularly immune checkpoint inhibitors that otherwise show impressive response rates in brain metastasis today (Goldberg et al. 2016; Margolin et al. 2012; Tawbi et al. 2018). For these patient groups, an alternative antiedema therapy with anti-angiogenics can be particularly interesting. There is even preclinical evidence that anti-angiogenics can reshape the immunological brain tumor environment in a way that would favor immunotherapies (Scholz et al. 2016).

Finally, in brain metastasis research, there is an increasing body of evidence that pharmaceutical agents given in a preventive schedule can have a much higher efficacies than given therapeutically when large brain metastases have already formed (Ilhan-Mutlu et al. 2016; Kienast et al. 2010; Lin et al. 2013; Palmieri et al. 2014). Together these data indicate (1) that antimetastatic effects of systemic drugs can be brain-specific, and therefore

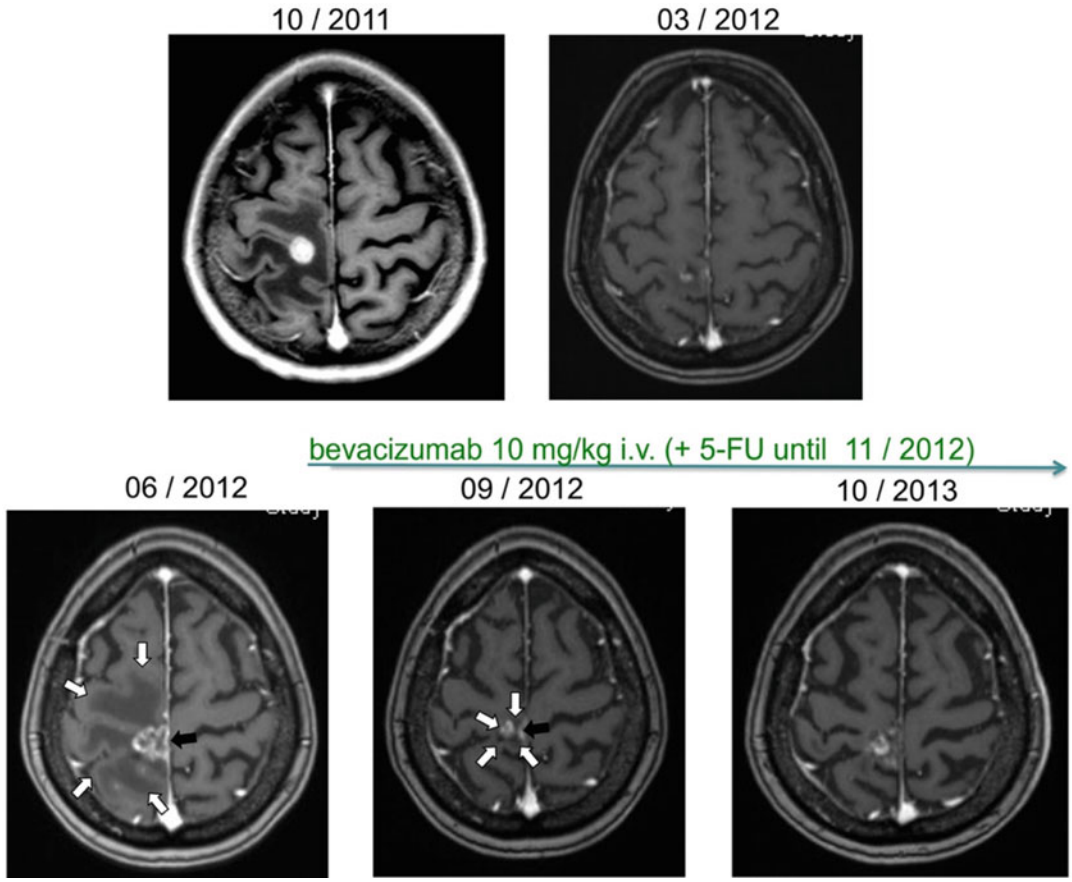

Fig. 3 Clinical benefit from bevacizumab in a patient suffering from a heavily-pretreated brain metastasis. 10/2011: Brain metastasis resection; 12/2011: recurrence; 1/2012: radiosurgery (18 Gy); 6/2012: metastasis recurrence and/or radionecrosis (black arrow), with extensive perifocal brain edema (white arrows); both was greatly reduced after initiation of bevacizumab therapy (9/2012, 10/2013). Clinically, the patient suffered from a high-grade paresis of the left leg which prevented independent walking in 6/2012, which prompted initiation of bevacizumab therapy (plus 5-FU until 11/2012). Shortly after that, the patient regained the ability to walk until 9/2012, with a residual minor paresis of the leg from this point of time one. In 10/2013, the patient was still in good neurological condition

BM prevention needs to be specifically studied; (2) that the preclinical models used can faithfully predict effectiveness (and lack thereof) in patients; and (3) that it might be possible to lower established doses of drugs manifold to achieve strong BM preventive effects, making long-term application of those low-dose schedules medically and financially feasible. Since *preventing* the outgrowth of brain-arrested cancer cells to symptomatic brain metastases by nonneurotoxic, systemic drugs has the potential to make a real difference for cancer patients, this avenue of research appears highly promising from a translational point of view. In this chapter, the case is made that anti-angiogenics could be particular interesting drugs in this respect, with indications for a brain-specific preventive effect of sub-clinical low doses in lung adenocarcinoma.

Cross-References

▶ Anti-angiogenic Targeting in Metastatic Colorectal Cancer Therapy

- ▶ Combination of Anti-angiogenics and Other Targeted Therapies
- ▶ Inhibition of Tumor Angiogenesis in the Treatment of Lung Cancer
- ▶ Imaging Tumor Angiogenesis
- ▶ The Value of Anti-angiogenics in Breast Cancer Therapy

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Part XVIII

Anti-angiogenics in Multiple Myeloma

The Value of Anti-angiogenics in Multiple Myeloma Therapy

Thomas Moehler, Dirk Hose, Mindaugas Andrulis, Anja Seckinger, and Hartmut Goldschmidt

Contents

Introduction 641
Multiple Myeloma and Angiogenesis 641
Proangiogenic Environment Through Plasma Cell Proliferation, Angiogenic Cytokines, and Hypoxia 641
Angiostatic Factors 642
Vasculogenesis 644
Exosomes and miRNA 644

Relevance of the Bone Marrow Microenvironment for Myeloma Progression and Angiogenesis 644

Biomarkers and Imaging of Angiogenesis in Multiple Myeloma 645
Gene Expression Data 645
Tissue-Derived Biomarkers 645
Blood-Borne Factors 645
Imaging 645

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Anti-angiogenic Activity of Approved Drugs for Multiple Myeloma 646

Immunomodulatory Drugs: Molecular Mechanisms of Action and Anti-angiogenic Activity 647

Immunomodulatory Drugs: Clinical Application and Use 647

Proteasome Inhibitors: Anti-angiogenic Activity and Clinical Use 647

HDAC Inhibitors 648

Clinical Trials with Anti-angiogenic Compounds 648
Anti-VEGF Approach: Anti-VEGF Antibodies and Multi-targeting TKIs 648
Targeting Hypoxia and HIF-1α Inhibition 649
PI3 Kinase Inhibitors 650

Future Directions 650
Nanoparticles and Materials Development 650
New Targets 650

Cross-References 652

References 652

Abstract

Bone marrow angiogenesis is a hallmark of multiple myeloma and an important component of the profound myeloma-induced changes of the microenvironment. Bone marrow angiogenesis evaluated histologically or via dynamic contrast-enhanced magnetic resonance imaging (dceMRI) is a prognostic marker for survival for multiple myeloma (MM) patients. Normal and malignant plasma cells express angiogenic factors including vascular endothelial growth factors (VEGF) and are able to induce angiogenesis. The constitutive expression of angiogenesis factors by plasma cell accumulation of malignant plasma cells and increasing hypoxia is considered as initial drivers for bone marrow angiogenesis and remodeling of the bone marrow microenvironment in myeloma. Bone marrow angiogenesis may then be further accelerated by sequential loss of angiogenic inhibitors and/or aberrant expression of angiogenic factors during disease progression. Several novel agents such as immunomodulatory drugs (IMiDs), proteasome inhibitors, and histone deacetylase (HDAC) inhibitors approved for myeloma exert anti-angiogenic activity. Several considerations are expected to further develop anti-angiogenic treatment concepts in multiple myeloma: angiogenesis dependency and the "critical" angiogenesis-related drivers may vary and are dependent on the stage of myeloma development from monoclonal gammopathy of unknown significance (MGUS) up to symptomatic and therapy-resistant MM. Promising new agents targeting bone marrow angiogenesis are currently evaluated in early clinical studies including inhibitors of the hepatocyte-growth-factor pathway. Achieving clinical relevant effects might require the combination of angiogenesis inhibitors as well as rational combination with standard of care treatments.

It is expected that additional compounds with the ability to inhibit pathological bone marrow angiogenesis will be integrated into the therapeutic armamentarium for multiple myeloma in the future.

Keywords

Multiple myeloma · Angiogenesis · Immunomodulatory drugs · Bortezomib · Biomarkers

Introduction

Multiple Myeloma and Angiogenesis

Progression of multiple myeloma is associated with a significant increase in bone marrow vascularization and microcirculation as evidenced by histological as well as imaging studies (Fig. 1) (Moehler et al. 2001, 2004; Vacca et al. 1994). Increased bone marrow microvessel density as assessed in bone marrow histology of patients with symptomatic multiple myeloma carries a poor prognosis.

Proangiogenic Environment Through Plasma Cell Proliferation, Angiogenic Cytokines, and Hypoxia

Normal plasma cells and myeloma cells express pro- and anti-angiogenic/angiostatic factors with the net result of a proangiogenic environment that leads to a proliferation of endothelial cells and development of microvessels (Vacca et al. 1994; 2006; Hose et al. 2009b).

In fact, bone marrow plasma cells of normal donors were able to elicit significant angiogenic response in contrast to memory B-cells (Hose

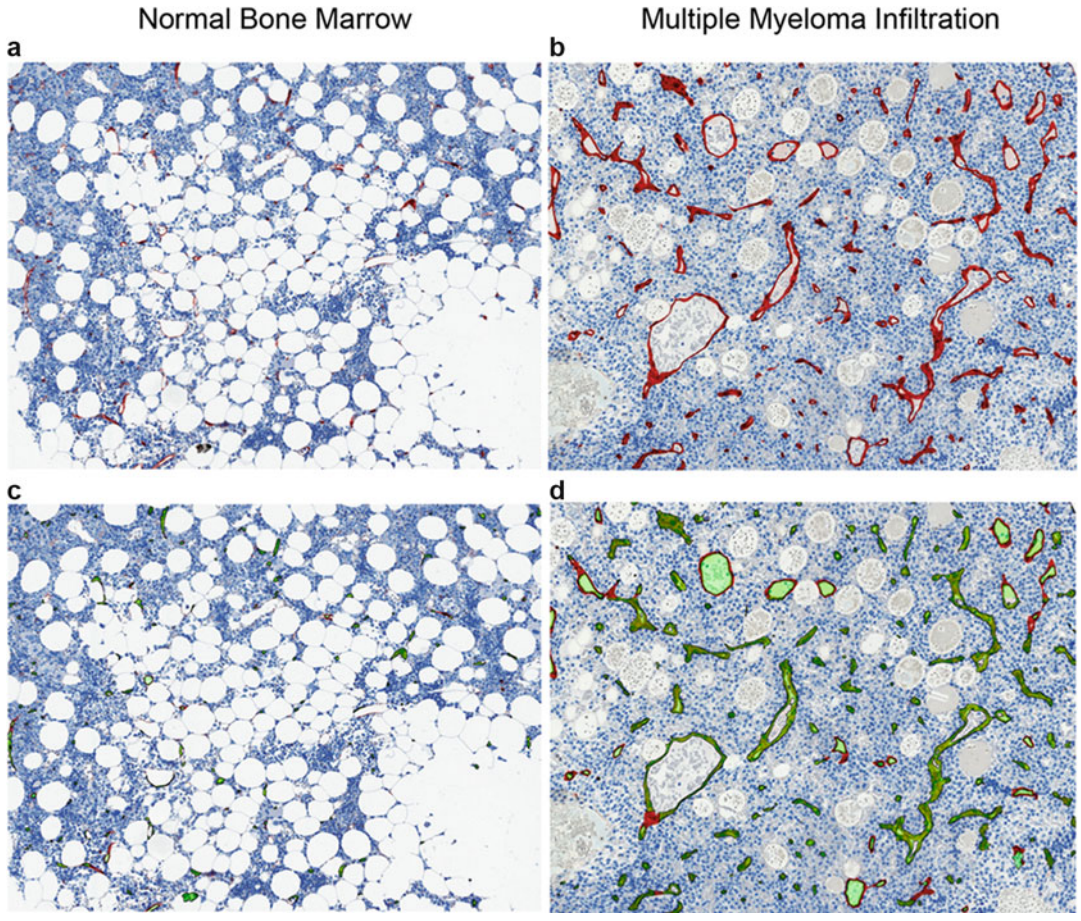

Fig. 1 Microvessel density in bone marrow infiltrated by multiple myeloma. An example of comparative analysis of microvessel density in biopsy of normal bone marrow compared to bone marrow with extensive myeloma infiltration (100× original magnification). The density of capillaries (**a**, **b**) CD34 immunostaining, (**c**, **d**) respective markup images from quantitative microvessel analysis performed with Aperio algorithm is considerably higher in bone marrow areas occupied by neoplastic plasma cells

et al. 2009b). A comprehensive analysis of gene and protein expression of angiogenesis-relevant factors comparing plasma cells of healthy volunteers to patients with MGUS and multiple myeloma revealed that plasma cells have a constitutive expression of a range of pro-angiogenic cytokines such as vascular endothelial growth factor-A (VEGF-A). VEGF-A as a central angiogenic factor is already expressed in 92% of normal bone marrow plasma cells and also abundantly expressed in myeloma cells (Hose et al. 2009b). Constitutive expression of angiogenic cytokines at the level of normal plasma cells was demonstrated for adrenomedullin (ADM), interleukin-6 (IL-6), placental growth factor (PGF), hepatoma-derived growth factors (HDGF), interleukin-8, and others (Fig. 2) (Kocemba et al. 2013; Hose et al. 2009b).

In addition to the angiogenic capacity that myeloma cells "inherit" from plasma cells, myeloma cells may acquire additional changes in terms of aberrant or increased expression of angiogenic factors, but may also involve the loss of anti-angiogenic factors (Hose et al. 2009b). Aberrant or overexpression of proangiogenic factors in myeloma cells compared to plasma cells are, for example, hepatocyte growth factor (HGF), interleukin-15 (IL-15), insulin-like growth factor-1 (IGF-1), transforming growth factor-α (TGF−α), heparanase (HNSE), angiopoietin-1 (Ang1), and connective tissue-derived growth factor (CTGF) (Fig. 2) (Vacca and Ribatti 2006; Hose et al. 2009b). Several of the listed molecular systems were evaluated in more detail: the activation of the Ang/Tie2 loop in myeloma cells was previously described as a relevant system in myeloma-induced angiogenesis (Giuliani et al. 2003). Fibroblast growth factor-2 (FGF-2) is another relevant angiogenic factor promoting bone marrow angiogenesis which is produced by myeloma cells but also by other cells in the bone marrow microenvironment (Kumar et al. 2004). The APRIL/B-cell maturation antigen (BCMA) system was extensively validated and confirmed in the relevance for myeloma pathogenesis (Moreaux et al. 2005, 2009).

Therefore the accumulation of clonal myeloma cells leading to a relevant increase of the concentration of angiogenic factors in the bone marrow has to be considered as an important initial driver for angiogenesis. Furthermore, increasing plasma cell density in the bone marrow leads to hypoxia which further promotes the expression of angiogenesis-related genes through increased expression of hypoxia-inducible factor-1α (HIF-1α) (Zhang et al. 2016).

Angiostatic Factors

The aspect of loss of angiostatic factors is considered as a critical step in cancer angiogenesis (Folkman 1995).

There is evidence that comparing MGUS with MM, angiostatic activity is lower in the latter (Kumar et al. 2004). Vacca et al. have likewise demonstrated lower expression of the angiostatic factor semaphorin A in MM compared to MGUS (Vacca et al. 2006).

Downregulation and/or loss of expression of platelet factor 4 (PF-4), an angiostatic molecule (Maione et al. 1990), in 40% of myeloma compared to normal plasma cells was discovered as the most prominent change of angiostatic factors in the comprehensive angiogenesis analysis conducted by Hose et al. (2009b). The role of PF-4 as angiostatic factor for myeloma was confirmed as PF-4 was able to decrease angiogenesis and myeloma progression in experimental/preclinical models (Yang et al. 2011). In addition to the molecules mentioned above, serpin-F1/pigment epithelium-derived factor (PEDF) and tissue inhibitor of metalloproteinase-2 (TIMP-2) might have a role as angiostatic factors in regulating myeloma-related angiogenesis (Seki et al. 2013). Bone morphogenic protein-6 (BMP-6) was found to be expressed by plasma cells as well as myeloma cells and suppresses angiogenesis (Seckinger et al. 2009) (Fig. 2). Increased expression of BMP-6 was identified as a prognostic parameter for prolonged survival in patients with multiple myeloma (Seckinger et al. 2009).

The evaluation of angiostatic factors is more complex compared to the proangiogenic drivers as many angiostatic molecules are actually derived from cleavage of cell surface receptors

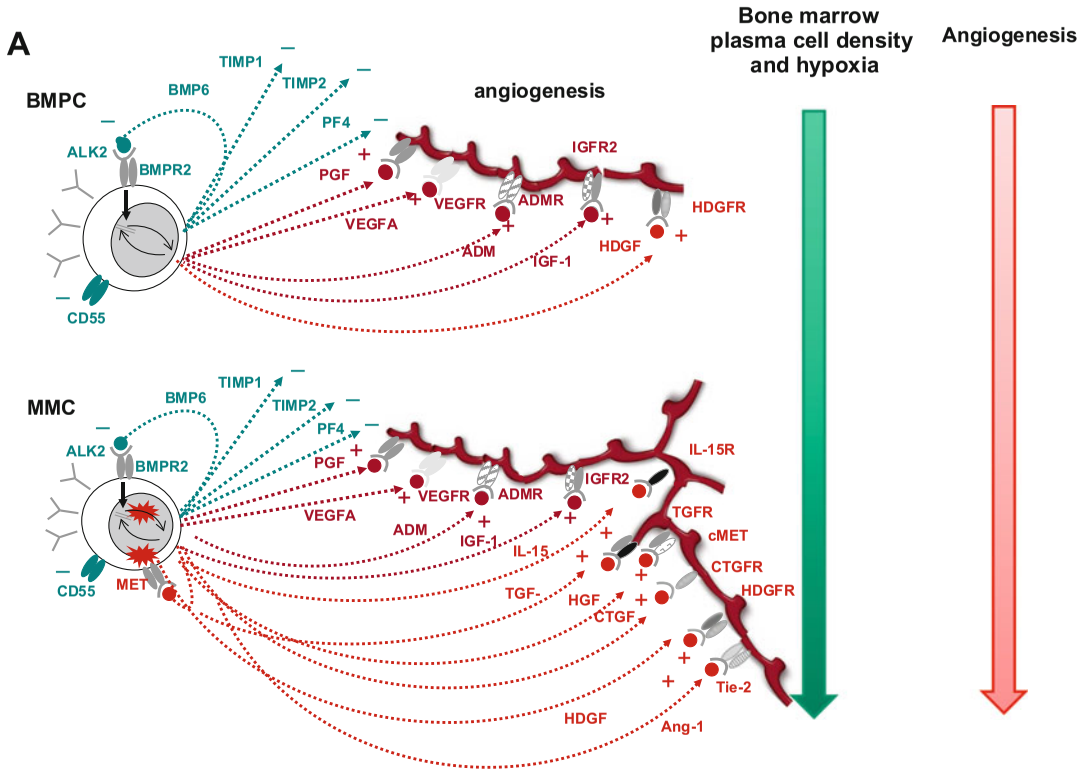

Fig. 2 Schematic representation of network of angiogenic and angiostatic factors (prominent examples) for myeloma-induced bone marrow angiogenesis (Modified according to Hose et al. 2009b). (**a**) Normal bone marrow plasma cells produce a range of angiogenic (*red arrows*) and angiostatic (*green*) factors. Accumulation of plasma cells and increasing hypoxia generate a proangiogenic environment (*arrows*). Cytokines and receptors expressed by angiogenic endothelial cells are shown. (**b**) Transformation into a myeloma plasma cell results in aberrant or increase in pro-angiogenic factors that further promote bone marrow angiogenesis. *Abbreviations*: *angiostatic factors*, bone morphogenic protein-6 (*BMP6*), tissue inhibitor of metalloproteinases-1 (*TIMP-1*), tissue inhibitor of metalloproteinases-2 (*TIMP-2*), platelet factor 4 (*PF4*); *angiogenic factors*, placental growth factor (*PGF*), vascular endothelial growth factor-A (*VEGF-A*), adrenomedullin (*ADM*), insulin-like growth factor-1 (*IGF-1*), hepatocyte growth factor (*HGF*), connective tissue-derived growth factor (*CTGF*), interleukin-15 (*IL-15*), transforming growth factor-α (TGF-α), hepatoma-derived growth factor (*HDGF*), angiopoietin-1 (*Ang-1*), angiopoietin receptor (*Tie-2*), and endothelial receptors of cytokines are displayed by adding "R" to the cytokine acronym

or extracellular matrix proteins (Limaverde-Sousa et al. 2014). Further examples include NK-4, a structural homologue to angiostatin, which was found to counteract HGF-induced myeloma progression in vitro as well as in a murine model (Du et al. 2007).

Vasculogenesis

A very interesting aspect is that myeloma cells are able to attract circulating endothelial precursor cells to develop bone marrow capillaries in a process of postnatal vasculogenesis (Moschetta et al. 2016).

Exosomes and miRNA

By regulating the expression of target genes, miRNAs control diverse cellular functions such as proliferation, differentiation, angiogenic activity, and apoptosis (Kong et al. 2012). Specific miRNAs have been associated with the different stages of plasma cell dyscrasias and correlated with the myeloma phenotype as well as cytogenetic profile and survival (Lionetti et al. 2009; Seckinger et al. 2015b). Among miRNAs deregulated in myeloma, miR-199a-5p is of relevant interest because it directly targets HIF-1α and thereby influences angiogenesis (Sayed and Abdellatif 2010). Raimondi et al. have demonstrated that miR-199a-5p downregulates HIF-1a and expression of angiogenic cytokines such as VEGF, IL-8, and FGF-2. miR-199q-5a treatment of myeloma cells in an in vivo murine model led to suppressed experimental myeloma progression and increased survival of mice compared to the control group (Raimondi et al. 2014). miR-mimetics have now emerged as therapeutic tools (Di Martino et al. 2016).

Research in myeloma pathogenesis has focused on the aspect that malignant transformation leading to increased clonal plasma cell proliferation and decreased apoptosis occurs early during development of MGUS (Klein et al. 2011). This initial transformation event leads to progressive accumulation of clonal plasma cells and increased bone marrow hypoxia that result in expression of angiogenic cytokines increasingly stimulating angiogenesis. Over time myeloma then develops additional angiogenic factors and loss of anti-angiogenic molecules as an angiogenesis "boost."

The relevance of angiogenesis for various cancer indications including hematological malignancies may vary considerably. The bone marrow has a special pathophysiology with fenestrated endothelial cells and a distinct microenvironment as outlined below in more detail. Whereas the role of novel microvessels for cell nutritional support might be less relevant compared to solid tumors or lymphomas, endothelial cells activated by myeloma cells play an active role in creating the microenvironment which is essential for the development of myeloma.

Many authors have therefore highlighted the relevance for treating the myeloma microenvironment as a major therapeutic concept for myeloma therapy, and the activity of the currently approved compounds relies on a combined effect on myeloma cells as well as the myeloma microenvironment (de la Puente et al. 2014; Moreau and Touzeau 2015; Lehners et al. 2016).

Relevance of the Bone Marrow Microenvironment for Myeloma Progression and Angiogenesis

The considerations described in the previous paragraphs focus mainly on the interaction of myeloma with endothelial cells. In the bone marrow, the cellular interactions are more complex. Different from other cancer types, myeloma cells are – as their normal counterpart – already located in "their" bone marrow microenvironment.

The interaction of myeloma cells with the bone marrow microenvironment is a key aspect of the pathophysiology of this disease (Bianchi and Munshi 2015). Within the bone marrow microenvironment, a vascular niche and an osteoblastic niche are distinguished. Myeloma cells enter the bone marrow niche and attach to bone marrow stromal cells in close proximity to endothelial cell microvessels and osteoblasts. Under physiological conditions the bone marrow vascular niche is responsible for nurturing the hematopoietic stem cells (Ribatti et al. 2015).

The bone marrow niche and its interaction with myeloma cells therefore have a special

importance in the development of multiple myeloma. A bi-/multidirectional effect is initialized by myeloma cells that lead to profound changes in the microenvironment compared to normal bone marrow (Bianchi and Munshi 2015).

Inflammatory cells and osteoblasts/osteoclasts support the angiogenesis process and contribute thereby to the pathophysiology of multiple myeloma (Vacca and Ribatti 2006; Ribatti and Vacca 2009). The relevance of bone marrow microenvironment for progression has been demonstrated for other bone marrow-specific malignancies such as acute and chronic leukemias as well (Krause et al. 2013).

Recently the role of exosomes in the cell-cell communication was described. Exosomes and miRNA contained in exosomes are important components in the regulation of progression of myeloma (Wang et al. 2016a; Ohyashiki et al. 2016; Roccaro et al. 2013), and aspects relevant for increased angiogenesis are described in the previous section of this chapter.

Preclinical and clinical research, focusing on the myeloma microenvironment, has significantly contributed to the development and approval of several novel agents with anti-angiogenic properties. Anti-angiogenesis strategies and biomarkers for angiogenesis in multiple myeloma are now reviewed in the following chapters.

Biomarkers and Imaging of Angiogenesis in Multiple Myeloma

Recent reviews have summarized currently available blood-borne or tissue-derived biomarkers and imaging technologies to evaluate angiogenic potential of cancer cells (Cidon et al. 2016; Hose et al. 2009b). Biomarkers and imaging of angiogenesis are expected to play an important role for the development of anti-angiogenic compounds in the future.

Gene Expression Data

Hose et al. have identified a set of 158 angiogenesis relevant genes expressed in myeloma cells (Hose et al. 2009b).

Tissue-Derived Biomarkers

Bone marrow histology has mainly focused on determination of microvessel density and angiogenesis index. Microvessel density is correlated with survival in myeloma and with disease-free survival in patients with solitary plasmocytoma (Kumar et al. 2002, 2003; Rajkumar et al. 2000; Sezer et al. 2000). Bone marrow biopsies can assess a range of factors. Therefore bone marrow biopsies can provide a better guidance on the actual expression of cytokines by myeloma cells (Ribas et al. 2004). Figure 1 exemplifies results of endothelial staining of a bone marrow section of multiple myeloma patient compared to normal bone marrow.

Blood-Borne Factors

Peripheral blood angiogenic cytokine levels such as bFGF, VEGF, angiopoietin-2 (Ang-2), HGF, and others are increased in patients with multiple myeloma compared to healthy volunteers and correlate with advanced disease and adverse prognosis (Neben et al. 2001a; Medinger et al. 2015; Pour et al. 2010; Seidel et al. 1998; Borset et al. 1996). A significant specific down-modulation of peripheral levels of angiogenic cytokines during thalidomide therapy of myeloma patients could not be detected (Neben et al. 2001b), indicating that thalidomide effects on myeloma are not mediated by an effect on cytokine secretion from the bone marrow microenvironment.

VEGF serum levels were found to be predictors of response to treatment in POEMS syndrome (Misawa et al. 2015).

Imaging

Various imaging technologies are under evaluation to detect the tumor microcirculation or the expression of receptors specifically particular in angiogenic endothelial cells (Chen et al. 2016).

Only a selected number of clinical studies have evaluated imaging of angiogenesis and microcirculation in multiple myeloma. Most data are currently available for dceMRI. Importantly,

histological assessment of bone marrow microvessel density correlated closely with dceMRI microcirculation parameters (Nosas-Garcia et al. 2005).

Increase in bone marrow microcirculation as detected by dceMRI microcirculation parameters was recently found to be prognostic for overall survival of symptomatic multiple myeloma patients (Merz et al. 2016).

In addition, dceMRI microcirculation changes induced by novel agents and chemotherapy could be monitored and are prognostic significant for overall and progression-free survival in multiple myeloma patients (Merz et al. 2015). Increased microcirculation detected by dceMRI correlates with local vertebral complications such as vertebral collapse in lumbar manifestations of myeloma bone disease (Merz et al. 2016; Scherer et al. 2002).

Several other MRI-related technologies are available to evaluate microcirculation and bone marrow angiogenesis. Arterial spin labeling MRI has been shown to be an early response predictor in multiple myeloma (Fenchel et al. 2010).

Anti-angiogenic Strategies in Multiple Myeloma

Judah Folkman and coworkers have initialized the strategy and search for developing angiogenesis inhibitors for cancer and other indications (Folkman 1971) with some being explored clinically (Jayson et al. 2016).

Therapeutic targets with anti-angiogenic potential were recently reviewed and can be summarized as follows: inhibition of angiogenic growth factors (1), blockade of angiogenic growth factor receptors (2), inhibition of angiogenic growth factor receptor signaling (3), interference with endothelial cell adhesion to extracellular matrix (4) or inter-endothelial adhesion (5), matrix proteases (6), transcription factors (7), morphogenic signals (8), and treatment with angiostatic factors (9) (Jayson et al. 2016).

It can certainly be considered as a major breakthrough for clinical angiogenesis research that anti-VEGF therapies have entered standard of care in many cancer indications since VEGF was discovered in the 1980s (Jayson et al. 2016). Since then a total of 12 therapeutic products which block VEGF signaling at various steps achieved regulatory approval: monoclonal anti-VEGF antibodies (Avastin, ramucirumab), VEGF-receptor 1/2 fusion protein (aflibercept), and several multi-targeted VEGF-R-specific tyrosine kinase inhibitors (TKIs) (Ricci et al. 2015; Zhao and Adjei 2015; Jayson et al. 2016; Wang et al. 2015).

Surprisingly, none of the agents targeting other aspects of the angiogenesis process have entered the clinic in oncology up to now. The current viewpoint is that cancer has developed multiple mechanisms to initialize and maintain angiogenesis; therefore multiple mechanisms of resistance to anti-angiogenic therapy exist (Ribatti 2016; Jayson et al. 2016).

Concepts to use anti-angiogenic agents in therapy of malignancies had initially focused on solid cancer indications, but over time a growing body of evidence indicates a relevance for a broad range of hematological malignancies as well (Moehler et al. 2004; Ribatti et al. 2001, 2013).

At this point in time, none of the anti-VEGF therapies that are part of treatment strategies in solid cancer indications are approved for multiple myeloma. Current status and further perspectives of the VEGF-pathway strategy as well as other anti-angiogenic strategies currently explored for multiple myeloma are described and discussed in the section "Future Directions" below.

In contrast, compounds that target the myeloma cell microenvironment more broadly – and in this respect – are recognized inhibitors of angiogenesis and myeloma-endothelial cell interaction which were successfully developed. Therefore, the next chapter describes the anti-angiogenic activity of proteasome inhibitors, immunomodulatory drugs (ImiDs), and histone deacetylase (HDAC) inhibitors.

Anti-angiogenic Activity of Approved Drugs for Multiple Myeloma

For several compounds currently approved for therapy of multiple myeloma, e.g., ImiDs, proteasome inhibitors, and HDAC inhibitors,

direct and indirect effects on bone marrow angiogenesis were demonstrated. Obviously, the direct cytotoxic effects on myeloma cells decrease the angiogenesis stimulus. In addition for several of these compounds, a direct anti-angiogenic activity has been shown as detailed below (Moreau and Touzeau 2015; Laubach et al. 2016; Moehler et al. 2016).

Immunomodulatory Drugs: Molecular Mechanisms of Action and Anti-angiogenic Activity

Thalidomide and other compounds in this class (e.g., lenalidomide [LEN] and pomalidomide) block angiogenesis in part by a direct effect on vascular cells during the process of angiogenesis (D'Amato et al. 1994). The molecular mechanism of action of thalidomide is described as via binding to cereblon. Cereblon is part of the multiprotein complex cullin-4-containing E3 ubiquitin ligase complex $CRL4^{CRBN}$ (Ito et al. 2010). Binding of IMiDs to cereblon is reported to have two major consequences: firstly, endogenous substrates of cereblon are blocked such as the transcription factor MEIS2, and secondly, binding of other proteins such as Ikaros (IKZF1) and Aiolos (IKZF3) to cereblon within $CRL4^{CRBN}$ is facilitated/enhanced. Ubiquitination of Ikaros and Aiolos leads to their degradation and inactivation by the S26-proteasome and ultimately induces an antiproliferative effect (Berndsen and Wolberger 2014; Cai and Yang 2016). Research on the immunomodulatory and anti-angiogenic effects of thalidomide and IMiDs suggests IMiD binding to cereblon is responsible for the immunomodulatory activity (Gandhi et al. 2014). Confirmatory data for the endothelial cells are however currently missing.

Further mechanisms of action may include the ability of IMiDs to down-modulate bFGF and other angiogenic factors by changing intracellular protein turnover and ubiquitination machinery within myeloma cells (Zhu et al. 2011).

Immunomodulatory Drugs: Clinical Application and Use

Thalidomide was the first IMiD that gained approval by FDA for patients with relapsed and refractory multiple myeloma in 2006. Since then thalidomide has received FDA and EMA approval for the first-line indication in combination with dexamethasone (dex). Lenalidomide/dex and pomalidomide/dex combinations were approved for patients with one or at least two prior therapies, respectively. Evidence for clinical activity for thalidomide, lenalidomide, and pomalidomide as maintenance therapy after induction/consolidation was recently presented in a meta-analysis (Wang et al. 2016b). A significant improvement of progression-free survival with lenalidomide or thalidomide maintenance therapy was described. The advantage of len/thal maintenance therapy was observed independent of a prior high-dose chemotherapy and autologous stem cell transplantation (ASCT). Palumbo et al. conducted a randomized study which compared ASCT as part of a multi-agent regimen in first line to a non-ASCT approach and provided evidence that ASCT combined with IMiDs is superior to IMiDs alone (Palumbo et al. 2014).

In addition to its therapeutic activity in multiple myeloma, lenalidomide is also approved for patients with relapsed or refractory mantle cell lymphoma and myelodysplastic syndrome with a 5q-deletion. Additional indication may follow as it was demonstrated that thalidomide and lenalidomide have therapeutic effects in Crohn's disease, rheumatoid arthritis, sarcoidosis, and other indications (Diamanti et al. 2015).

IMiDs therefore play an important role in the therapeutic strategy for myeloma patients.

Proteasome Inhibitors: Anti-angiogenic Activity and Clinical Use

Bortezomib was the first-in-class proteasome inhibitor developed for multiple myeloma and achieved first approval as first-in-class compound for multiple myeloma in 2003. Bortezomib is a dipeptide boronic acid proteasome inhibitor

which reversibly inhibits the chymotrypsin-like activity of the 20S-proteasome subunit. The net effect of proteasome inhibition with bortezomib is the accumulation of intracellular regulatory proteins, e.g., IκB, leading to down modulation of NfκB and other transcription factors decreasing the activity of many other proteins (Sanchez-Serrano 2006). As IMiDs, bortezomib acts indirectly on angiogenesis by its cytotoxic activity on myeloma cells and thus reducing the angiogenic stimulus inferred by these. Secondly, bortezomib downregulates the expression of angiogenic cytokines by myeloma cells and decreases the direct adhesion of myeloma cells to bone marrow stromal as well as endothelial cells (Hideshima et al. 2011). In addition to these effects on myeloma cells, bortezomib was found to inhibit endothelial function and the angiogenesis process directly. In vitro experiments demonstrated that bortezomib in concentrations that were achievable in the clinical setting was able to significantly downregulate proliferation, formation of network structures in in vitro angiogenesis assays, and suppress angiogenic cytokine secretion (Roccaro et al. 2006).

Osteoclasts stimulated by myeloma cells are considered to be important contributors to the proangiogenic bone marrow environment. Bortezomib and other proteasome inhibitors have positive effects on the overall bone marrow microenvironment. In particular, proteasome inhibitors suppress activity of osteoclast and instead support proliferation of osteoblasts as well as overall bone formation (Accardi et al. 2015; Giuliani et al. 2006). Therefore suppression of osteoclast activity may contribute to the overall anti-angiogenic effects of bortezomib (Roccaro et al. 2006).

Since the initial FDA approval of bortezomib in 2003 for multiple myeloma, several other proteasome inhibitors were approved by FDA and EMA (carfilzomib, ixazomib). Additional proteasome inhibitors are currently in clinical development. Regarding their anti-angiogenic activities, there is no data available that their mode of action would significantly differ between the available proteasome inhibitors (Accardi et al. 2015; de la Puente et al. 2014).

HDAC Inhibitors

Acetylation and deacetylation of histone proteins are important epigenetic mechanisms for the regulation of gene expression. HDAC inhibitors are recognized to have multiple effects on tumor cells and endothelial cells (Deroanne et al. 2002; Gahr et al. 2015). HDAC inhibitors were primarily developed for their effects to induce tumor cell cytostasis, differentiation, and apoptosis (de la Puente et al. 2014). Additional research has demonstrated that HDAC inhibitors down-modulate angiogenic cytokine expression of tumor cells but also exert a direct anti-angiogenic effect on activated endothelial cells, e.g., by inhibition of endothelial VEGF-R expression (Deroanne et al. 2002; Heider et al. 2006).

The HDAC inhibitor panobinostat was recently approved by FDA for patients with relapsed and refractory multiple myeloma in combination therapy with bortezomib, and EMA approval is awaited in near future.

Clinical Trials with Anti-angiogenic Compounds

Anti-VEGF Approach: Anti-VEGF Antibodies and Multi-targeting TKIs

Multi-targeting TKIs such as sorafenib were investigated in multiple myeloma cells as well as in in vivo myeloma model and induced responses with increased survival in murine models (Kharaziha et al. 2012; Ramakrishnan et al. 2010). Kumar et al. have reported on a combination of sorafenib as multi-targeted VEGF-R TKI with bortezomib in a mixed population of patients with relapsed and refractory cancer indications, and one patient with renal cell cancer developed a partial response (Kumar et al. 2013).

In patients with relapsed and refractory follicular lymphoma, the combination of rituximab and bevacizumab was found to improve the progression-free survival in a randomized proof-of-concept study (Hainsworth et al. 2014). In this study 60 patients were randomized to receive

single-agent rituximab or a combination of rituximab/bevacizumab. The combination therapy improved the median progression-free survival from 10.7 month in the control group receiving rituximab to 20.7 months in the group treated with the bevacizumab/rituximab combination. In addition, the numerical overall survival was superior for the combination with 73% at 4 years compared to 53% in the control group which was not significant, but the study was not powered for overall survival.

In patients with relapsed and refractory multiple myeloma, bevacizumab was tested in combination therapy with bortezomib compared to bortezomib as single agent in the AMBER study which enrolled 102 patients (White et al. 2013). The bevacizumab combination therapy was well tolerated in this patient population with some expected bevacizumab class effects reported more frequently in the combination arm as expected. Patients in the bevacizumab/bortezomib combination arm had an increased overall response rate of 51% versus 43.4% ($p = 0.04$). However, median progression-free survival and overall survival were not statistically significantly different.

A phase II study which evaluated bevacizumab single agent against bevacizumab plus thalidomide in relapsed or refractory myeloma patients showed one patient in the single arm cohort ($n = 6$) with highest expression of VEGF in myeloma cells achieving stable disease; two patients in the bevacizumab/thalidomide combination arm developed an objective response, and three patients in this cohort ($n = 7$) developed stable disease (Somlo et al. 2011).

As VEGF-pathway-related TKIs have been successful in other cancer entities, several multi-targeting VEGF-receptor-specific TKIs (pazopanib, vandetanib, sorafenib, and SU5416) were explored in relapsed and refractory myeloma patients in studies enrolling 15–28 patients (Kovacs et al. 2006; Prince et al. 2009; Zangari et al. 2004; Yordanova et al. 2013). Yordanova et al. have reported on two partial responses in patients with relapsed/refractory myeloma treated with sorafenib/dexamethasone (Yordanova et al. 2013). In the other studies, the patients developed stable disease as the best response.

The results of these clinical studies are not surprising as single-agent angiogenesis inhibitors are expected to stabilize tumor growth or delay progression rather than inducing short-term tumor regression. In fact, approvals of VEGF-pathway inhibitors (either antibodies or TKIs) in other cancer indications have been mainly based on prolongation of progression-free survival and overall survival.

Overall, in multiple myeloma, therapeutic strategies targeting the VEGF-pathway particularly in single-agent studies have not achieved clinical relevant success so far. Therefore, further trials might thus aim at integrating VEGF-inhibitors in multi-agent combination regimen or evaluating the better integration of biomarkers. Promising opportunities for drug development of angiogenesis inhibitors including VEGF-pathway inhibitors are further discussed below.

Targeting Hypoxia and HIF-1α Inhibition

As outlined above, hypoxia and activation of HIF-1α are considered an important driver of myeloma-induced angiogenesis. HIF-1α inhibition has emerged as an interesting target for myeloma therapy, and significant effects are reported in murine in vivo model as well as in cell culture experiments (Storti et al. 2013, 2016; Hose 2009a; Hose et al. 2010).

TH-302/evofosfamide is a hypoxia-activated cytotoxic prodrug. TH-302 was evaluated in a phase I/II clinical study in relapsed and refractory myeloma patients and with clinical activity noted including patients with partial and minimal response (Laubach 2014). Pharmacological inhibitors of HIF-1α are currently under development (Viziteu et al. 2016). ENZ-2968 is an antisense oligonucleotide inhibitor of HIF-1α who has entered the clinical development stage (Jeong et al. 2014). ENZ-2968 was able to suppress HIF-1α-protein and mRNA levels obtained by intra-tumoral tumor biopsies during treatment and down-modulated microcirculation detected

by dceMRI. These results indicate that a HIF-1α antisense strategy appears to be feasible in the context of clinical studies.

Inhibition of Heparanase and Heparin-Binding Growth Factors

The role of heparanase for angiogenesis is known for many years and summarized in reviews (Fux et al. 2009; Cassinelli et al. 2013). Heparanase is a β-d-glucuronidase which cleaves heparan sulfate chains attached to extracellular matrix or the cell membrane. In the context of multiple myeloma, heparanase has probably several roles. Heparanase binds to the heparin sulfate side chains of syndecan-1 (CD138) and releases heparin-binding (myeloma) growth factors which in turn bind to their cytokine receptors or activate endothelial cells, e.g., heparin-binding endothelial growth factor (HBEGF), a proliferation-inducing ligand (APRIL), amphiregulin (AREG), and the neuregulins.

Pro-heparanase bound to syndecan-1 also supports the clustering of syndecans and thereby is able to activate myeloma cells. Moreover, heparanase induces shedding of syndecan-1 and thereby promotes myeloma proliferation and angiogenesis (Purushothaman et al. 2010). Syndecan-1 is able to bridge and couple VLA-4 and VEGF-R2 and supports migration and angiogenesis (Jung et al. 2016).

The chemically modified non-anticoagulant heparin analogue roneparstat (SST0001) is able to inhibit heparanase and disrupt the heparanase/syndecan-1 axis. Roneparstat was found to have anti-myeloma activity in vitro and in animal models (Ritchie et al. 2011; Pala et al. 2016). Roneparstat is currently investigated in a phase I study in multiple myeloma.

PI3 Kinase Inhibitors

PI3K inhibitors (De et al. 2013) block angiogenesis and cytokine-activated tumor progression and are a recognized therapeutic concept for multiple myeloma (Ikeda et al. 2010) currently entering phase I trials. Examples comprise SF1126 which was successfully evaluated in a preclinical model as single agent and was found to augment the therapeutic activity of bortezomib (De et al. 2013).

Future Directions

The approval of VEGF-pathway inhibitors for many cancer indications was a significant contribution of angiogenesis research to cancer therapy. A number of other molecules have failed to achieve clinical relevant effects clinically. There are still many opportunities and molecular entities available to further explore the potential to achieve therapeutic effects of anti-angiogenic compounds for myeloma patients. This chapter is focused to summarize some of the promising avenues currently explored but can certainly be not completely exhaustive of the topic.

Nanoparticles and Materials Development

Kyphoplasty and radiotherapy are common interventions to control local complications as bone pain and bone fractures in patients with multiple myeloma (Kasperk et al. 2012). Angiogenic "hot spots" may be present in terms of focal myeloma lesions with higher risk for progression and local complications (Merz et al. 2016). Further research is required to determine if microcirculation imaging by dceMRI may guide radiotherapy as primary or secondary prophylaxis for bone complications and fractures. Inserting material that facilitates the local release of compounds with anti-angiogenic activity during kyphoplasty is therefore an attractive idea that may lead to clinical effects (Striegler et al. 2015).

New Targets

Novel thalidomide analogues are developed with a modified chemical structure and improved anti-angiogenic properties. The discovery of IMiDs as ubiquitination-E-ligase inhibitors will potentially impact on the development of novel IMiD candidates (Beedie et al. 2015).

As the mechanism of angiogenesis in myeloma is complex, there is research ongoing on the role of different cytokines and cell/extracellular matrix and cell/cell interactions. Multiple factors – so far not been explored for clinical use – may be involved in regulating angiogenesis such as HGF, APRIL, CTGF, interleukin-15 and interleukin-20, angiopoietin (Ang), placental growth factor (PGF), and adrenomedullin (ADM). Confronted with this network of angiogenic cytokines elaborated by myeloma cells and the other bone marrow cells, it is an important task to identify those with special relevance for myeloma-related angiogenesis.

In this context, HGF is considered a target as it was found to be aberrantly expressed in myeloma compared to normal plasma cells (Hose et al. 2009b; Kocemba et al. 2013), being increased in the serum of myeloma patients (Seidel et al. 1998; Borset et al. 1996,) and forming an autocrine loop (Ferrucci et al. 2014). The cMET-specific tyrosine kinase inhibitor (TKI) tivantinib has demonstrated activity in human myeloma cell lines. In preclinical experiments on myeloma cells, it was recognized that $cMET^{low}$ cell lines did not respond to the cMET kinase inhibitor amuvatinib (Phillip et al. 2013). The humanized anti-HGF antibody ficlatuzumab was recently evaluated in patients with advanced cancer indication, including four patients with multiple myeloma, and was associated with a mild to moderate toxicity profile and induced stable disease in 44% of the patients (no objective responses achieved) (Patnaik et al. 2014). The development of anti-HGF is more advanced in other cancer indications, and a trend in overall survival was reported in patients with gastric cancer who received rilotumumab combined with chemotherapy compared to the chemotherapy control group (Iveson et al. 2014). Furthermore, cMET expression in tumor tissue was a strong predictor of response and clinical benefit to rilotumumab therapy (Doshi et al. 2015). Taken together, anti-HGF/cMET therapeutic strategies appear viable for multiple myeloma in particular if cMET and/or HGF expression is present on myeloma cells.

The B-cell maturation antigen (BCMA)/APRIL system is involved in the pathogenesis of multiple myeloma as well as other hematological malignancies by promoting cell proliferation and induction of angiogenesis, e.g., by inducing the expression of proangiogenic cytokines in myeloma cells (Moreaux et al. 2009). An antibody against APRIL was able to suppress myeloma proliferation in human-SCID chimeric models (Tai et al. 2016). In addition, bispecific antibodies targeting BCMA have shown promising preclinical activity (Seckinger 2015a).

Given the redundancy of angiogenic factors present in the bone marrow of multiple myeloma patients and the comparably low impact of single-agent inhibition, a simultaneous targeting of these factors might prove the most promising strategy.

It is known since decades that the tumor angiogenic response is subject to profound changes during transition from primary tumor lesion to metastasis and late stage-advanced disease. Therefore, anti-angiogenic treatments have been shown to have certain "windows of opportunity." Their activity was for most compounds restricted to specific developmental stages of cancer development (Bergers et al. 1999). This concept has not been widely embraced in clinical anti-angiogenesis research up to now.

Multiple myeloma could be an ideal clinical model as patients with malignant plasma cells albeit not suffering from fully developed symptomatic myeloma are frequently recognized prior to development of symptomatic disease and followed over many years.

Importantly there is a growing interest in the development of effective strategies to prevent transition of clinical asymptomatic forms of plasma cell disease as smoldering myeloma into symptomatic myeloma (Mateos et al. 2013). Research in this area has been greatly facilitated by the identification of risk factors for progression in patients with smoldering myeloma (Rajkumar et al. 2014; Neben et al. 2013). Clinical studies in high-risk smoldering myeloma appear to be an attractive option for anti-angiogenesis targets which are expressed at early stages of the disease. Based on risk assessment and composition of the myeloma bone marrow microenvironment, specific angiogenesis inhibitors for various phases of

the disease as first line, refractory, relapsed, and maintenance should be considered and explored.

At present it appears that a range of pro-angiogenic molecules expressed already in bone marrow plasma cells (e.g., VEGF, adrenomedullin ADM, IGF, and PGF) may be especially suited for intervention at early stage, with additional target aberrantly being expressed in myeloma cells as HGF and hepatoma-derived growth factor (Figs. 1 and 2) (Hose et al. 2009b).

In this respect, it is expected that it will not be one anti-angiogenic compound in a "one-size-fits-all" approach but may require the combination of anti-angiogenic molecules to block redundancy of angiogenesis factors in bone marrow microenvironment. Likewise, combinatorial approach of anti-angiogenesis agents with approved targeting agents should further be explored. The evaluation of vascular-targeting strategies for anti-angiogenesis agents to the bone marrow microenvironment is another interesting area of research that could improve therapeutic efficacy and decrease adverse events of angiogenesis inhibitors (Hida et al. 2016).

Cross-References

- ▶ Biomarkers for Anti-angiogenic Therapy
- ▶ Imaging Tumor Angiogenesis
- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis

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Part XIX

Anti-angiogenics in Human Metabolism

The Implication of Anti-angiogenic Treatment of Malignancies on Human Metabolism

Nina Obad and Rolf Bjerkvig

Contents

Introduction 662
Mechanisms of Resistance 663
Hypoxia and Necrosis 663
Hypoxia and Metabolism 664
Hypoxia and Invasion 665
Metabolic Adaptations to Anti-angiogenic Therapy 666
Conclusion 668
Cross-References 669
References 669

Abstract

As angiogenesis is one of the hallmarks of cancer, a high number of therapeutic strategies have been developed to target the formation of new blood vessels in a growing tumor. Even though clinical efficacy has been documented in some cancer types, it is highly acknowledged that tumors develop resistance to anti-angiogenic therapy. A well-characterized response to anti-angiogenic therapy is the development of intratumoral hypoxia. This, in turn, may lead to specific adaptations in cellular metabolism in order for tumor cells to grow in a nutrient- and oxygen-deprived microenvironment.

The presented chapter describes key features of metabolic adaptations in response to induced hypoxia following anti-angiogenic therapy. Importantly, anti-angiogenic therapy can lead to metabolic reprogramming toward anaerobic metabolism where glycolysis is uncoupled from oxidative phosphorylation. This in turn points at potential metabolic targets that may be of importance for the

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development of combinatorial treatment principles. Moreover, due to intra- and intertumoral heterogeneity, the challenge lies in identifying tumor subtypes that might respond to antimetabolic therapies. Thus, antimetabolic therapies might leverage future anti-angiogenic therapies.

Keywords
Anti-angiogenic therapy · Hypoxia · Invasion · Resistance · Metabolism · Metabolic adaptation

Introduction

Angiogenesis is characterized by the sprouting of endothelial cells from preexisting vessels and is considered to be the principal mechanism of vascularization during development and tumor growth. In addition to angiogenesis, tumors can acquire oxygen and nutrients by other mechanisms, including vasculogenesis (de novo formation of new vessels from endothelial precursor cells), vessel co-option (use of preexisting blood vessels), and vascular mimicry (formation of tube-like structures from tumor cells, independent of endothelial cells) (Soda et al. 2011; Jain and Carmeliet 2012).

Under normal conditions, the process of neovascularization is tightly regulated. In solid tumors, which require an increasing blood supply to sustain growth and survival, angiogenesis is increased, a process that frequently results in chaotic vessels with a leaky architecture.

The induction and maintenance of angiogenesis is described as a multistep process, often referred to as the angiogenic switch. The switch is driven by an imbalance between pro- and anti-angiogenic factors (Hanahan and Folkman 1996). Key growth factors and receptors (underlined) involved in angiogenesis include vascular endothelial growth factors (VEGFs)/*VEGFR1–3*, angiopoietins (ANG-1 and -2)/*TIE 1/2*, fibroblast growth factors (FGFs)/*FGFR1–4,* and hepatocyte growth factor (HGF)/*c-Met* (Welti et al. 2013). In particular, the VEGFs have been regarded as key regulators of both physiological and pathological neovessel formation. The VEGF family consists of five members (VEGF-A, -B, -C, -D, and PIGF). VEGF-B, -C, -D, and PIGF have mainly been shown to have a function during embryonic angiogenesis, lymph angiogenesis, and vasculogenesis, whereas VEGF-A has been considered to be the primary driver of angiogenesis in many aggressive solid tumors (Ferrara 2002; Hartenbach et al. 1997).

The expression of VEGF is primarily regulated by the oxygen tension. Hypoxia induces VEGF expression via hypoxia-inducible factor HIF-1α (see below) (Semenza 2002b). Furthermore, several major growth factors including FGF, PDGF, and TGF-α/β have been shown to upregulate VEGF (Ferrara et al. 2003). VEGF-A is secreted by the tumor cells and primarily binds to VEGFR-2 on the endothelial cell surface which in turn activates several downstream signaling pathways such as the MAP kinase and PI3K/AKT pathways, thereby increasing vascular permeability, endothelial cell migration, and mobilization of endothelial precursor cells (Norden et al. 2009).

In contrast to VEGF, ANG-2 works as an antagonist of ANG-1 and will, in the absence of VEGF, cause vessel regression (Lim et al. 2004).

Interestingly, it has been shown that ANG-2 works with VEGF to facilitate endothelial cell proliferation and migration and that ANG-2/TIE signaling may reduce vascular pericyte coverage (Augustin et al. 2009).

The net result of these processes is a highly disorganized tumor vasculature, consisting of multiple vessel subtypes ranging from capillaries to big fenestrated sinusoids and glomeruloid vessel structures, causing an abnormal, reduced, or sluggish blood flow and increased vascular permeability and interstitial fluid pressure (Nagy et al. 2009). This leads to edema, which in turn represents a barrier for drug delivery (Jain 1994). The inefficient tumor vasculature will lead to a poor oxygenation of tumors (hypoxia), which is considered as an important mechanism underlying treatment resistance to radio- and/or chemotherapy.

Mechanisms of Resistance

Anti-angiogenic compounds have been shown to be effective in numerous preclinical models as well as in phase I/II clinical trials. This has led to FDA approval of several anti-angiogenic compounds (many that represent multikinase inhibitors) that mostly target VEGF and associated signaling mechanisms (Table 1). However, it should be emphasized that many of the anti-angiogenic tyrosine kinase inhibitors also show off-target effects. Even though a high number of anti-angiogenic compounds have been developed, phase III clinical trials have shown limited efficacy on overall survival, yet with some improvement in progression-free survival.

Many mechanisms of resistance have been proposed. These mainly relate to the pathophysiological nature of tumor blood vessels that may be stimulated by VEGF independent mechanisms. Such mechanisms include (i) stimulation of myeloid cells that may trigger VEGF independent angiogenesis (Ferrara 2010), (ii) involvement of tumor associated fibroblasts (TAFs) that may stimulate angiogenesis under VEGF inhibited conditions (Crawford et al. 2009), and (iii) alternative mechanisms such as vessel co-option, vascular mimicry, intussusception, and vasculogenesis (Chen and Chen 2014; Manotis 1999; Donnem et al. 2013).

In addition, compelling evidence has been provided supporting the notion that anti-angiogenic therapy leads to increased intratumoral hypoxia. This in turn causes metabolic responses that may play vital roles in the adaptation of tumors to anti-angiogenic therapy (Keunen et al. 2011; Fack et al. 2015). Such responses may affect and stimulate both tumor cells and endothelial cells (McIntyre and Harris 2015).

Hypoxia and Necrosis

The presence of hypoxic and necrotic regions within tumors is associated with an aggressive tumor growth and a poor prognosis (Hockel et al. 1999, Lu and Kong 2010). Frequently, rapidly proliferating tumor cells exist within an

Table 1 FDA-approved angiogenic inhibitors (2015)

Approved indication	Generic name	Trade name	Function
Kidney cancer	Axitinib	Inlyta	Tyrosine kinase inhibitor; VEGFR1–3, c-KIT, and PDGFR inhibitor
Colon, kidney, lung, certain cervical, ovarian, fallopian tube, and peritoneal cancers	Bevacizumab	Avastin	VEGF inhibitor
Thyroid cancer	Cabozantinib	Cometriq	Tyrosine kinase inhibitor; C-Met and VEGFR2 inhibitor
Certain types of thyroid cancer	Lenvatinib	Lenvima	Multikinase inhibitor
Kidney cancer, soft tissue sarcomas, gastrointestinal stromal tumors	Pazopanib	Votrient	Multikinase inhibitor
Lung and stomach cancers	Ramucirumab	Cyramza	VEGFR2 receptor antagonist
Colorectal cancer, gastrointestinal stromal tumors	Regorafenib	Stivarga	Multikinase inhibitor
Kidney cancer, thyroid cancer	Sorafenib	Nexavar	Multikinase inhibitor
Gastrointestinal stromal tumors, pancreatic cancer, and kidney cancer	Sunitinib	Sutent	Multikinase inhibitor
Thyroid cancer	Vandetanib	Caprelsa	Multikinase inhibitor
Colorectal cancer	Ziv-aflibercept	Zaltrap	VEGF trap
Lung cancer	Nintedanib	Vargatef	Multikinase inhibitor

environment where an ineffective, irregular microvasculature is present. This leads to a lower oxygen tension and hypoxia.

The pO_2 in many solid tumors is usually less than18 mmHg, compared to normal tissues where the pO2 levels usually are above 30 mmHg (Vaupel et al. 2007; Hockel and Vaupel 2001; Brown and Wilson 2004). Also, there are large regional differences within tumors where the oxygenation levels may range from normoxia to extreme hypoxia (Aquino-Parsons et al. 1999). Such differences can affect various clonal cell populations within solid tumors and are therefore regarded as important factors in shaping their intratumoral heterogeneity.

The most important protein involved in the hypoxic response is the transcription factor HIF-1. It is composed of two subunits, HIF-1α and HIF-1β, where its activity is mainly dependent on the α subunit (Bardos and Ashcroft 2004). Both subunits are constitutively expressed at the transcriptional level. Under normoxic conditions, HIF-1α is ubiqutinated through interaction with the Von Hippel-Lindau (VHL) tumor suppressor protein and is degraded by the proteasome. This degradation depends on an oxygen-dependent hydroxylation of at least one of two proline residues (Jaakkola et al. 2001; Ivan et al. 2001).

Under hypoxia, HIF1-α is stabilized and translocated to the nucleus where it activates DNA promoter regions known as hypoxia response elements (HREs). HREs induce the transcription of several hypoxia-responsive genes that help the cells to adapt to hypoxia, most importantly VEGF.

HIF-1α activity in tumor cells can, in an O_2-independent manner, also be induced by specific genetic alterations mediated by oncogene gain or tumor suppressor gene loss of function. Oncogene activation may increase HIF-1α expression (Jiang et al. 1997) by activating the mTOR pathway (Zhong et al. 2000). This can lead to an mTOR-dependent translation of HIF-1α mRNA into protein (Laughner et al. 2001). Tumor suppressor gene loss of function may also increase HIF-1α levels by other mechanisms. For instance, it has been shown that both the PI3-K/Akt and MAPK signaling pathways can induce HIF-1α expression under normoxic conditions. In addition, HIF-1α expression can by induced by microenvironmental conditions such as accumulation of nitric oxide (Quintero et al. 2006) and reactive oxygen species (Gao et al. 2007).

The regulation of metabolism is also one of the principal functions of HIF-1α, where it mediates a transition from oxidative to glycolytic metabolism through the regulation of glucose transporter-1 (Glut-1), carbonic anhydrase IX (CAIX), lactate dehydrogenase-A (LDHA), and phosphoglycerate kinase (PGK) (Semenza 2012).

Necrosis is defined as an accidental cell death due to lack of environmental stimuli. It is a defining feature in many tumors, not necessarily related to tumor size, suggesting that the presence of necrosis is regulated by other factors in addition to the vascular supply (Raza et al. 2002). Pseudopalisades are dense cellular arrangements that, for some tumors (glioblastomas), surround necrotic areas. In these, it has been proposed that intravascular thrombosis leads to hypoxia that in turn induces a migration front of tumor cells (pseudopalisades), whereas nonmigrating cells die and remain as necrotic deposits. Finally, glomeruloid vascular proliferation occurs as a response to high levels of VEGF secreted by the hypoxic tumor cells (Brat et al. 2004).

As shown for necrosis, hypoxia and HIF expression both correlate with poor prognosis. As HIF-1α is essential for both vascular and metabolic adaptations to hypoxia, inhibiting HIF should be regarded as a promising treatment strategy.

Hypoxia and Metabolism

For cancer cells to proliferate, they must be able to produce biomass, including nucleotides, amino acids, and lipids. Proliferating cells require energy in the form of adenosine triphosphate (ATP). In order to survive and grow in oxygen- and nutrient-deprived microenvironments, tumors adapt their metabolism. Reprogramming of energy metabolism has, as a consequence, been recognized as an emerging *hallmark of cancer* (Hanahan and Weinberg 2011). Several mutations in key cancer genes (p53, Ras, Myc) promote glucose uptake in cancer

cells (Mayers and Vander Heiden 2015). In addition to increased glucose uptake, proliferating cancer cells metabolize glucose differently (Kroemer and Pouyssegur 2008). In normal cells, glucose is converted into pyruvate when oxygen is present. Pyruvate then enters the tricarboxylic acid (TCA) cycle to generate ATP through oxidative phosphorylation (OXPHOS). In cancer cells, pyruvate is converted to lactate even when oxygen is abundant, termed aerobic glycolysis or the Warburg effect (Warburg et al. 1927). Even though glycolysis is less efficient in generating ATP compared to OXPHOS (2 versus 36 ATP per glucose molecule), there are several advantages of enhanced glucose uptake in cancer cells. By not only relying on OXPHOS, the cells can live in conditions with an unstable oxygen supply.

Importantly, tumors can metabolize glucose through the pentose phosphate pathway (PPP). The primary role of PPP is to generate nicotinamide adenine dinucleotide phosphate (NADPH), which protects the cell from oxidative stress. NADPH also promotes the production of ribose-5-phosphate for nucleotide generation (Agnihotri and Zadeh 2016). Finally, generating lactate through glycolysis acidifies the microenvironment, which may favor invasion and suppress anticancer immune responses (Mayers and Vander Heiden 2015).

As mentioned above, many solid tumors show an increase in glycolysis compared to normal tissues (Oudard et al. 1996). The metabolic adaptation that leads to this is influenced by several environmental and genetic factors as described above. HIF-1α is stabilized under hypoxia leading to an adaptation to metabolic stress by inducing several glycolytic enzymes (Fig. 1). Hexokinase (HK) is the first enzyme in glycolysis. Normal tissues, such as the brain, express HK1, whereas tumor cells preferably express HK2. HK2 is a highly regulated enzyme and is transcriptionally regulated by HIF-1α, glucose, insulin, and glucagon, among others (Pedersen et al. 2002). Xenograft studies have shown that depletion of HK2 leads to an inhibition of glycolysis, with a concomitant increase of normal oxidative respiration leading to apoptosis (Wolf et al. 2011a, b). Hypoxia also prevents the entry of pyruvate into the TCA cycle through activation of pyruvate dehydrogenase kinase 1 (PDK1). Instead, pyruvate is broken down to lactic acid whereupon it is removed by MCT4.

The enzyme LDHA catalyzes pyruvate into lactate leading to an accumulation of lactate in the cytosol, thus uncoupling glycolysis from the TCA cycle (Agnihotri and Zadeh 2016). High levels of LDHA in cancers have been reported to be associated with poor survival (Koukourakis et al. 2014, Giatromanolaki 2006). Several studies have also shown that LDHA can play an important role in tumor invasion (Colen et al. 2011; Seliger et al. 2013). LDHA can be downregulated in IDH1 mutant tumors such as gliomas, which might contribute to the increased survival in patients with this mutation (Chesnelong et al. 2014). LDHA is considered as a safe therapeutic target, as an inherited mutation, which leads to loss of LDHA, only causes mild symptoms of exertional myopathy (Kanno et al. 1988). Thus, inhibiting lactate metabolism might be a promising approach in cancer therapy.

Hypoxia and Invasion

Invasion is perceived as a multistep process, where tumor cells migrate through the extracellular matrix (ECM) and degrade ECM proteins by involving numerous proteases such as serine, cysteine, aspartyl, and metalloproteases (MMPs). Many studies have shown an upregulation of MMPs in tumors (Koul et al. 2001; Brown and Murray 2015). For instance, it has been shown that the PI3K/AKT pathway increases MMP activity in the cells at the invasion front of the tumor, giving them increased proteolytic capabilities (Kubiatowski et al. 2001). Hypoxia has also been linked to invasion through HIF-mediated transcription of MMPs (Brat et al. 2004). HIF-1α may also promote invasion through an activation of the actin-bundling protein fascin (Zhao et al. 2014). Fascin is usually expressed in membrane protrusions (invadopodia) that may facilitate invasion through the extracellular matrix (Li et al. 2010).

Other important mediators of invasion are the RhoGTPases, which regulate the cytoskeletal dynamics, cell adhesion, and migration (Vega and Ridley 2008; Fortin Ensign et al. 2013). Also, the transforming growth factor ß (TGF-β) may support tumor cell migration by increasing MMP levels (Joseph et al. 2013). There may also be a direct link between the expression of RhoGTPases and HIF-1α expression, yet the exact mechanisms of action are not clear (Turcotte et al. 2003). Several other mechanisms whereupon HIF can mediate invasion exist, underpinning the complexity of HIF expression as a mediator of tumor cell invasion and metastasis. For instance, HIF-1α regulates the expression of key genes (c-Met, CXCR4, RIOK3, and LOX) all of which have shown to be associated with metastasis (De Bock et al. 2011; Singleton et al. 2015).

Metabolic Adaptations to Anti-angiogenic Therapy

The deregulation of cellular energetics, as a hallmark in cancer, is primarily based on the acknowledgment that tumors may undergo substantial metabolic reprogramming during progression (Semenza 2014).

Three vascular responses to anti-angiogenic therapy have been described in the clinic:

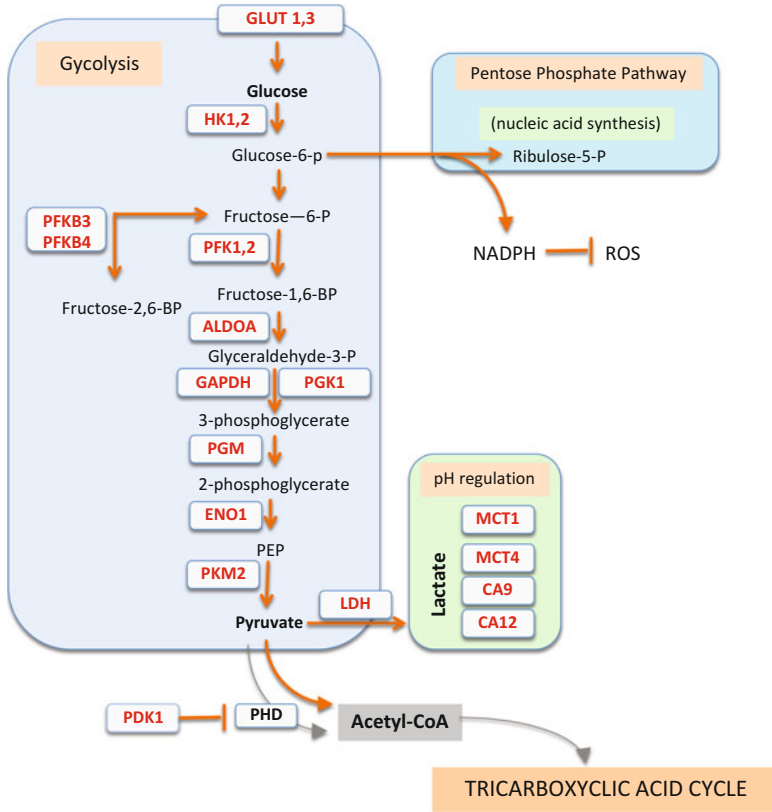

Fig. 1 Putative metabolic processes that may be upregulated in response to hypoxia. Enzymes and proteins (*red)* show increased expression or activity under hypoxia. Arrows (*orange*) denote an increased flux following anti-angiogenic therapy caused by a reduced perfusion *GLUT* glucose transporters, *HK* hexokinase, *PFK* phosphofructokinase, *ALDOA* aldolase A, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *PGK1* phosphoglycerate kinase 1, *PGM* phosphoglycerate mutase, *ENO1* enolase 1, *PKM2* pyruvate kinase M2, *LDHA* lactate dehydrogenase, *MCT* monocarboxylate transporter, *CA* carbonic anhydrase, *PDK1* pyruvate dehydrogenase kinase 1, *PFKFBP* phosphofructokinase bisphosphatase

(i) reduced perfusion, (ii) no perfusion response, and (iii) increased perfusion (referred to as vascular normalization) (Batchelor et al. 2013). Although the effect of antivascular therapy may differ depending on the tumor type and their biological characteristics, the most frequently reported observation in the clinic is reduced perfusion with an induction of hypoxia (Hattingen et al. 2011; Yopp et al. 2011). It has been shown, in preclinical models as well as in the clinic, that bevacizumab treatment causes a reduction in tumor perfusion with an increased HIF-1α and CA9 expression (Keunen et al. 2011; Yopp et al. 2011).

Metabolic genes that may be upregulated following anti-angiogenesis-mediated hypoxia include those involved in glycolysis such as GLUT1, GLUT3, HK1/2, PFK1/2, ALDOA, GADPDH, PGK1, PGM, ENO1, PKM2, and LDHA, as well as those involved in pH regulation (MCT1, 4 and CA9/12) (Favaro et al. 2011). Hypoxia mediated by anti-angiogenic treatment can also reduce oxidative phosphorylation by inhibiting pyruvate to enter the TCA cycle. This can occur by an upregulation of pyruvate dehydrogenase kinase (PDK1) with a concomitant inhibition of pyruvate dehydrogenase (PDH), where pyruvate instead is broken down to lactic acid and extruded by MCT4 (Fig. 2) (Brahimi-Horn et al. 2011).

Hypoxia also leads to an acidification of the microenvironment with the production of lactate from glycolysis (Parks et al. 2013). In this context, specific pH regulatory proteins show increased activity, such as the monocarboxylate transporters (MCT1 and 4) which carry molecules such as lactate and pyruvate across biological membranes. Recently, it has been demonstrated in preclinical breast cancer models that the multikinase inhibitors nintedanib and sunitinib caused an initial regression of tumor growth followed by a resumed growth in the absence of angiogenesis. Detailed analysis showed that the resumed growth was mediated by an upregulation of glycolysis with a differential expression of MCT1 and 4, where in particular genetic ablation of MCT4 (responsible for lactate transport) overcame therapeutic resistance (Pisarsky et al. 2016).

Even though glycolysis is upregulated following anti-angiogenic treatment, it does not

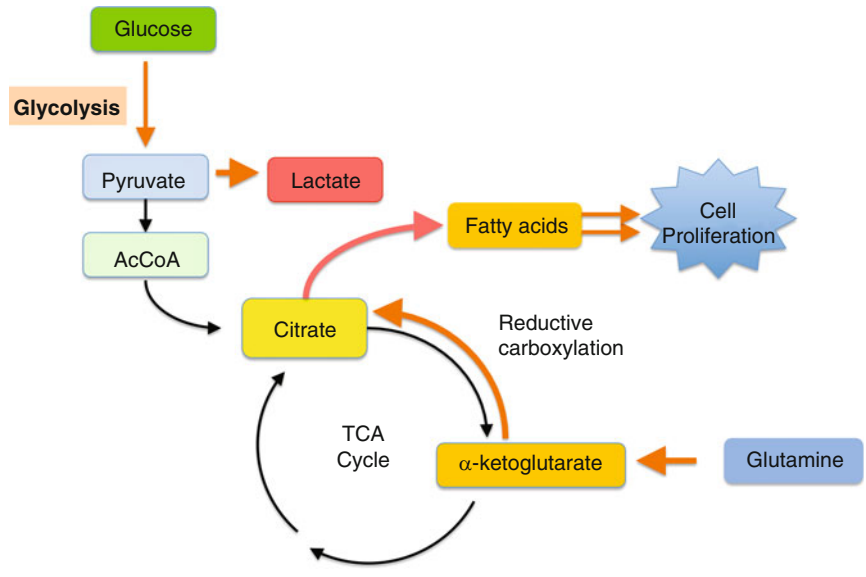

Fig. 2 Hypoxia can lead to an increased glutamine uptake where α-ketoglutarate derived from glutamine is used to replenish intermediates of the TCA cycle instead of pyruvate. This process may involve a reductive carboxylation of α-ketoglutarate to citrate, which in turn can be used for lipid synthesis required for tumor growth (*orange arrows* depict the reverse pathway)

necessarily imply that mitochondria are not active (Sun and Denko 2014). For instance, hypoxia may lead to an increased glutamine uptake where α-ketoglutarate derived from glutamine is used to replenish intermediates of the TCA cycle instead of pyruvate. This process may involve a reductive carboxylation of α-ketoglutarate to citrate (Fig. 2) (Fendt et al. 2013). The citrate produced can then be used for lipid synthesis, which is required for tumor growth (Fig. 2) (Sun and Denko 2014).

Also, the pentose phosphate pathway (PPP) may be upregulated following anti-angiogenic treatment, since it has been shown that stabilization of HIF-1α increases expression of genes involved in the PPP (Riganti et al. 2012). The PPP generates nucleotides as well as NADPH, which represents an important reducing agent required for lipid, nucleotide, and amino acid synthesis. This pathway is also important for ROS protection (Favaro et al. 2012).

By performing metabolic flux analysis in human glioblastoma xenograft models, it has been shown that bevacizumab treatment leads to an increased influx of glucose into the tumors, with a subsequent increase in LDHA and lactate levels. In addition, key metabolites associated with the TCA cycle were reduced following treatment. This was also verified by MRS and ^{18}F-FMISO and 18F-FDG PET analysis, confirming an increased hypoxia, glucose uptake, and lactate levels in the treated tumors. The LC-MS analysis also showed decreased levels of L-glutamine, supporting previous reports indicating that under hypoxia, glutamine is used for macromolecule synthesis (Fack et al. 2015; Metallo 2012). Also, reduced levels of glutathione were observed following treatment, suggestive of an induction of oxidative stress (Fack et al. 2015). Also here an upregulation of key PPP enzymes was observed. Adding to the complexity of metabolic reprogramming, it has recently been shown that a switch between PPP and glycolysis may occur during hypoxic stress in several cancer cell types as well as in nonneoplastic cells, and that the connection between the PPP and proliferation and glycolysis and migration may be independent of oxygen levels (Kathagen-Buhmann et al. 2016).

Although glycolysis is a much less efficient metabolic pathway, it has been proposed that the constitutive upregulation as seen in most human cancers gives the tumors a growth advantage in nutrient-deprived, hypoxic environments (Gatenby and Gillies 2004), a hypoxic environment that may be augmented following anti-angiogenic treatment.

Conclusion

Several preclinical and clinical studies show that anti-angiogenic therapy leads to decreased perfusion with a concomitant induction of hypoxia. Thus, it is likely that a combinatorial approach, targeting hypoxic adaptation mechanism together with anti-angiogenic therapy, can provide an improved therapeutic effect. For instance, it has been shown that knockdown of HIF-1α reduces the growth of neuroblastoma xenografts when combined with anti-angiogenic therapy (Hartwich et al. 2013).

It has also been observed, in preclinical models, that the PDK inhibitor dichloroacetate (DCA) can increase oxidative phosphorylation and enhance the effect of bevacizumab treatment in glioblastomas (Kumar et al. 2013). DCA has also been shown to overcome sorafenib resistance in hepatocellular carcinoma xenografts where the treatment leads to increased ROS and ATP levels and reduced lactate levels (Shen et al. 2013). However, at present it is still an open question if DCA treatment in conjunction with anti-angiogenic therapy will work in the clinic.

Also, inhibition of CA9, a central pH regulatory enzyme, has been shown to enhance the effect of bevacizumab therapy in glioblastoma and colon xenografts (McIntyre et al. 2012).

Recently, targeting altered metabolic pathways in cancer has emerged as a promising approach, attracting interest from various pharma companies. For instance, the small molecule inhibitor CB-839 that inhibits glutaminase has been developed. CB-839 catalyzes the conversion of glutamine to glutamate, which when converted to α-ketoglutarate may drive the TCA cycle in reverse to make citrate for de novo fatty acid synthesis

(Fig. 2). Other emerging drugs targeting metabolic pathways include CPI-613 (a pyruvate dehydrogenase inhibitor), PFK-158 (a PFKB3 inhibitor), DTP-348 (a CA9 inhibitor), AZD3965 (a MCT1 inhibitor), TVB-2640 (targeting fatty acid synthesis) as well as multiple inhibitors targeting HIF-1α. Several of the drugs mentioned above are at present in phase I/II clinical trials.

It should be emphasized that targeting cancer metabolism is a complex issue where different pathways may work in concert within a heterogeneous tumor. For many tumors, the challenge lies in selecting the subtypes that may respond to antimetabolomic therapy. In the context of anti-angiogenic therapy, however, there is a clear indication pointing at a concerted upregulation of glycolysis, indicating that such therapies can drive tumors in a certain metabolic direction. Therefore, targeting glycolytic pathways in conjunction with anti-angiogenic therapy may show future promise.

Cross-References

- ▶ Mechanisms of Anti-angiogenic Therapy
- ▶ Mechanisms of Tumor Angiogenesis
- ▶ Pathology of Tumor Angiogenesis

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Index

A
Abiraterone, 580
ABTAA, 57
Acidosis, 54
Adherens junctions, 93, 101
Aerobic glycolysis, 665
Aflibercept, 380, 386–387, 427, 585, 599
Age-related macular degeneration, 219
Androgen receptor, 581
Angiogenesis, 92, 122, 128, 164, 184, 186, 212, 235–236, 279, 283, 294, 295, 297, 300, 301, 314, 315, 317, 319, 351, 360, 380–381, 417, 450–451, 466, 499, 502, 504–505, 570, 580–584
  agents, 548
  clinical studies on, 424
  DCE-MRI (*see* Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI))
  EPCs in tumor angiogenesis, 187
  experimental and clinical diagnostic modalities for, 418–421
  extracellular matrix in, 190–192
  inhibitors, 217–218, 550, 646, 649
  integrin in, 195
  MRI, 282–284
  multiple myeloma (*see* Multiple myeloma)
  in pancreatic adenocarcinoma, 421–423
  PET scanning, 285
  in pNET, 423–424
  regression of blood vessels and endogenous inhibitors, 19–20
  signaling, 185–187
  sprouting, 6
  switch, 4, 11, 20–25, 417, 424, 450, 550, 662
Angiogenesis, gastroesophageal cancer
  Ang1, 230, 241
  Ang2, 231, 232, 235, 236–239, 241–243, 317, 371
  angiopoietin–Tie2, 398–399
  apatinib, 407
  bevacizumab, 403
  biological background, 396–397
  biomarker research, 402
  integrins, 399
  platelet-derived growth factor, 398
  ramucirumab, 406
  regorafenib, 407
  sorafenib, 407
  sunitinib, 406–407
  tumor stroma, cellular components of, 399–400
  vascular endothelial growth factor, 397–398
Angiopoietin (Ang), 15, 20, 55, 530, 583
  Ang1, 597
  Ang2, 316, 318, 371, 439
  in developmental lymphangiogenesis, 138–140
  growth factors, 229
  in human cancer, 243–245
  in inflammation and vascular remodeling, 233
  in metastasis, 238
  receptor, 38
  in tumor growth and angiogenesis, 235
  in tumor lymphangiogenesis and lymphatic metastasis, 144–146
Angiopoietin-Tie, 597
Angiostatin, 20, 642–644
Anti-angiogenesis, 19, 20, 212, 314–315, 319, 328–330, 466
  agents, 52, 53, 57
  drugs, 95, 294, 296
  See also Metronomic chemotherapy
Anti-angiogenics
  brain metastases prevention, 629–631
  ovarian cancer (*see* Ovarian cancer therapy, anti-angiogenics)
  palliative benefits, 632
  pragmatic use of, 633
  therapy of brain metastases by, 631–633
  treatment, 651
Anti-angiogenic therapy, 237, 244, 452–456, 491, 666–668
  extracellular matrix remodeling, inhibition of, 190
  metastatic breast cancer, 516
  non-metastatic breast cancer, 521
  PDAC (*see* Pancreatic ductal adenocarcinoma (PDAC))
  pNETs (*see* Pancreatic neuroendocrine tumors (pNETs))
  tumor blood vessels, regression of, 195
  tumor vasculature and microenvironment, normalization of, 198–202

D. Marmé (ed.), *Tumor Angiogenesis*,
https://doi.org/10.1007/978-3-319-33673-2

Anti-angiogenic therapy (*cont.*)
tyrosine kinase inhibitors, 186
vascular endothelial growth factor inhibitors, 186
vascular progenitor cells, inhibition of, 187–190
Anti-angiogenic transcription factors
forkhead transcription factors, 80, 81
kruppel-like factors 2 / 4, 82
nuclear factor-erythroid 2 p45-related factor, 82
p53 transcription factor, 82, 83
recombining binding protein suppressor of hairless, 84
Anti-VEGF-A/VEGFR2 therapy, 52–53
Apatinib, 407–408
Apopotosis, 648
Arbeitsgemeinschaft Gynaekologische Onkologie (AGO), 535
Arresten, 19
Ascites, 530, 538, 539
Atezolizumab, 369, 504, 595
AVAglio trial, 615
Avastin®, *see* Bevacizumab
Axitinib, 491, 572

B
Basement membrane (BM), 54, 188, 190, 197, 199
Basic fibroblast growth factor (bFGF), 499, 598
Bevacizumab, 53, 186, 198, 201, 214–215, 294, 296, 303, 314, 315, 317, 351, 353, 354, 362, 380–386, 399, 402–404, 426–427, 441, 467, 479, 480, 499, 516–519, 521, 530, 553–558, 574, 584–585, 599, 613, 615–620, 649
with cisplatin and radiation therapy, 480–481
in firstline therapy, 531–532
locally advanced HNSCC, 480
with radiation therapy and both chemotherapy and anti-EGFR therapy, 482
with radiation therapy and chemotherapeutic regimens, 481
in recurrent, platinum-resistant ovarian cancer, 532–533
in recurrent, platinum-sensitive ovarian cancer, 532
recurrent and metastatic HNSCC treatment, 483–485
Biomarkers, 645–646
blood-based, 297
circulating endothelial and endothelial progenitor cells, 298–299
circulating tumor cells, 299
cytokines and pro-angiogenic factors, 297–298
definition, 294
DNA hypermethylation, 301
drug toxicities as, 302
genetic alterations, 300
imaging-based, 302–303
metabolic, 297
microenvironmental, 296–297
microRNAs, 301–302
neutrophil-to-lymphocyte ratio, 299
pro-angiogenic pathway activation, 295–296
single nucleotide polymorphisms, 300–301
Bladder cancer, 594
Bleeding, 353, 355, 499
Blood-brain barrier (BBB), 39, 98, 126
Blood flow, 15, 35, 42
Blood perfusion, 95
Blood vessels, 34, 38, 40
formation, 4–6
Bone marrow
histology, 641, 645
niche, 644
Bone morphogenic protein-6 (BMP-6), 642
Bortezomib, 647, 649
Bradykinin, 125
Brain macrometastasis, 629
Brain metastases
breast cancer, 632
colorectal cancer, 632
incidence, 628
lung cancer, 631–632
palliative benefits by anti-angiogenics, 632
prevention with anti-angiogenics, 629–631
renal cancer, 632
therapy by anti-angiogenics, 631–633
BRCA1/2 mutation, 540
Breast cancer, 268, 295, 296, 298, 300, 516
metastatic, 516
non-metastatic, 521
Brivanib, 388
Brivianib, 440

C
Cabozantinib, 573, 585–586, 600
Cadherins, 124, 191, 192
cAMP, 75
cAMP Response Element-Binding (CREB), 75
cAMP-response element (CRE), 75
Cancer, 43, 44
biliary tract, 364
breast, 365
colorectal, 362, 363, 367, 369
non-squamous non-small lung, 364
ovarian, 371
Cancer associated fibroblasts, 268
Cancer stem cells (CSCs), 316, 318
Canstatin, 19
Capecitabine, 336
Carboplatin, 499, 505
Cardio-vascular system, 458
Castration resistant prostate cancer (CRPC), 580
CD117 antigen, 451
Cediranib, 355, 389, 469, 502, 518, 538, 558, 614
Cell adhesion receptors, 190–192
Cereblon, 647
Cervical cancer
angiogenesis in, 548–552
clinical relevance, 552–558
metastatic, 547, 552, 553
persistent, 550, 553

pre-invasive changes, 546
recurrent, 552, 553
risk factors, 547
screening, 546
treatment options, 547–548
Cervical epithelium, 546
Cervix uteri, 547
Cetuximab, 362, 370, 380
Chemokines, 13
Chemotherapy, 381–384, 396, 402, 403, 405, 407, 530, 575
Child-Pugh score, 439
Cisplatin, 337, 500, 505
Claudins, 101, 124, 125
Clear cell renal cell carcinoma (ccRCC), 295, 296, 300
Clinical trials, 362, 366, 382, 384, 388
c-MET, 440
Colorectal cancer (CRC), 269, 296, 314, 319, 353
incidence, 380
tumour angiogenesis, 380
Conversion therapy, bevacizumab, 383
CREB, *see* cAMP Response Element-Binding (CREB)
Cyclophosphamide, 332
Cytokines, 642, 644, 645, 648, 650, 651
Cytotoxic drugs, 328–330
Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), 575

D
DC-101, 352
Delta-Notch signalling, 74
Development and physiological homeostasis, 219
Diapedesis, 101
DNA damage, 350
Docetaxel
with aflibercept, 585
with bevacizumab, 584
with lenalidomide, 587
with thalidomide, 587
DOG1, 451
Doppler flow measurement, 419
Doublestrand breaks, 350
Dovitinib, 598
Doxorubicin, 466
Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI), 645, 650
advantages, 279
biological relevance, 280–281
and derived parameters, 280
methodology, 279
VEGF inhibitors, 281

E
E6 and E7 oncogenes, 550
Edema, 40, 44, 54
Elpamotide, 426
Endocan, 596
Endorepellin, 19
Endostatin, 19
Endothelial barrier, 102
Endothelial cell(s), 34, 39, 92, 184, 185, 187, 188, 191, 192, 194, 197, 199, 200, 316–319, 351
proliferation, 7, 9–13
Endothelial cell-cell junctions, 99
adherens junctions, 101–102
tight junctions, 100
VE-cadherin, 102
Endothelial junctions, 40–42, 107–113
Endothelial nitric oxide synthase (eNOS), 127
Endothelial polarity, 109
Endothelial progenitor cells (EPCs), 4, 6, 13, 14, 258, 317, 319
in anti-angiogenic treatments, 189–190
in cancer diagnosis and prognosis, 189
in tumor angiogenesis, 187
and tumor micro environment, 187–189
Endothelial quiescence promoting transcription factors, *see* Anti-angiogenic promoting transcription factors
Endothelial-to-mesenchymal transition, 107
Endothelium, 229, 240
Enzalutamide, 580
Ephrin/EPH, 13–14
Epidermal growth factor (EGF), 12
Epidermal growth factor receptor (EGFR), 502
signaling, 360
Epithelioid mesenchymal tumor, 451
ERG, 75, 76
Erlotinib, 364, 370, 502
Erythropoietin, 314
Etoposide, 337, 505
ETS (E-26 transformation specific) transcription factors, 75, 76
European Medicines Agency (EMA), 533
European Organisation for Research and Treatment of Cancer (EORTC), 469
Everolimus, 574, 600
Exons, 452
Exosomes, 317, 644
Extracellular matrix (ECM), 6, 8, 11, 13, 17, 19, 25, 190–195, 314, 317, 319, 644, 650, 651

F
Famitinib, 388
FDA-approved angiogenic inhibitors, 663
[^{18}F]Fluorodeoxy-glucose (FDG), 456
FGF3 mutations, 594
Fibroblast-derived growth factor (FGF), 11, 12, 15, 129, 162, 424, 443, 583
FGF-2, 163, 314, 317, 318, 642
mutations, 594
Fibrous stroma, 422
FIGO stage, 531, 532
Firstline treatment, 534, 537
Fistula, 355
Flavonoids, 195–197

5-Fluorouracil, 314, 319, 382
FMS-related tyrosine kinase 1 (*FLT1*), 581
Focal adhesion kinase (FAK), 37
FOLFIRI, 380
Folfirinox, 416
FOLFOX, 380
Forkhead box O (FOXO), 80
  FOXO1, 57, 230
Forkhead transcription factors, 80, 81
Free OH· radicals., 350
French Sarcoma Group, 470
Fruquintinib, 388

G
Gain-of-function mutation, 452
Gastric cancer, 296
  angiopoietin–Tie2, 399
  and biomarker research, 402
  integrins, 399
  platelet-derived growth factor, 398
  tumor stroma, cellular components of, 399
  vascular endothelial growth factor, 397
Gastrointestinal stromal tumours (GISTs), 450
  anti-angiogenic therapy, 452
  incidence, 451–452
  mutations, 452
  NF1-associated, 452
  paediatric, 452
  PDGRFA, 452
  SDH-deficient, 452
  wild-type, 452
GATA family, 76, 78
Gemcitabine/cisplatin, 500, 599
Genetic instability, 54
Genomic instability, 318
German Interdisciplinary Sarcoma Group (GISG), 470
Glioblastoma, 296, 297, 316, 318, 353, 355, 610
Glucose transporter type 1 (GLUT1), 78
Glucose uptake, 664
Glycocalyx, 39
Glycolytic metabolism, 664
G-protein coupled receptors, 127
Granulocyte-colony stimulating factor (G-CSF), 314, 317
Granulocyte macrophage-colony stimulating factor (GM-CSF), 314, 317
Groupe d'Investigateurs Nationaux pour l'Étude des Cancers Ovariens (GINECO), 540

H
Haemangiogenesis carcinomatosa and metastases, 266–267
Hazard ratio (HR), 531, 537, 539
HDAC inhibitors, *see* Histone deacetylase (HDAC) inhibitors
Head and neck carcinoma, 479, 480, 482, 485, 490, 491
Heme oxygenase 1, 597
Hemorrhages, 104
Heparanase, 642, 650
Heparin-binding epidermal growth factor-like (HB-EGF), 598
Hepatocellular carcinoma, 318, 319, 436
Hepatocyte growth factor (HGF), 162, 439, 642, 644, 645, 651, 652
Hepatoma arterial-embolization prognostic score (HAP), 437
Heterogeneity, 54
Hexokinase (HK), 665
HIF-1α, 55, 548
Histamine, 125
Histamine receptors, 127
Histone deacetylases (HDAC), 551
  inhibitors, 648
Human epidermal growth factor receptor 2 (HER2), 365
Human papilloma-virus (HPV), 546
  high-risk types 16 and 18, 547
Hyper-permeable vessels, 54
Hypothyroidism, 458
Hypovascular, 417, 421, 422
Hypoxia, 9, 13, 20–23, 52, 54, 57, 59, 60, 64, 316–317, 350, 352, 422, 424, 551, 641–642, 649–650, 662
  HIF-1α, 351, 570, 642, 644, 649, 662
  intratumoral, 663
  microenvironments, 581
  tumor angiogenesis, 21
Hypoxia-inducible factor (HIF), 21, 23, 78, 570
Hypoxia response elements (HREs), 664
Hypoxic inducible factors (HIF), 262

I
Iatrogenic Cushing syndrome, 632
Icrucumab, 601
Ifosfamide, 466
Imaging
  angiogenesis (*see* Angiogenesis)
  emerging technologies, 286–287
  uses, 278
Imatinib, 453–454
IMiDs, *see* Immuno-modulatory drugs (IMiDs)
Immune checkpoint inhibitors, 541, 575
Immuno-modulatory drugs (IMiDs)
  clinical application and use, 647
  molecular mechanisms of action and anti-angiogenic activity, 647
Immunomodulatory substance, 426
Immunotherapy, 366, 541
Indirect effect, 350
Inflammation, 111, 438
Inflammatory cells, 317
Inflammatory cytokines, 127
Integrins, 5, 13, 14, 21, 191–192, 194–195, 240–241
Interferon α (IFN-α), 19, 574
Interferon β (IFN-β), 19
Interleukin-1β (IL-1β),-4,-12,-18, 19
Interleukin-2, 574
Interstitial hypertension, 54
Intraocular neovascular disorders, 219
Intratumoral heterogeneity, 299, 303, 664

Intratumoral oxygen tension measurements, 422
Intravasation, 516
Intravesical chemotherapy, 594
Intravital microscopy (IVM), 418
Intussusception, 4, 7–8
  angiogenesis, 74
  microvascular growth (IMG), 317, 318
Ipilimumab, 368, 575
Irinotecan, 383
Ischemia, 355

J
Junctional adhesion molecules (JAMs), 101, 125
  JAM-B, 108

K
KIT gene, 451
  mutation, 452
KRAS-gene, 421
Kruppel-like factors (KLF) 2/4, 82

L
Lactate, 665
Lactate dehydrogenase A (LDHA), 78, 665
Lamellipodia, 78
Lapatinib, 366
Leakage
  cells, 43
  molecules, 43
  vascular (*see* Vascular leakage)
Lenalidomide, 587–588
Lenvatinib, 389, 518, 573
Linifanib, 389, 440
Liquid biopsy, 459
Lower oxygen tension, 664
Lung cancer, 258, 296, 300, 498
Lymph-angiogenesis, 158, 163, 595
Lymphangitic carcinomatosis, 170
Lymphatics, 158
  capillaries, 160
  development, 138, 140, 142, 144
  endothelial cells, 159
  metastasis, 144–146, 163
  valve morphogenesis, 142–143
  vasculature, 159
  vessel(s), 39, 41, 44
  vessel hyaluronan receptor-1, 162
Lymph node metastases, 168
Lymphocytes, 317
Lymphogenous spread, 164
Lymphovascular invasion, 168
LYVE-1, 162

M
Macrophages, 351
  migration inhibitory factor, 597
Maintenance therapy, 385–386, 531, 532, 537, 539
Malignant pleural mesothelioma, 506
Masitinib, 456
Maspin, 550, 551
Matrix metalloproteinase (MMP), 12, 20, 21, 26, 522, 598
  MMP-9, 351, 423
MAX dimerization protein (MAD), 79
Medulloblastomas, 611
MEK/MAPK kinase, 451
Melanoma, 317, 319
Meningioma, 611, 618
Metabolic reprogramming, 666
Metabolic responses, 663
Metabolism, 664–665
Metastasis, 54, 238–239
Metastasized colorectal cancer, 388, 390
Metastatic breast cancer, 516–519
Metastatic colorectal cancer, 214–215
  aflibercept, 387
  bevacizumab, 381
  brivanib, 388
  cediranib, 389
  famitinib, 388
  fruquintinib, 388
  lenvatinib, 389
  linifanib, 389
  motesanib, 389
  nintedanib, 388
  ramucirumab, 387
  tivozanib, 389
  trebananib, 389
  vandetanib, 390
  vatalanib, 390
Methotrexate, vinblastine and adriamycin (MVAC), 594
Methotrexate, 336
Metronomic chemotherapy, 520–521
  anti-angiogenic mechanism, 331
  capecitabine, 336
  cisplatin, 337
  cyclophosphamide, 332
  etoposide, 337
  goals, 330
  methotrexate, 336
  resistance to, 340
  taxanes, 337
  tegafur, 336
  temozolomide, 336
  trofosfamide, 332
Microcirculation, 641, 645, 646, 650
Microenvironment, 367, 417, 421, 423, 642, 644–645, 648
Micrometastases, 521
MicroRNAs (miRNAs), 582, 644
Microvascular density (MVD), 421, 424
Microvessel density, 422, 595
Mimicry, 96
Mitochondria, 668
Mitogenic promoter, 422
Mitotic catastrophe, 350
MMPs, *see* Matrix metalloproteinase (MMP)
Monoclonal antibodies, 360, 467, 615
Monoclonal gammopathy of unknown significance (MGUS), 642, 644

Motesanib, 389, 503
mTOR inhibitors, 574
Multi-kinase inhibitors, 534
  cediranib, 538
  nintedanib, 536
  pazopanib, 535
  sorafenib, 536
Multiple myeloma
  angiostatic factors, 642
  anti-angiogenic strategies, 646
  anti-VEGF approach, 648–649
  blood-borne factors, 645
  bone marrow microenvironment, 644
  exosomes and miRNA, 644
  gene expression data, 645
  HDAC inhibitors, 648
  heparanase and heparin-binding growth factors, 650
  hypoxia and HIF-1α inhibition, 649
  imaging, 645–646
  immuno-modulatory drugs, 647
  nanoparticles and materials development, 650
  PI3 kinase inhibitors, 650
  plasma cell proliferation, angiogenic cytokines and hypoxia, 641
  proteasome inhibitors, 647
  tissue-derived biomarkers, 645
  vasculogenesis, 644
Muscle invasive bladder cancer (MIBC), 594
Mutations
  activating, 451
  FGF3, 594
  GISTs, 452
  PDGFRA, 452
  point, 457
MYC-associated protein X (MAX), 79
Myeloid-derived suppressor cells (MDSCs), 317
Myocyte enhancer factor-2 (MEF2), 82
Myofibroblasts, 268

N
Nanobody, 371
N-cadherin, 124
Necrosis, 438, 664
Neo-angiogenesis, 516, 530
Neovascularization, 4, 6, 12, 13, 17, 20, 23–24
Netrins, 13, 15
Neuroendocrine tumor, *see* Pancreatic neuroendocrine tumors (pNETs)
Neurofibromatosis type 1 (NF1)-associated GIST, 452
Nilotinib, 455
Nintedanib, 388, 504, 535–536, 601
Nivolumab, 369, 504, 575
Non-metastatic breast cancer, 521–522
Non-muscle invasive BC (NMIBC), 594
Non-small-cell lung cancer (NSCLC), 498
Notch, 6, 18–19
Notch signaling pathway, 94
Nuclear factor-erythroid 2 p45-related factor (NRF2), 82
Nuclear factor (NF)-κB, 79

O
Occludin, 125
Off target effects, 458
Olaparib, 540
Oligodendroglial tumors, 611
Onartuzumab, 217
Osteoblasts/osteoclasts, 645
Ovarian cancer, 298, 302
  anti-angiogenics, bevacizumab (*see* Bevacizumab)
  immunotherapies, 541
  multi-kinase inhibitors (*see* Multi-kinase inhibitors)
  PARP inhibitors, 540
Overall survival (OS), 531, 532, 535, 536, 541
Overexpression, 417, 422, 642
Oxaliplatin, 380
Oxidative phosphorylation (OXPHOS), 665
Oxygenation, 352, 355
Oxygen-enhancement ratio (OER), 350

P
p53
  pathway, 421, 550
  transcription factor, 82, 83
Paclitaxel, 470, 499
Palliative cancer-therapy, 336, 337
Pancreatic adenocarcinoma
  experimental studies, 422–423
  pathophysiology, 421–422
Pancreatic ductal adenocarcinoma (PDAC), 416, 417
  animal models of, 418
  antibodies and targeted proteins, 426–427
  characteristics of, 419, 420
  and focal chronic pancreatitis, 420
  interferon-alpha, 426
  IVM methods, 418
  kinase inhibitors, 427–428
  treatment in, 416
  up-regulation of HIF-1α mRNA expression, 422
  vaccination studies, 425
  VEGF, 421, 422
Pancreatic intraepithelial neoplasias (PanIN), 421
Pancreatic neuroendocrine tumors (pNETs)
  clinical studies, 424
  experimental studies, 423–424
  hypervascularized structure, 417
  kinase inhibitors, 428
  pathophysiology, 423
Panitumumab, 363, 380
Paracellular permeability, 123–124
  adherens junctions, 124–125
  adhesion proteins, 123

cadherins, 124
tight junctions, 124, 125
VE-cadherin, 124
Parenchymal damage, 355
PARP inhibitors, *see* Poly(ADP-ribose) polymerase (PARP) inhibitors
Pathophysiology, 645
Pazopanib, 187, 355, 368, 455, 469, 491, 517, 535, 558, 572, 600
Pembrolizumab, 504
Pemetrexed, 500
Pentose phosphate pathway (PPP), 665, 668
Peptibody, 530, 539
Perforation, 355
Pericyte, 6, 8, 15, 17, 54, 184, 194, 199, 200, 261, 315–316
proliferation, 581
Peri-tumoral desmoplastic reaction, 420
Permeability, 38–39, 123
Phosphorylation, 125
Physiological angiogenesis, 4, 11, 18, 19, 24, 26
PI3K/Atk pathways, 451
Pigment epithelium-derived factor, 19
Placenta growth factor (PlGF), 218, 314, 442
Plasma cells, 642, 644, 651
Plasmalemma vesicle-associated protein (PLVAP), 146
Platelet derived endothelial growth factor (PDGF), 16–17, 315, 424, 427, 438, 439, 450, 582
PDGFRα, 450–452
PDGFRβ, 56, 450, 582
Platelet endothelial adhesion molecule, 101
Platelet factor 4 (PF-4), 20, 642
Platinum-resistant disease, 532, 533
Platinum-sensitive recurrent disease, 532, 538, 539
pNETs, *see* Pancreatic neuroendocrine tumors (pNETs)
Pneumonitis, 355
Point mutations, 457
Poly(ADP-ribose) polymerase (PARP) inhibitors, 540–541
Polyoma virus middle T-antigen (PyMT), 236
Positron emission tomography (PET), 282, 285–286, 456
Postnatal vasculogenesis, 318
Postoperative residual tumor, 531
pRb, 550
Precision oncology, 294
Preclinica evidence, anti-angiogenesis and radiotherapy, 351–352
Preclinical studies, 361, 365
Predictive biomarkers, 294, 296, 298, 522
Predictive factors, 501, 522
Pro-angiogenic factors, 5, 7, 9, 11, 12, 16, 17, 20, 23, 25
Pro-angiogenic transcription factors
cAMP, 75, 76
ETS, 75, 76
GATA, 76, 77
hypoxia-inducible factors, 78
MYC, 79
nuclear factor, 79
YAP/TAZ, 78
Pro-angiongenic factors, 417
Programmed death-1 (PD-1), 541
PD-1 ligand (PD-L1), 541
PD1-PDL1 blockade, 458
Progression-free survival (PFS), 531, 533, 535, 540
Prostate cancer, 258, 580
Proteasome inhibitors, 647–648
Proteinase families, 12
Protein kinase A (PKA), 76
Protein kinase C (PKC), 76
Pseudopalisades, 664

Q
Quality of life, 531, 533, 540

R
Radical cystectomy, 594
Ramucirumab, 217, 380, 387, 396, 404–406, 442, 503, 518, 601
Receptor tyrosine kinase (RTK), 162, 548
Recombining binding protein suppressor of hairless (RBPJ), 84
Regorafenib, 380, 436, 441, 454–455, 469, 601
Renal cancer, 295
Renal cell carcinoma (RCC), 295
angiogenesis, 570–572
anti-angiogenic drugs, 572
bevacizumab, 574
chemotherapy, 575
classification, 570
diagnosis, 570
everolimus, 574
interferon-α, 574
interleukin-2, 574
metastatic, 570
temsirolimus, 574
tyrosine kinase inhibitors, 572
Resistance, 315, 317, 319, 614, 663
adaptive, 583–584
innate, 584
to treatment, 457
Response evaluation criteria in solid tumors (RECIST), 456
Retinopathy, 44

S
Selectins, 191, 192
Selection for transarterial chemoembolisation treatment (STATE), 437
Selectivity, 538
Self-seeding paradigm, 165
Semaphorins, 9, 13, 14
Sentinel lymph node, 168
Serum/glucocorticoid regulated kinase (SGK), 81
Sieving, 39, 42

Signal transducer, 36
Single-nucleotide polymorphisms, 522
Skin reactions, 459
Slits, 14
Small cell lung cancer (SCLC), 505
Small molecule tyrosine kinase inhibitors, 360
Smooth muscle, 160
Soft tissue sarcomas, 466
Sorafenib, 186, 216, 427, 428, 436, 455–456, 469, 485–486, 487, 502, 517, 519–520, 536, 573, 585, 600
Sprouting angiogenesis, 4, 6–7, 74, 79, 317
Squamous cell cancer, 490
Squamous cell carcinoma, 478
  head and neck, 479
Stalk cells, 7, 11, 18
Succinate-dehydrogenase (SDH)-deficient GIST, 452
Sunitinib, 187, 216, 319, 355, 368, 454, 468, 489, 502, 505, 520, 538–539, 558, 572, 585, 600
  locally advanced HNSCC, 490
  recurrent and/or metastatic HNSCC, 489–490
Suramin, 517
Survival, 642, 644, 645, 647, 648, 651
Systemic chemotherapy, 470–471

T
Targeted therapy, 365, 372, 530, 532
Tasquinimod, 586–587
Taxanes, 337
Tegafur, 336
Temozolomide, 336
Temsirolimus, 369–370, 574
Thalidomide, 587, 645, 647, 649, 650
Thrombospondin, 599
  TSP-1, 17, 19, 550
  TSP-2, 19
TIE receptors
  as critical regulator of collecting lymphatic vessels, 140–141
  in lymphatic *vs.* blood vessel formation, 141
  Tie1, 229, 231, 233, 241, 242
  Tie2+ monocytes (TEMs), 317
  Tie2, 38, 56, 229, 231, 233, 239, 241
  Tie, 232, 241
Tight junctions (TJs), 100–101, 125–126
Time to disease progression (TTDP), 443
Time to extrahepatic spread or vascular invasion, 443
Tip cells, 6, 7, 9, 11, 13, 16, 93
Tissue-inhibitor of metalloproteinase-2 (TIMP-2), 642
Tivozanib, 389, 573
TNP-470, 552
Tortuous vessels, 54
Trabectedin, 466
Transarterial chemoembolization (TACE), 436
Transcellular permeability, 123
Transcription factors
  anti-angiogenic, 80–84
  pro-angiogenic, 75–80
Transforming growth factor-beta (TGF-β), 17–18, 421
Transmembrane protein, 124
Trastuzumab, 365
Trebananib, 389, 539
Trebaninib, 371
Tremelimumab, 368
Tricarboxylic acid (TCA) cycle, 665
Trofosfamide, 332
Tubulin binding agents (TBAs), 197
Tumor associated fibroblasts (TAFs), 317
Tumor-associated macrophages, 58
Tumor blood vessel, 185, 187, 195–198
Tumor cell dissemination, 530
Tumor edema, 112
Tumor growth, 314, 316, 319
Tumorigenesis, 450
Tumor immune microenvironment, 147
Tumor lymphangiogenesis, 136–138
  angiopoietins, 144–146
Tumor microenvironment, 54
Tumor vasculature, 4, 20, 24, 52, 54, 59, 60, 66, 96
Tumor vessel, 6, 9, 14, 21, 24–25
Tumor vessel normalization
  as anti-angiogenic, 59
  anti-tumor immune cells, 60
  clinical application challenges, 65
  conventional chemotherapeutic drugs and radiation therapy, 60
  disadvantages, 61–63
  immune cells, 58–59
  metastasis, 60
  pericyte coverage and, 56–57
  translational implications, 63–65
  tumor endothelium, 56
Tumstatin, 19
Tunica adventitia, 260
Tunica intima, 260
Tunica media, 260
Tyrosine kinase, 517
  receptors, 450
Tyrosine kinase inhibitor (TKI), 186–187, 315, 316, 318, 452, 456–457, 468, 560, 561, 572–574, 614, 615, 618
  cediranib, 538
  pazopanib, 535
  sorafenib, 536–537

U
Ubiquitination-E-ligase inhibitors, 650
Urothelial carcinoma, 594
US Food and Drug Agency (FDA), 186, 214, 365, 532, 533
Uterine cervical cancer, 547, 550, 552

V
Vaccination, 425–426
Vandetanib, 354, 355, 366, 390, 490–491, 502, 601
Vascular barrier, 122

Vascular co-option, 318
Vascular disrupting agents (VDAs)
  categories of, 196
  in combination therapy, 198
  flavonoids, 196
  history of, 196
  as monotherapy, 197–198
  resistance to, 198
  tubulin binding agents, 197
Vascular endothelial cadherin, 8, 13, 16, 37, 40, 41, 56, 102–106
Vascular endothelial growth factor (VEGF), 6, 8, 9, 12, 13, 15, 19, 23, 35–38, 52, 74, 92, 125, 128–130, 158, 186, 187, 190, 197, 200, 202, 212, 236, 239, 244, 262, 295, 298, 300, 302, 314, 316, 318, 319, 380, 417, 419, 421, 424, 426, 439, 450, 466, 498, 552, 571, 642, 645, 646, 649, 650
  angiogenic inhibitors, 217–218
  and Ang2 signaling, 371–372
  anti-angiogenic therapy, 130
  with cancer immunotherapy, 218
  and cetuximab, 362–363
  and CTLA4 blockade, 368
  C-Src, 125
  and erlotinib, 364–365
  and HER2-directed therapy, 365–366
  isoforms, 212
  and panitumumab, 363–364
  pathway, 186, 530, 548
  and PDL1 / PD1 blockade, 368–369
  protein inhibitors, 216–217
  receptor, 213–214, 295, 298, 300, 302, 388–390, 467
  receptor inhibitors, 560
  small molecule inhibitors of VEGFR2 tyrosine kinase, 216
  VEGF-A, 266, 582
  VEGF-B, 266
  VEGF-C, 158, 172, 266, 595
  VEGF-D, 158, 172, 266
  VEGFR1, 129, 264, 438, 581, 582
  VEGFR2, 35, 37, 52, 129, 172, 265, 422, 425, 427, 582
  VEGFR3, 129, 172, 266
  VEGF-Trap, 316
  VEGF-VEGFR signaling pathway, 9–12
Vascular endothelial growth factor (VEGF) inhibitors, 186
  DCE MRI assessment, 281
  and immune system, 283
  mode of action, 283
Vascular endothelial protein tyrosine phosphatase (VE-PTP), 239
Vascular extracellular matrix, 190–191
Vascular guidance, 6, 9, 13, 18
Vascular hijacking, 258
Vascular homeostasis, 92
Vascularization, 641
Vascular leakage, 97–99
  and cancer, 43
  and myocardial infarction, 44
  in ocular diseases, 44
Vascular normalization, 198, 201–202, 315, 316, 667
Vascular permeability, 109–111, 122
  cell-cell junctions, 123
  endothelial barrier, 123
  inflammatory cytokines, 127
  paracellular permeability, 123
  pathological angiogenesis, 127
  physiological angiogenesis, 127
  physiological conditions, 126
  regulation, 126–127
  transcellular permeability, 123
Vascular remodeling, 15, 104
Vasculature, 122
Vasculogenesis, 4–6, 74, 92, 122, 187, 190
Vasculogenic mimicry, 4, 8, 258, 318
Vatalanib, 390, 455, 618
Vesicles-vacuolar organelles (VVO), 40, 98, 123
Vessel growing, 4, 13, 15, 24
Vessel guidance, 13, 25
Vessel normalization, 198, 200–201, 329, 331
Vessel remodeling, 15–19
Vessel stabilization, 7, 15, 24
Vincristine, 319
Vinorelbine, 337
von Hippel-Lindau (VHL) gene, 21, 22, 215, 262, 296, 300, 570, 571

W
Warburg effect, 665

X
XELOX, 381

Y
YAP/TAZ, 79, 80

Z
Zona occludens family proteins (ZO), 100