1. Introduction

The ability of solid tumors to sustain growth beyond a few millimeters in size is dependent on their capacity to acquire nutrients and oxygen and to dispose of metabolic waste products and carbon dioxide through the formation of new blood vessels from pre-existing ones [1]. This process, termed angiogenesis or neovascularization, is considered one of the essential hallmarks underlying cancer development and metastasis [2]. During early tumor progression, angiogenesis is activated when the balance between pro- and anti-angiogenic signals is tilted in favor of neovascularization within the tumor microenvironment, facilitating mobilization, proliferation, migration and increased survival of endothelial cells from normally quiescent vasculature [3]. The precise mechanisms behind this ‘angiogenic switch’ may differ between tumor types, but include oncogene-driven (such as Ras and Myc) or hypoxia-driven expression of pro-angiogenic factors, and peritumoral infiltration and activation of immune cells stimulating the angiogenesis process [2,4]. Interestingly, tumor-associated endothelial cells can actively attract circulating bone marrow-derived precursor cells to angiogenic sites where they integrate into newly formed vessels as pericytes or endothelial cells [5]. Although controversial, angiogenesis may also progress through vascular mimicry in which tumor cells differentiate to form de novo vascular channels that resemble endothelial tubes [6].
### Article highlights.

- Tumor angiogenesis is a hallmark of cancer, but anti-angiogenic treatment has not yet met its potential clinically.
- Although endothelial cells are non-malignant, their intracellular signaling pathways are extensively deregulated with profound crosstalk between different signaling cascades.
- Resistance against anti-angiogenic agents can arise due to the inherent redundancy in intracellular signaling pathways regulating angiogenesis and the ability of tumor cells to adapt to a hypoxic microenvironment.
- An individualized approach based on tumor biopsies is needed to identify which signaling pathways are deregulated in any particular patient, both upfront and later when the treatment fails.
- Distinct Vegf or Vegfr-1 single nucleotide polymorphisms (SNPs), hypoxia and circulating plasma VEGF levels have been suggested as predictive biomarkers of bevacizumab response based on retrospective analyses of clinical trials, but need to be prospectively validated in larger cohorts.
- Novel therapeutic strategies within anti-angiogenic therapy include the use of the integrin inhibitor cilengitide in combination with radio- and chemotherapy for the treatment of glioblastoma multiforme (GBM), targeting of VEGFR-3 on developing tumor endothelium and the use of drug ‘cocktails’, such as combined VEGFR-2 and MET inhibition, to counteract alternative signaling and drug resistance when VEGF/VEGFR-directed therapy is given.

This box summarizes key points contained in the article.

Furthermore, subpopulations of cancer cells with stem-like properties have recently been reported to differentiate into bona fide endothelial cells in both leukemia and several solid malignancies, although the quantitative contribution of this phenomenon seems small [7]. Thus, it is becoming increasingly evident that tumor-associated angiogenesis is the net result of a complex interplay within the tumor microenvironment between cancer cells, stimulated vascular and perivascular cells, recruited myeloid and mesenchymal stem cells and activated fibroblasts [4,8].

The developing tumor vasculature displays considerable heterogeneity and consists of a broad range of vessel subtypes which differs markedly from the normal, physiological vasculature with respect to organization, structure and function. Tumor vessels are characterized by excessive capillary sprouting, complex and widespread vessel branching, distorted and enlarged vessels, inconsistent blood flow, leakiness and increased levels of endothelial cell proliferation and apoptosis [9]. As a result of such malfunctioning vessels, the tumor microenvironment is inadequately nourished, leading to hypoxia, acidic pH and increased interstitial fluid pressure (IFP) with accompanying edema development [10]. This further accelerates the angiogenic response, creating a self-reinforcing vicious cycle that may compromise the therapeutic efficacy of both radio- and chemotherapy. The traditional rationale for anti-angiogenic therapy is that abrogation of new vessel formation and/or disruption of existing vessels will restrain cancer progression and metastasis by essentially starving the tumor of its nutrients [11].

Although controversial, some preclinical and clinical findings indicate that ‘vessel normalization’, a process in which immature tumor vessels are pruned and the remaining vessels normalize and mature following anti-angiogenic treatment, may improve delivery of chemotherapeutic drugs, reduce shedding of metastatic cells into the circulation, and improve the metabolic microenvironment by reducing tumor hypoxia [10]. Even though tumor vessel normalization is observed after anti-angiogenic therapy, it is at present heavily debated if such a normalization will lead to increased tumor perfusion, since reduced perfusion also has been observed in some tumor types after therapy [12].

As listed in Table 1, angiogenesis inhibitors can interfere with most of the critical steps in the neovascularization process, and several of these agents have been studied in clinical trials over the last decade. Bevacizumab, a recombinant humanized monoclonal IgG1 antibody against VEGF-A (VEGF), was the first molecular targeted anti-angiogenic agent approved for use in combination with chemotherapy and cytokine therapy in the treatment of various advanced solid tumors. First-generation oral small-molecule tyrosine kinase inhibitors (TKIs) such as sorafenib, sunitinib and pazopanib inhibit a broad range of molecular targets, such as VEGFR-1 – 3, platelet-derived growth factor receptor (PDGFR), KIT and FLT-3 among others. They have already been approved as single agents in the treatment of metastatic renal cell carcinomas (RCC), and are currently being explored as both monotherapy and in combination with chemotherapy in several other advanced malignancies. The second-generation TKI axitinib inhibits the VEGFRs more potently than the earlier TKIs, possibly augmenting the therapeutic effect while reducing the adverse effects caused by off-target activity, thus increasing the therapeutic window [13]. Furthermore, new targets involved in both angiogenesis and tumor cell growth have been identified, such as the hepatocyte growth factor (HGF) receptor MET, which is targeted clinically by the combined MET/anaplastic lymphoma kinase (ALK) TKI crizotinib [14]. Figure 1 summarizes the mechanisms of action of the anti-angiogenic agents discussed above.

Despite a decade of clinical trials with angiogenesis inhibitors, the results in humans have to a large extent been disappointing in terms of improving patient survival. It has clearly not been a ‘cure for cancer’, and drug resistance has been a definite problem clinically. At the same time, some cancer patients clearly benefit from anti-angiogenic therapy, exemplified by a subset of patients with metastatic melanoma who had stable disease for 15 – 20 months while on bevacizumab [15]. However, the biomarkers to identify these individuals beforehand have so far been missing. In this review, the authors will discuss the complexity of growth promoting signaling pathways in the tumor endothelium,
FGFR: Fibroblast growth factor receptor; mAb: Monoclonal antibody; PDGFR: Platelet-derived growth factor receptor.

and address some key issues in resistance to angiogenesis inhibitors. Furthermore, they will elaborate on how to identify tumors that are likely to respond to anti-angiogenic therapy and highlight new therapeutic strategies that have emerged recently.

2. The spiderweb: key angiogenic signaling pathways and their interaction

There is an immense number of signaling pathways involved in tumor angiogenesis, which is extensively reviewed elsewhere [4]. Due to its role as a survival factor for endothelial cells, the VEGF and VEGF family of receptors seems to be crucial for endothelial cell growth and neovascularization [16]. Their importance in development is demonstrated by the fact that embryonic lethality occurs in mice even if a single VEGF allele is deleted [17]. Furthermore, the majority of angiogenesis inhibitors that have been investigated in clinical trials of human cancer have targeted VEGF-VEGF receptors, either alone or as one of several targets. VEGF is produced by tumor cells, as well as tumor-associated stromal cells, and elicits its major pro-angiogenic properties by binding to its corresponding receptor tyrosine kinase (RTK) VEGFR-2 expressed by endothelial cells and bone marrow-derived cells [18,19]. This initiates several signaling cascades, such as phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) and phospholipase C gamma (PLCγ)-protein kinase C (PKC)-Raf-mitogen-activated protein kinase kinase (MEK)-mitogen-activated protein kinase (MAPK), to facilitate increased vascular permeability as well as increased endothelial cell proliferation, migration and survival [20].

Similar to VEGF-mediated signaling, binding of the fibroblast growth factors (FGFs) to their RTKs FGF receptors 1–4 (FGFR1–4) on the surface of endothelial cells activates the PLCγ-PKC-Ras-Raf-MAPK and PI3K-AKT-mTOR pathways, suggesting the possibility of considerable crosstalk between VEGF/VEGFR and FGF/FGFR signaling. Overexpression of VEGF and FGF by human tumor cells is independently associated with a poor outcome [21-23], and combined inhibition of VEGF- and FGF-mediated signaling accordingly improves angiogenesis inhibition and impairs tumor growth in vivo compared with blocking VEGFR-2 alone [24,25]. Platelet-derived growth factors (PDGFs) regulate vessel maturation as well as the recruitment of pericytes and smooth muscle cells to the vasculature, and their activity is mediated by two RTKs, PDGFR-α and PDGFR-β [26]. Importantly, signaling through the PDGFRs activate a broad range of intracellular pathways including PLCγ-PKC-MAPK, PI3K-AKT and Ras-Raf-MEK, resulting in pericyte precursor cell proliferation and migration [27]. A combined anti-angiogenic therapy targeting the PDGF and VEGF receptors decreases the IFP of malignant tumors in vivo [28], indicating that such dual inhibition could be employed to improve the delivery of cytotoxic drugs. However, tumor cells also promote erythropoietin (EPO) production in perivascular cells via PDGF-PDGFR signaling to stimulate erythropoiesis and tumor oxygenation [29], indicating that PDGF inhibition could in fact worsen tumor hypoxia.

Recent evidence suggests that the angiopoietin (Ang)/Tie2 pathway is critical for tumor angiogenesis [30,31]. In the presence of VEGF, Ang-2 facilitates sprouting angiogenesis by interrupting Ang-1-mediated vessel normalization/stabilization, causing impaired pericyte coverage, vessel destabilization and increased

Table 1. Anti-angiogenic agents and their targets in the angiogenesis process.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Endothelial target</th>
<th>Relevance in anti-angiogenic therapy</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cilengitide</td>
<td>αvβ3 integrin</td>
<td>Inhibits endothelial cell migration and blocks endothelial cell–cell interactions and endothelial cell–matrix interactions</td>
<td>[98]</td>
</tr>
<tr>
<td>Endostatin</td>
<td>α5β1 integrin</td>
<td>Inhibits endothelial proliferation/migration and induces apoptosis by antagonizing the FAK/c-Raf/MEK/ERK1 pathway</td>
<td>[117]</td>
</tr>
<tr>
<td>Tumstatin</td>
<td>αvβ3 integrin</td>
<td>Inhibits endothelial cell proliferation and induces apoptosis by blocking protein synthesis</td>
<td>[61]</td>
</tr>
<tr>
<td>ALT4 mF4-31C1 mAb</td>
<td>VEGFR-3</td>
<td>Disrupts the integrity of newly formed vessels by targeting endothelial sprouts during the early phases of angiogenesis</td>
<td>[104]</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>VEGF</td>
<td>Induces endothelial apoptosis</td>
<td>[18]</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>PDGFR</td>
<td>Inhibits pericyte adherence to endothelial cells</td>
<td>[4]</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>VEGFR-1 – 2</td>
<td>Inhibits endothelial apoptosis</td>
<td></td>
</tr>
<tr>
<td>Pazopanib</td>
<td>FGF</td>
<td>Inhibits endothelial proliferation</td>
<td></td>
</tr>
<tr>
<td>Crizotinib</td>
<td>MET</td>
<td>Inhibits endothelial cell growth and prevents tumor cell invasiveness</td>
<td>[35]</td>
</tr>
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FGFR: Fibroblast growth factor receptor; mAb: Monoclonal antibody; PDGFR: Platelet-derived growth factor receptor.
vascular permeability [32,33]. Ang-2 binding to Tie2 triggers PI3K-AKT-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Ras-Raf-MEK activity, promoting endothelial cell survival and proliferation/migration, respectively. Tipping the balance from Ang-2-mediated neovascularization to Ang-1-mediated vessel maturation is therefore a potential therapeutic strategy to stop angiogenesis and improve tumor perfusion and drug delivery [34].

Of increasing interest is the interplay between various signaling pathways, where there is significant overlap on downstream key regulator proteins such as mTOR and MAPK. This means that several endothelial growth factors can function in parallel to stimulate angiogenesis via the same pathways, which is demonstrated by the VEGF-VEGFR-2 and HGF-MET pathways, both converging on the AKT-mTOR and Ras-MAPK signaling axes in endothelial cells (Figure 2A) [35,36]. On one hand, this suggests that combined inhibition of VEGFR-2 and MET is necessary to block angiogenesis efficiently, and on the other hand that targeting downstream hubs like mTOR and MAPK can block the response to several pro-angiogenic receptors simultaneously.

Ligands and RTKs involved in neovascularization are present on many different cell compartments within a malignant tumor, introducing another level of complexity to angiogenesis regulation. Cancer-associated fibroblasts are the major source of the MET ligand HGF, emphasizing the important pro-angiogenic role of non-malignant cell populations within the tumor microenvironment [37]. Apart from endothelial cells, MET is also present on tumor cells, and MET-induced signaling facilitates endothelial cell growth by inducing VEGF expression and impairing thrombospondin-1 (TSP-1) expression in the tumor cells [38]. This VEGF-TSP-1 imbalance...
mediated by MET suggests that the HGF-MET pathway may contribute to the angiogenic switch within the tumor microenvironment. This further suggests that the angiogenic switch is not mediated by acquisition of mutations alone, but is regulated by the interplay between malignant and non-malignant cell populations in the tumor microenvironment. In line with this, the recently published data by the authors suggest an important role of heat shock protein 27 (HSP27) in controlling the angiogenic switch in a human tumor dormancy model by regulating the secretion of both VEGF and bFGF from tumor cells [39].

The extensive interplay between various signaling pathways and cell compartments in the tumor can be seen as a spiderweb; i.e., a network of many intertwining communication lines. Accordingly, the analysis of a single pathway is too simplistic, and more comprehensive assays where all the key players are analyzed simultaneously are needed in order to assess how the angiogenesis process should be targeted. This is a feasible approach today, either by analysis of upregulated growth factors in interstitial fluid extracted from the tumor [40] or as kinase assays where tumor biopsies are analyzed to assess which receptor kinases are deregulated [41].

3. Tales of the expected: resistance to anti-angiogenic therapy

Anti-angiogenic therapy was originally thought of as a therapy ‘resistant to drug resistance’ [42]. It was assumed that drugs directed at the non-malignant part of the cancer; such as, the endothelial cells, would target the ‘Achilles’ heel of cancer’ since these cells have a stable genome and lack the evading mechanisms inherent in cancer cells. Although controversial, angiogenesis may also progress through vascular mimicry in which tumor cells contribute to the formation of vascular channels that resemble endothelial tubes [6,43-50]. This phenomenon, if relevant, would facilitate acquired resistance to angiogenesis inhibitors similar to therapy designed to target the malignant cell population.

Based on clinical experience and the results of clinical trials to date, it is quite evident that resistance to anti-angiogenic treatment occurs. This resistance broadly falls into two major categories: resistance due to factors within the non-malignant part of the tumor and resistance related to the malignant cells.

Redundancy represents an inherent part of all biological systems. If one signaling pathway necessary for cell growth is
blocked, alternative pathways can be upregulated to replace it, leading to acquired drug resistance. This can occur even if endothelial cells harbor a stable and normal genome, and there is an emerging body of data focusing on such cellular plasticity as a reason for drug resistance [1,24,51,52]. For instance, anti-VEGFR-2 therapy failed both in preclinical tumor models and in human gliomas due to upregulation of FGF, PDGF as well as several other signaling molecules [24,51,53]. Furthermore, there is extensive crosstalk between signaling pathways important for tumor angiogenesis, which can circumvent the inhibition of one growth factor receptor [4,54]. However, alternative signaling can be prevented as exemplified by resistance to anti-VEGFR-2 therapy which is avoided if an antibody to another member of the VEGF family, PlGF, is added [55]. Since many signaling pathways converge on the same downstream signaling molecules, another way of avoiding resistance due to signaling crosstalk is to target downstream hubs, such as mTOR and MAPK, if the signaling converges downstream of the receptors [4].

Apart from acquired resistance to angiogenesis inhibitors, upfront resistance due to the inherent characteristics of a particular malignant tumor can also occur [1]. A malignant tumor can simply lack the dependency on a particular angiogenic signaling pathway, in which case a single-target angiogenesis inhibitor will fail [56,57]. This is very evident in RCC, where most patients respond, but close to 20% progress directly on bevacizumab [58,59]. Furthermore, tumor cells and other components of the tumor microenvironment, such as myeloid cells and stromal cells, produce a multitude of pro-angiogenic growth factors, exposing endothelial cells to the essential growth factors needed [4]. Inhibition of a single cytokine is therefore in many cases not enough to block the angiogenesis process. Furthermore, tumor-associated endothelial cells differ from endothelial cells outside of the tumor, as they have numerous genes upregulated [60]. This provides the endothelial cells with alternative growth factor receptors and downstream signaling pathways if one receptor is blocked, pointing again at the necessity of drug combinations or ‘cocktails’ to counteract angiogenesis [1]. Accordingly, whereas the angiogenesis inhibitors bevacizumab and tumstatin had a minor anti-tumor effect individually, the combination profoundly inhibited tumor growth in 786 RCC [61].

The other major resistance mechanism is derived from the inherent plasticity of the malignant cells themselves. The tumor microenvironment is characterized by hypoxia, which the cancer cells have adapted to and survive in [4]. Hypoxia also promotes a more aggressive genotype, selecting for cancer cells harboring TP53 inactivating mutations [62,63]. There is increasing evidence that anti-angiogenic therapy can lead to metabolic changes from oxidative respiration to glycolysis [12] where adenosine triphosphate (ATP) for DNA synthesis and cell growth is provided by the pentose phosphate shunt. Thus, the requirement for oxygen and neovascularization is reduced. Alternatively, if the neovascularization process is halted, cancer cells can grow along pre-existing blood vessels to obtain adequate oxygen and nutrient supply, so-called vascular co-option [64].

Yet another avoidance mechanism has been observed in malignant brain and pancreatic tumors where angiogenesis inhibition switches the cancer from solid tumor growth into diffuse infiltrative growth to obviate the need for neovascularization (Figure 2B – C) [12,65]. This invasive phenotype seems related to epithelial-to-mesenchymal transition and can be counteracted by HGF/MET inhibition [14,66]. Infiltrative growth is further facilitated by fibroblasts activated by the tumor cells to secrete serine and matrix metalloproteinases to enable endothelial and tumor cell invasion [37]. In line with this, novel strategies are warranted where tumor cell migration or glycolysis are targeted when such resistance mechanisms develop.

A major controversy in the field of angiogenesis inhibition is whether endothelial cells harbor mutations that will contribute to drug resistance. At present, data in support of such a phenomenon are limited [1]. Therefore, endothelial genetic alterations are probably not the basis for drug resistance, and they should retain a partial sensitivity to anti-VEGF therapy. This is supported by a recent clinical study showing that bevacizumab had a significant therapeutic effect when added to chemotherapy in second-line treatment, beyond progression on bevacizumab in the first-line setting [67]. This could imply that the drug resistance develops against the chemotherapeutic agent(s) which bevacizumab is combined with, and that a therapeutic benefit from anti-VEGF treatment can still be derived if another type of chemotherapy is added in the second-line setting.

Apart from the malignant cells a tumor consists of a wide variety of non-malignant cell populations, such as endothelial cells, leucocytes, fibroblasts and bone marrow-derived endothelial precursors [1]. All of these cell populations are known to be involved in tumor angiogenesis, although their individual importance is a matter of discussion. Cancer-associated fibroblasts can secrete stromal cell-derived factor-1 (SDF-1) to recruit bone marrow-derived precursor cells to the growing neovascularure [68]. However, the recruitment of bone marrow-derived endothelial progenitors to the tumor neovascularure has been reported to be either relevant or irrelevant quantitatively [69-71]. Thus, the therapeutic gain from preventing the recruitment of bone marrow-derived cells in human cancer remains to be determined. Furthermore, pro-angiogenic growth factors can be secreted by various cell populations within a malignant tumor. This is exemplified by VEGF which is secreted by malignant cells, endothelial cells and fibroblasts within the tumor, indicating that several cell compartments need to be targeted simultaneously to stop VEGF production [72]. Accordingly, if VEGFR-2 is blocked on the endothelium, VEGF can still be produced by tumor cells and cancer-associated fibroblasts to stimulate angiogenesis via VEGF-R1 and VEGF-R3 [73].

Since resistance to angiogenesis inhibitors clearly exists, improved therapeutic strategies need to be developed. There
are several potential strategies for improvement. One is to obtain upfront tumor biopsies in order to determine which pro-angiogenic pathways are upregulated, followed by a tailor-made treatment to the individual patient [74]. Also, targeting signaling molecules far upstream in the pathways, such as ligands or RTKs, probably increases the possibility of escape mechanism due to crosstalk between pathways and alternative signaling further downstream. Therefore, drugs which target central, downstream hubs, such as mTOR and MAPK, might theoretically be less prone to trigger escape mechanisms. The potential for mTOR inhibition to prevent resistance in breast cancer cells is elegantly demonstrated in the recent BOLERO-2 trial where the rapalogs everolimus was added to the aromatase inhibitor exemestane [75]. However, resistance can occur even during mTOR inhibition due to a high cellular autophagy capacity [76]. Furthermore, the targeting of such central signaling molecules is clearly associated with toxicity issues, probably due to interference with the many pathways necessary for normal tissue homeostasis [75].

Finally, certain growth factors are clearly more critical than others for endothelial cell survival. In tumor blood vessels with incomplete pericyte coverage, VEGF withdrawal causes endothelial cell apoptosis, pointing at VEGF as a critical survival factor [16]. Thus, targeting VEGF should be considered as a backbone whereupon other therapies are added. Also, since tumor angiogenesis is the net result of the balance between pro- and anti-angiogenic endogenous proteins, the focus should not only be directed at the inhibition of activators such as VEGF, PDGF and FGF, but also at activating the natural inhibitors such as TSP-1, and collagen-derived degradation products such as endostatin and tumstatin [77]. These endogenous anti-angiogenic compounds are categorized as ‘direct’ angiogenesis inhibitors. Instead of ‘indirect’ angiogenesis inhibition of ligands and receptors, these direct-acting agents induce endothelial cell apoptosis via simultaneous inhibition of several signaling pathways [1]. Thus, it has been suggested that ‘direct’ angiogenesis inhibitors would be less prone to resistance [1]. However, the use of such endogenous angiogenesis inhibitors still needs to be refined, in particular with respect to stability and solubility of the drugs if administered to humans [77].

4. Finding the needle in the haystack: predictive biomarkers

Clinically, it is well known that some patients have a much more favorable response to anti-angiogenic therapy than others, carrying the same malignancy. The search for predictive biomarkers in order to identify the patients who will benefit from treatment beforehand has therefore been extensive. A large number of potential biomarkers have been studied, but so far none has been successfully identified and more research is needed [78].

An obvious approach is to assess whether tumors that are heavily vascularized are more sensitive to angiogenesis inhibitors than those that are not. This has been investigated by using immunohistochemistry where blood vessels are stained and counted in vascular hot spots using the microvessel density (MVD) method, or non-invasively by functional magnetic resonance imaging (fMRI) looking at tumor perfusion. Apart from its prognostic value in some studies, the MVD of malignant tumors has no value in predicting who will respond to anti-angiogenic therapy, and neither has fMRI at the moment [78,79].

Being the main target of anti-angiogenic therapy, VEGF and VEGFRs have been extensively studied as biomarkers. For VEGF there has been a large number of studies with varying results, but measuring this growth factor is fraught with a multitude of potential pitfalls [79,80]. A particular problem is the low stability of VEGF, whereas soluble VEGFR-1 seems to be more robust and could be a better biomarker for angiogenesis inhibitor response [81,82]. However, using careful analysis recent findings support a potential role for plasma VEGF as a predictive marker for the therapeutic benefit of adding bevacizumab to chemotherapy in metastatic breast cancer and advanced non-small cell lung cancer (NSCLC) [83]. Moreover, distinct Vegf single nucleotide genetic polymorphisms (SNPs) in a retrospective analysis of the E2100 trial have been shown to predict improved outcome in terms of prolonged overall survival (OS) and less grade 3/4 hypertension in metastatic breast cancer patients receiving bevacizumab and paclitaxel [84]. Vegf SNPs have also been identified to be potentially predictive in assessing outcome in metastatic colorectal patients treated with irinotecan-based chemotherapy plus bevacizumab [85]. Recently, a SNP in Vegfr-1 was also found to predict for poor response to bevacizumab in two clinical trials where the anti-VEGF antibody was combined with chemotherapy [86].

Although controversial, the onset of hypertension during bevacizumab treatment has been associated with improved outcome in patients with advanced NSCLC [87], metastatic colorectal cancer [88], RCC [89] and melanoma [90]. In particular, increased diastolic blood pressure seems to define who will respond to angiogenesis inhibitors [89,91]. It has been suggested that the development of hypertension during bevacizumab treatment could reflect decreased nitric oxide synthesis due to a more effective blockade of VEGF signaling, translating into improved patient outcome [92].

Given the modest effect of anti-angiogenic therapy in most cases, good biomarkers are clearly needed, and the above findings are promising. Yet, the potential clinical utility of these biomarkers needs to be prospectively validated in larger cohorts.

5. New kids on the block: novel treatment principles

The integrin family of cell-matrix adhesion receptors is expressed as heterodimers at the cell surface of endothelial
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cells, and orchestrate sprouting angiogenesis by integrating angiogenic signals between the ECM compartment and the intracellular signaling pathways regulating migration, invasion, survival and proliferation [4]. Notably, overexpression of the integrin αvβ3 occurs on both the angiogenic endothelial cells and the cancer cells whereas it is absent on normal endothelial cells [93,94], providing a strong rationale for targeted therapy in an effort to halt cancer progression and metastasis. Accordingly, abrogation of αvβ3-mediated signaling by either cyclic peptides or antibodies blocks angiogenesis and induces tumor regression in vivo [93,95]. The synthetic pentapeptide cilengitide antagonizes αvβ3 and αvβ5 activity by blocking integrin-ligand binding, and consequently decreases angiogenesis in vivo and impairs orthotopic brain tumor growth in preclinical models [96,97]. A recent multicenter pilot study demonstrated that cilengitide is well tolerated when administered in combination with standard radiotherapy with concurrent and adjuvant temozolomide in patients with newly diagnosed glioblastoma [98]. The addition of cilengitide also increased progression-free survival (PFS) at 6 months and the median survival rate compared with historical controls treated with radiotherapy and temozolomide alone. Interestingly, this therapeutic benefit seemed more prominent in patients with epigenetic silencing of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT), which is a significant predictor of temozolomide response. It has therefore been suggested that cilengitide may cause vascular normalization that subsequently improves the delivery and thereby the cytotoxic efficacy of temozolomide chemotherapy predominantly in GBM patients without MGMT expression. An ongoing randomized Phase III trial (CENTRIC, EORTC 26071-22072, NCT00689221) compares the addition of high-dose cilengitide with temozolomide chemotherapy to the standard regimen of temozolomide and radiation in newly diagnosed GBM patients with a methylated MGMT promoter.

Until recently, little attention has been given to VEGFR-3, the third member of the VEGFR family. The lack of viable VEGFR-3 knockout mice has contributed notably to this state. Vegfr-3 gene inactivation leads to defects in arterial-venous remodeling of the primary vascular plexus and causes lethality by embryonic day (E) 10.5 indicating a crucial role for VEGFR-3 in physiological (developmental) angiogenesis [99]. It has been shown that VEGFR-3 is expressed in all endothelial cells during early embryogenesis and after E10.5, VEGFR-3 expression becomes confined to the lymphatic vessels [100] and certain fenestrated blood capillaries [101]. VEGF-C and VEGF-D are the only known ligands of VEGFR-3 and induce lymphangiogenesis in adults, the formation of new lymphatic vessels from pre-existing ones, in a mode similar to angiogenesis. VEGFR-3 expression is a valuable tool to distinguish between vascular and lymphatic endothelium in adult tissues. However, VEGFR-3 expression is reinduced in blood vascular endothelial cells in some tumors [102] and chronic inflammatory wounds [103]. Spurred by this observation it has been proposed that anti-angiogenic therapies directed at VEGFR-3 might become effective, especially in targeting vessels that are resistant to VEGF or VEGFR-2 inhibitors [104,105]. Indeed, by using ALT4, an anti-VEGFR-3 monoclonal antibody, the growth of C6 rat glioblastoma and PC-3 human prostate adenocarcinoma xenografts in mice was inhibited by affecting the integrity of tumor blood vessels, leading to microhemorrhages [106]. Similar results were obtained by using mF4-31C1, another anti-VEGFR-3 monoclonal antibody. The growth of treated tumors was inhibited and blood vessel density significantly decreased, and the tumor stroma was characterized by increased hypoxic and necrotic areas, however, with no signs of microhemorrhages [105]. The combination of anti-VEGFR-2 and anti-VEGFR-3 antibodies markedly decreased the vascular surface area and growth of LNM35 human large-cell carcinoma of the lung and B16 mouse melanoma tumors, compared with VEGFR-2 inhibition alone [104]. These results show that VEGFR-3 is indispensable not only during normal development, but also for pathological tumor angiogenesis. However, the mechanism by which VEGFR-3 regulates this process is not fully understood. Prominent expression of VEGFR-3 within tumors has been seen in endothelial sprouts indicating specificity during the early phases of angiogenesis [104]. Sprouting angiogenesis involves the specification of endothelial cells into tip and stalk cells controlled by Notch signaling [107,108]. It should also be emphasized that gradients of VEGF induce single endothelial cells to become leading tip cells of emerging angiogenic sprouts. In particular delta-like 4 (Dll4)-Notch1 signaling regulates the formation of appropriate numbers of tip cells to control vessel sprouting [109], and this process can further be regulated by microRNAs [110,111]. Thus, tip cells respond to VEGF guidance signals whereas stalk cells proliferate to form the vascular network [108]. Filopodia that extend from tip cells may initiate vessel fusion provided that they make contact with each other. It has been shown that the whole process is chaperoned by Tie2α and non-ribosomal peptide-1 (NRP1)α macrophages, which express growth factors and proteolytic enzymes [112]. Surprisingly, tip cells of endothelial sprouts within tumors demonstrated prominent VEGFR-3 expression in the filopodial extensions. Furthermore, VEGF-Cα macrophages were present at sites of sprout fusion, implicating VEGF-C as a key factor in the assembly of the vascular network. Disruption of tip cell fusion points and ineffective angiogenesis has been observed in both Vegf haploinsufficient and macrophage-deficient apop mice [104,113]. It has been shown that genetic or pharmacological but not antibody-mediated inactivation of VEGFR-3 leads to increased number of tip cells and vessel hyperplasia in vivo [104]. Similar findings have been observed after Notch signaling pathway disruption. Thus, it has been proposed that VEGF-C secreted by macrophages activate VEGFR-3 expressing tip cells to turn on Notch target genes, facilitating an assembly of vascular network. Thus, the presence of VEGFR-3 on tumor blood
endothelial cells illustrates the complexity of the process of tumor angiogenesis. Effective inhibition of tumor progression may require the inactivation of multiple angiogenic targets and emerging preclinical data clearly indicate that VEGFR-3 might become one of them.

The HGF/SF-MET signaling pathway has also been shown to be important in the development of tumor-associated angiogenesis and early tumor progression by stimulating the proliferation and migration of both endothelial cells and tumor cells. Recent findings have demonstrated that MET is expressed in tumor-derived exosomes, i.e., actively released small endosomal vesicles, from patients with advanced metastatic melanoma, and increased levels of MET expression in shed exosomes has been associated with more extensive metastasis [114]. Notably, metastasis was aided through improved vascular permeability and angiogenesis mediated by bone marrow-derived cells expressing high levels of MET, indicating that MET signaling contributes to several cancer hallmarks such as angiogenesis, invasion, and metastasis. Abrogation of MET expression in the tumor-derived exosomes accordingly impaired the pro-metastatic properties of the bone marrow-derived cells, thus providing a rationale for MET targeting in patients with advanced metastatic melanoma. MET targeting agents currently being studied include competitors of MET/HGF binding, monoclonal antibodies aimed at HGF and MET and small molecule TKIs [115]. Moreover, concomitant inhibitors of VEGFR and MET signaling such as XL184 (also known as cabozantinib) have demonstrated anti-tumor efficacy in clinical trials for a broad range of solid malignancies [35]. Dual inhibition of VEGFR and MET signaling could represent a potential therapeutic strategy in those tumors where there is a shift toward a more invasive phenotype following VEGF-targeted therapy such as bevacizumab, induced by hypoxia-driven MET upregulation (Figure 2B - C) [35]. Interestingly, a recent study demonstrated how anti-VEGF therapy actually increases MET signaling and epithelial-to-mesenchymal transition by removing a break protein on the VEGFR-2/MET receptor heterocomplex [116]. Besides pointing at a potential resistance mechanism against anti-VEGF therapy, this emphasizes why combined VEGFR/MET inhibition could be an important therapeutic strategy.

6. Conclusion

Dr. Judah Folkman initiated the field of tumor angiogenesis research more than 40 years ago [11]. Since then, angiogenesis has been identified as a hallmark of cancer, and although malignant cells can grow in a non-angiogenic manner, solid tumors are to a large extent angiogenesis dependent. However, the quest for efficient angiogenesis inhibitors has so far not resulted in drugs which completely and permanently halt neovascularization. The clinical trials with bevacizumab have clearly demonstrated that there is no mono-dependency on VEGF for tumor angiogenesis. Still, targeting VEGF signaling seems to be a common denominator for all therapies to date which have caused a survival benefit in human cancer. This indicates that therapy directed at VEGF or VEGFR-2 should be an integrated part of future drug cocktails that are designed. Anti-angiogenic agents such as bevacizumab and several TKIs have during the recent years been approved for the treatment of several advanced solid cancers in combination with chemotherapy or as single agents based on Phase III clinical trials. Although significant, the improvement in PFS is only a few months, and in many cases without a corresponding increase in OS. Accordingly, there is an imminent need for predictive markers that can be used to identify potential responding patients upfront, since many do not respond to treatment and can still contract serious and potential lethal side effects from the treatment.

It has become clear that angiogenesis regulation is much more complex than what was first anticipated. Malignant tumors contain a multitude of growth factors being secreted to stimulate the endothelium, causing numerous growth promoting signaling pathways to be upregulated. This needs to be appreciated in order to design methodology to analyze the tumors on an individualized basis to decide which drugs to use. Furthermore, the signaling network within the tumor endothelium is not stable, and like all biological systems, the cells adapt when anti-angiogenic therapy is given. Therefore, tumors need to be reanalyzed when the treatment fails to assess which alternative signaling pathways have been upregulated to understand how continued angiogenesis or a switch to non-angiogenic tumor growth can be prevented.

7. Expert opinion

Despite 40 years of intense research in cancer angiogenesis and more than a decade of clinical trials with angiogenesis inhibitors, the promised potential of cancer cure is still beyond the horizon. At the same time, a lot more is known today about the many intricate and redundant signaling cascades promoting tumor neovascularization. Thus, the time has come to design clinical trials in an individualized manner based on the profile of angiogenic and anti-angiogenic cytokines and dominant signaling pathways of each tumor. Furthermore, we need to acknowledge that resistance against angiogenesis inhibitors frequently develops as a result of alternative intracellular signaling in the tumor endothelium when one pathway is blocked, suggesting that therapeutic strategies targeting several key signaling pathways simultaneously need to be developed. Alternatively, targeting key downstream signaling hubs like mTOR might block resistance due to alternative signaling if they converge on the same downstream molecules. One should also realize that the tumor microenvironment consist of other cell types, apart from endothelial cells and tumor cells, which contribute to angiogenesis, so preventing myeloid cell activity or the recruitment of bone marrow-derived cells to the tumor
endothelium could have a therapeutic potential. Additionally, the inherent plasticity of the malignant cells and their capability to adapt to an infiltrative growth pattern following angiogenesis inhibition needs to be appreciated, and highlights the need to consider escape mechanisms outside the endothelial cell compartment.

Current evidence suggests that VEGF/VEGFR-mediated signaling is essential for endothelial cell survival, and targeting of VEGF should be considered the backbone on which new anti-angiogenic drugs are added. The implementation of pre-treatment biopsies to identify key angiogenic pathways seems warranted to design an individualized and tailored therapy. It is becoming increasingly evident from both pre-clinical and clinical findings that the therapeutic efficacy of anti-angiogenic agents is likely restricted to a subset of cancer patients with similar underlying tumor biology. Thus, careful patient selection in advance of anti-angiogenic treatment will be necessary in order to improve survival. To conclude, the authors suggest a switch from large trials where unselected tumors are treated with a given anti-angiogenic agent to small trials where tumors are mapped upfront for their upregulated pro-angiogenic pathways and where tumors with a similar profile are treated with the same tailor-made anti-angiogenic cocktail.

**Declaration of interest**

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