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The Great Escape; the Hallmarks of Resistance to Antiangiogenic Therapy

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Abstract—The concept of antiangiogenic therapy in cancer treatment has led to the approval of different agents, most of them targeting the well known vascular endothelial growth factor pathway. Despite promising results in preclinical studies, the efficacy of antiangiogenic therapy in the clinical setting remains limited. Recently, awareness has emerged on resistance to antiangiogenic therapies. It has become apparent that the

intricate complex interplay between tumors and stromal cells, including endothelial cells and associated mural cells, allows for escape mechanisms to arise that counteract the effects of these targeted therapeutics. Here, we review and discuss known and novel mechanisms that contribute to resistance against antiangiogenic therapy and provide an outlook to possible improvements in therapeutic approaches.

I. Introduction

Angiogenesis, the formation of novel blood vessels from pre-existing ones, is indispensable for tumor progression and metastasis formation. Several decades ago, it was postulated that because tumors are angiogenesis dependent, inhibiting this process would be a means to combat cancer (Folkman, 1971). Since then, numerous studies have been published describing the process of tumor angiogenesis in more detail and identifying diverse pro- and antiangiogenic factors (reviewed by Griffioen and Molema, 2000; Carmeliet and Jain, 2011; Weis and Cheresh, 2011). Although these factors are in balance in healthy tissues, a shift toward proangiogenic factors, i.e., the angiogenic switch, marks the onset of tumor angiogenesis. This will cause activation of endothelial cells (EC) in local blood vessels, resulting in basement membrane and extracellular matrix degradation, EC migration and proliferation, and tube formation to form new vascular sprouts.

Therapeutic interference with tumor angiogenesis is expected to be an efficient means of anticancer treatment of a number of reasons. First, the target cells, i.e., the EC, are in direct contact with the blood, ensuring easy delivery of blood-borne therapeutics. Second, eradicating only a few EC will cause an "avalanche" effect by killing many tumor cells that depend on a single capillary. Third, EC are considered to be genetically stable cells, reducing the chance of acquired drug resistance. Finally, as EC throughout the body are generally quiescent, antiangiogenic therapy can be expected to have limited side effects because it targets only activated EC (Griffioen and Molema, 2000; Weis and Cheresh, 2011).

The discovery of vascular endothelial growth factor (VEGF) as one of the driving growth factors of angiogenesis (Leung et al., 1989) was key in the development of the first approved antiangiogenic therapeutic, the anti-VEGF antibody bevacizumab (reviewed by Ferrara et al.,

2004). More recently, compounds targeting the activity of angiogenic growth factor receptors, the tyrosine kinase inhibitors (TKIs), like sunitinib and sorafenib, were approved for clinical use (Kane et al., 2006; Goodman et al., 2007). These compounds have shown to benefit patients with cancer and angiogenic eye diseases. However, despite the expectations from preclinical investigations, clinical benefit has been relatively limited, resulting in mostly only enhanced progression-free survival and sometimes improvement in overall survival (Ebos and Kerbel, 2011). Although it was expected that angiogenesis inhibitors would be less sensitive to induction of resistance, it seems that there are several mechanisms resulting in decreased responsiveness to antiangiogenic drugs. In parallel to John Sturges' movie *The Great Escape*, where a high level of organization was necessary to escape a German prisoner of war camp, it appears that an intricate system of regulatory pathways is available to the tumor and its stromal components to resist the activity of a drug. In recent years, the development of resistance to antiangiogenic therapies has gained more and more attention (Bergers and Hanahan, 2008; Loges et al., 2010; Ebos and Kerbel, 2011; Clarke and Hurwitz, 2013). Here, we review proposed mechanisms of resistance to antiangiogenic therapies, discuss the consequences of resistance, and provide an outlook for improving the therapeutic benefit of antiangiogenic therapy.

II. Mechanisms of Resistance

Although initially hypothesized to be absent, the induction of resistance to antiangiogenic drugs comes in many different flavors, similar to those described for chemotherapy. It seems that most of the resistance mechanisms to antiangiogenic therapy are not genetic, or at least no clear genetic explanations are available. It has been suggested that this is the reason for resistance to

ABBREVIATIONS: AMD, age-related macular degeneration; ANG, angiopoietin; BMDC, bone marrow-derived cell; CAF, cancer-associated fibroblast; CCL, C-C motif ligand; CXCL, C-X-C motif ligand; CXCR, CXC receptor; EC, endothelial cell; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EPC, endothelial progenitor cell; EV, extracellular vesicle; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GBM, glioblastoma multiforme; HGF, hepatocyte growth factor; HIF, hypoxia inducible factor; HNSCC, head and neck squamous cell carcinoma; IL, interleukin; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; PCV, polypoidal choroidal vasculopathy; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PIGF, placental growth factor; RCC, renal cell carcinoma; SDF, stromal-derived factor; Sema3A, semaphorin 3A; S1P, sphingosine-1 phosphate; SNP, single nucleotide polymorphism; TGF, transforming growth factor; TIE, tyrosine kinase with immunoglobulin-like and EGF-like domains (ANG receptor); TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor.

be reversible and transient. In this section, the hallmarks of resistance to antiangiogenic drugs are discussed (Fig. 1A). We elaborate on different mechanisms that have been implied in antiangiogenic therapy resistance, both in clinical and preclinical settings. In addition, we discuss emerging mechanisms of resistance for which no clinical evidence has yet been presented but that are likely to become more apparent players in this phenomenon.

A. Redundancy in Growth Factor Signaling

Although VEGFs constitute the best known angiostimulatory protein family, EC activation and induction of angiogenesis can be triggered by numerous growth factors, including—but not limited to—angiopoietins (ANGs) (Fagian and Christofori, 2013), fibroblast

growth factors (FGFs) (Brooks et al., 2012), transforming growth factors (TGFs) (Pardali et al., 2010), and placental growth factor (PlGF) (Bergers and Hanahan, 2008; Carmeliet and Jain, 2011; Gacche and Meshram, 2014). Except for PlGF, which binds VEGF receptors, most angiogenic factors signal through specific transmembrane receptors, which are expressed on EC. This variety of growth factors culminates in a plethora of pathways that tumor cells can exploit to induce angiogenesis. Moreover, novel proangiogenic growth factors and receptors are still being discovered. For example, we recently identified PAI-1 as the target protein that mediates the antiangiogenic activity of 16K prolactin (Bajou et al., 2014). In addition, several members of the galectin protein family have been found

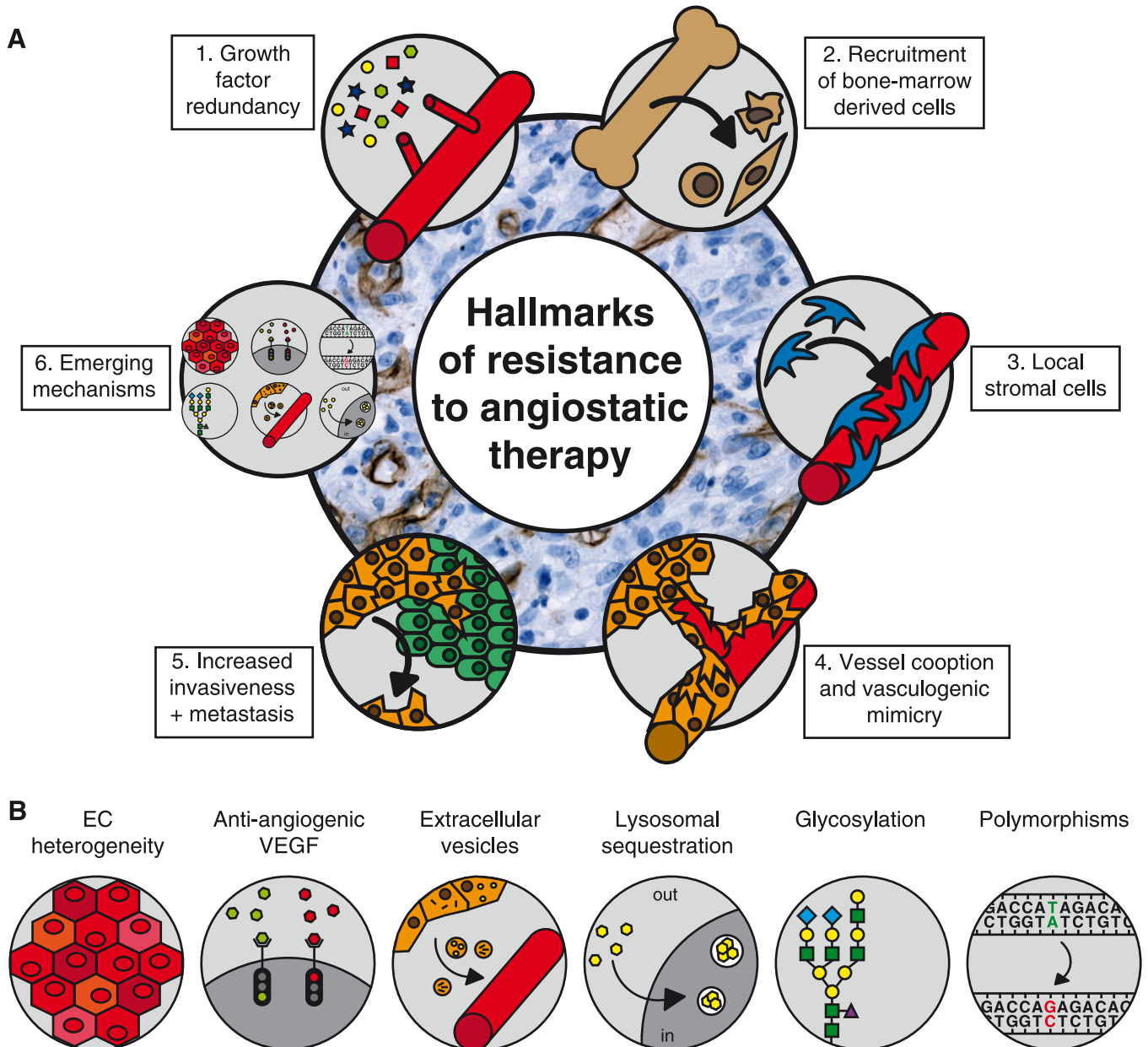


Fig. 1. The hallmarks of resistance to antiangiogenic treatment. (A) Five distinct mechanisms to overcome antiangiogenic treatment can be distinguished. The sixth group (B) comprises a growing number of emerging mechanisms contributing to loss of activity of antiangiogenic drugs.

to induce and facilitate angiogenesis (Thijssen et al., 2013). Other recent additions to the growing list of angiostimulatory growth factors and receptors include angiopoietin-like 7 (Parri et al., 2014), high-mobility group box 1 (van Beijnum et al., 2013), metabotropic glutamate receptor-1 (Speyer et al., 2014), soluble CD93 (Kao et al., 2012), and progestin (Najib et al., 2014).

All these findings extend the angiostimulatory toolbox of tumors and further illustrate the angiogenic potential that resides in malignant tissues. Moreover, the expression of many growth factors can be induced by hypoxia, which occurs as a result of the antiangiogenic therapy (Casanovas et al., 2005; Ebos et al., 2007, 2009; Fischer et al., 2007). Consequently, it can readily be anticipated that targeting a single angiogenic growth factor or its receptor will have only limited therapeutic effect, either because of intrinsic resistance due to redundancy in already activated pathways or because of acquired/evasive resistance by activation of alternative growth factor pathways. Indeed, several lines of evidence show that inhibition of a specific growth factor can induce the expression of others, both in preclinical models as well as in clinical trials. For example, Willett et al. (2005) reported that treatment of rectal cancer patients with the VEGF-targeting antibody bevacizumab significantly increased the plasma levels of PIGF already 12 days after treatment. Induction of PIGF after anti-VEGF therapy was also reported in other clinical studies (Motzer et al., 2006; Rosen et al., 2007). In a phase II study combining FOLFIRI with bevacizumab in metastatic colorectal cancer patients, Kopetz et al. (2010) found that the levels of several angiogenic factors increased before disease progression, including FGF2, PIGF, and hepatocyte growth factor (HGF). A comparable observation, i.e., increased FGF2 and PIGF, was made in glioblastoma patients treated with AZD2171 (cediranib), a pan-VEGF receptor tyrosine kinase inhibitor (Batchelor et al., 2007, 2010). Also in glioblastoma a growth factor-related escape mechanism was identified, i.e., the upregulation of the chemokine receptor CXCR4 on EC after induction of hypoxia (Zagzag et al., 2006). Evidence for this was also found in hepatocellular carcinoma in which both stromal-derived factor 1 (SDF1), also called C-X-C motif ligand 12 (CXCL12), and its receptor CXCR4 are overexpressed in sinusoidal EC, indicating an autocrine SDF1-CXCR4 activation loop (Li et al., 2007). Furthermore, an increase in circulating CXCL12 was linked to disease progression in hepatocellular carcinoma patients treated with sunitinib (Zhu et al., 2009). Other studies reported that increased VEGF and PIGF levels in response to sunitinib even occurred in non-tumor-bearing mice (Ebos et al., 2007; Griffioen et al., 2012). Although the latter suggests a systemic and tumor-independent response, at least after sunitinib treatment, it can still be anticipated that the elevated levels of PIGF play a role in resistance to anti-VEGF

therapy. This is supported by Fischer et al. (2007), who also observed increased PIGF levels in tumor-bearing mice after anti-VEGFR2 treatment.

As expected, acquired resistance is not limited to induction of PIGF. For example, in established tumors in RIP1-Tag2/Rag1^{-/-} mice (a pancreatic cancer model) prolonged treatment with an anti-VEGFR2 antibody induced only a transient tumor growth delay and a modest survival benefit (Casanovas et al., 2005). Although part of this limited response was attributed to vessel cooption (see section II.D.1), further analyses revealed an increased expression of several proangiogenic growth factors, including Ephrins, ANG1, and FGFs (Casanovas et al., 2005). Comparable results were described by Gyanchandani et al. (2013a) who performed whole genome microarray analysis on xenograft head and neck squamous cell carcinoma (HNSCC) tumors that were either responsive or had acquired resistance to anti-VEGF therapy (bevacizumab). They found increased expression of FGF2 and FGFR3 in the resistant tumors (Gyanchandani et al., 2013a). Interestingly, the FGF-mediated acquired resistance of HNSCC was different from intrinsic resistance, because this was linked to increased interleukin (IL)-8 expression. In contrast, tumors with acquired resistance showed reduced IL-8 expression (Gyanchandani et al., 2013a,b). Regarding IL-8 and resistance to anti-VEGF therapy, Batchelor et al. (2013) suggested that elevated IL-8 plasma levels might serve as a biomarker for evasion to treatment with the pan-VEGF TKI cediranib in patients with newly diagnosed glioblastoma (Batchelor et al., 2013). Huang et al. (2010) reported that increased IL-8 levels could serve as a predictive marker for intrinsic resistance to sunitinib treatment in patients with renal cell carcinoma (RCC). On the other hand, in the same study they showed that IL-8 expression was also increased in xenograft RCC tumors that acquired resistance to sunitinib (Huang et al., 2010). Thus, IL-8 might play a role in both intrinsic and acquired resistance to anti-VEGF therapy, but this appears to depend on the type of tumor or type of inhibitor. The increased IL-8 expression in VEGF-therapy resistant tumors has also been linked to a proinflammatory response that indirectly could induce angiogenesis by the recruitment of proangiogenic CD11b⁺ myeloid cells (Carbone et al., 2011). Finally, Cascone et al. (2011) found a role for epidermal growth factor (EGF) and FGF signaling in resistance to bevacizumab in xenograft lung tumor models. Interestingly, different vascular phenotypes were associated with either acquired or intrinsic resistance, with the latter showing clear features of vessel normalization (Cascone et al., 2011). A similar observation was made in Wilms' tumors, i.e., pediatric kidney cancer (Huang et al., 2004). This implies that therapeutic strategies to overcome resistance might be different for intrinsic and acquired resistance.

Not only soluble growth factors are induced by antiangiogenic therapy. The expression of endoglin (CD105), an endothelial-specific coreceptor for TGF- β , was reported to increase after anti-VEGF antibody treatment in a pancreatic cancer model (Bockhorn et al., 2003). Endoglin expression is upregulated during tumor angiogenesis and in proliferating EC (Duff et al., 2003), and it functions in facilitating TGF- β /ALK1 signaling (Lebrin et al., 2004; van Meeteren et al., 2012) as well as VEGF signaling (Liu et al., 2014b). Different studies indicate that endoglin is essential for angiogenesis (Bourdeau et al., 1999; Duff et al., 2003). To date, no clinical evidence is presented on the possible contribution of enhanced endoglin expression in the development of resistance to antiangiogenic therapy. However, it was demonstrated that targeting endoglin in combination with anti-VEGF therapy was more effective than single therapy in vitro (Liu et al., 2014a). In addition, the combination treatment was well tolerated in the clinical setting and even showed effect in VEGF therapy refractory patients (Gordon et al., 2014).

Altogether, current findings clearly show that redundancy in angiostimulatory signaling can underlie both intrinsic and acquired resistance to antiangiogenic therapy. It is also becoming evident that numerous angiogenic growth factors can be involved and that the specific resistance mechanisms depend on the type of cancer and the type of inhibitor.

B. Recruitment of Bone Marrow-Derived Cells

Infiltration of bone marrow-derived cells into the tumor tissue has been linked to tumor growth and angiogenesis (Coussens and Werb, 2002; Mantovani et al., 2008; Crawford and Ferrara, 2009). As described in the previous section, various studies have shown that blocking tumor vascularization by antiangiogenic therapy leads to release of proangiogenic factors, like PlGF, VEGF, ANG1, and FGFs, as well as cytokines such as granulocyte colony-stimulating factor (G-CSF), SDF1, and IL-8 (Casanovas et al., 2005; Ebos et al., 2007, 2009; Fischer et al., 2007). Many of these factors stimulate the recruitment of bone marrow-derived cells into the tumor environment, including monocytes/macrophages, myeloid-derived suppressor cells (MDSC), endothelial progenitor cells (EPC), and cancer-associated fibroblasts (CAF) (Orimo et al., 2005; Grunewald et al., 2006; Kalluri and Zeisberg, 2006; Crawford and Ferrara, 2009; Solinas et al., 2009; Capece et al., 2013). It has become evident that these cells can play a major role in the induction of resistance to antiangiogenic drugs.

1. Myeloid Cells. MDSC, also denoted as Gr1⁺ CD11b⁺ myeloid cells, are a mixed cell population consisting mainly of neutrophils but also of macrophages and dendritic cells with immunosuppressive and tumor promoting capacities (Shojaei and Ferrara, 2008a; Crawford and Ferrara, 2009). In cancer patients and

tumor bearing mice, an excessive production of MDSC has been described (Yang et al., 2004b; Serafini et al., 2006; Marigo et al., 2008; Diaz-Montero et al., 2009). Shojaei et al. (2007a) demonstrated that anti-VEGF treatment refractory tumors have an increased mobilization and infiltration of MDSC into the tumor tissue compared with treatment-sensitive tumors. The same study also showed that MDSC derived from resistant tumors are functionally different from those derived from treatment-sensitive tumors. Furthermore, treatment-resistant MDSC were able to sustain tumor growth in the presence of anti-VEGF antibodies (Shojaei et al., 2007a). An explanation for this could be that resistant tumors increase the expression of G-CSF (Shojaei et al., 2009). The increase in G-CSF leads to induction of Bv8 (prokinectin 2) expression in the bone marrow (Negri et al., 2007), which promotes survival and differentiation of myeloid progenitors. In addition, Bv8 induces the mobilization of progenitor cells from the bone marrow to the peripheral blood and ultimately their infiltration into tumor tissue (Shojaei et al., 2007b; Shojaei et al., 2008). Interestingly, in the MDSC population, neutrophils were found to produce VEGF and Bv8 (LeCouter et al., 2004; Ohki et al., 2005; Shojaei et al., 2008), and it has been suggested that MDSC-derived Bv8 directly promotes tumor angiogenesis, even when the VEGF signaling pathway is blocked (Shojaei et al., 2007b). Recently, a strong expression of Bv8 was also found in neutrophils infiltrating human tumors, supporting the observations made in animal models (Zhong et al., 2009).

In addition to a mediating role for neutrophils, involvement of T helper cells has been postulated to play a role in resistance to antiangiogenic therapy. In a recent preclinical study it was demonstrated that tumor infiltrating T helper type 17 (Th17) cells and interleukin-17 (IL-17) induced G-CSF expression via nuclear factor- κ B and extracellular-related kinase signaling, leading to recruitment of MDSC into the tumor tissue. Inhibition of Th17 cell function rendered previously resistant tumors sensitive to treatment with anti-VEGF antibodies again (Chung et al., 2013). All these observations suggest a role for MDSC in resistance to anti-VEGF therapy.

Other myeloid cells possibly implicated in resistance to antiangiogenic therapy are monocytes and/or macrophages. These cells are recruited to the tumor tissue by different cytokines, including VEGF, chemokine C-C motif ligand 2 [CCL2; also called monocyte chemoattractant protein-1 (MCP1)], and macrophage colony stimulating factor (Solinas et al., 2009; Capece et al., 2013). Tumor-associated macrophages secrete multiple proangiogenic growth factors, including TGF- β , VEGF-A, VEGF-C, epidermal growth factor (EGF), thymidine phosphorylase, and chemokines (CCL2 and CXCL8) (Hotchkiss et al., 2003; Lin et al., 2006; Murdoch et al., 2008; Schmidt and Carmeliet, 2010; Mantovani et al., 2013). In addition, macrophages secrete matrix metalloproteinases

(MMPs), which results in extracellular matrix degradation and release of matrix-sequestered growth factors that can promote angiogenesis and tumor growth (Bergers et al., 2000; Coussens and Werb, 2002; Huang et al., 2002; Mantovani et al., 2002). Macrophages also participate actively in vascular sprouting by functioning as "bridging cells" between two separate tip cells (Fantin et al., 2010; Schmidt and Carmeliet, 2010; Mantovani et al., 2013). From all this, it can be anticipated that macrophages can contribute to resistance to antiangiogenic therapy, but their exact role is still poorly understood. In different murine tumor models anti-VEGF therapy was shown to reduce macrophage infiltration (Salnikov et al., 2006; Dineen et al., 2008; Roland et al., 2009a,b; Lynn et al., 2010). However, because macrophages can be recruited by multiple growth factors and cytokines it can be anticipated that such inhibitory effects are only transient. In addition, the effects might depend on specific macrophage/monocyte subsets. For example, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2)-expressing monocytes/macrophages represent a distinct population that is recruited by hypoxia-inducible and tumor-secreted chemokines, including CXCL12 and ANG2 (De Palma et al., 2005; Murdoch et al., 2007; Venneri et al., 2007; Sica et al., 2012). These cells physically associate with tumor vessels and release proangiogenic growth factors including VEGF (De Palma et al., 2005; De Palma and Naldini, 2011). In preclinical models of mammary carcinoma and insulinoma, inhibition of ANG2 did not block recruitment of TIE2-expressing macrophages but hindered upregulation of their TIE2 receptor, resulting in a reduced production of proangiogenic growth factors and their association with blood vessels (Coffelt et al., 2010; Mazziari et al., 2011; Clarke and Hurwitz, 2013).

This clearly shows that these macrophages contribute to angiogenesis and thus possibly contribute to resistance to antiangiogenic therapy.

2. Endothelial Progenitor Cells. The main chemotactic factors for EPC are VEGF and SDF1 (Orimo et al., 2005; Grunewald et al., 2006; Crawford and Ferrara, 2009), which are released by endothelial cells and tumor cells but also by cancer-associated fibroblasts. Upon stimulation of the chemokine receptor C-X-C chemokine receptor-7 (CXCR7) by SDF1, EPC secrete proangiogenic cytokines and promote angiogenesis (Dai et al., 2011; Yan et al., 2012). Of note, CXCR7-SDF1 signaling was also found to regulate trafficking and homing of angiogenic mononuclear cells into areas of tumor growth and angiogenesis in multiple myeloma (Azab et al., 2014). In addition, EPC can differentiate into EC and incorporate into newly forming blood vessels (Rafii et al., 2002). It has been proposed that antiangiogenic therapy causes hypoxia, leading to activation of hypoxia inducible factor-1 α (HIF1 α) in tumor cells (Bergers and Hanahan, 2008).

Upon activation of HIF1 α , tumor cells secrete SDF1 and VEGF, which then might stimulate mobilization and recruitment of EPC and other bone marrow-derived cells (Ceradini et al., 2004; Du et al., 2008). The actual individual contribution of EPC and their role in resistance to anti angiogenic therapy, however, is still poorly understood and requires further investigation.

C. Local Stromal Cells

It has become clear that local stromal cells, which may also be derived from the bone marrow, can play a role in resistance to angiogenesis inhibitors as well. Two eminent examples of such cells, i.e., pericytes and cancer-associated fibroblasts, are highlighted below.

1. Pericytes. Pericytes—also known as Rouget cells, periendothelial cells, or mural cells—interact with EC and modulate vessel diameter, blood flow, and vessel permeability, regulate endothelial proliferation and differentiation, as well as stabilize the newly formed endothelial tubes (Teicher and Ellis, 2008). In the tumor vasculature, pericytes also support the functionality of blood flow (Shepro and Morel, 1993). Moreover, they protect EC from antiangiogenic therapies and have thus been implicated in clinical resistance to vascular targeting drugs (Bergers and Hanahan, 2008). The mechanism of pericyte recruitment to EC is still poorly understood, but platelet derived growth factor (PDGF) is key in the recruitment of these cells. As described by Abramsson et al. (2003), paracrine cosignaling via PDGF-B and PDGF receptor (PDGFR)- β plays a main role in this process, as well as in blood vessel maturation and stabilization. They showed that PDGF-B contains a special region, the so-called "retention motif," responsible for mediating binding to proteoglycans at the surface of EC that most probably enables the localization of PDGF-B at EC (Abramsson et al., 2003).

Pericytes can also act as EC proliferation suppressors, leading to more pronounced neovessel maturation (Orlidge and D'Amore, 1987). Several studies have shown that pericyte coverage of the microvasculature in the tumor increases after treatment with angiogenesis inhibitors. An early study showed that treatment of tumor bearing mice with recombinant ANG1 leads to a major increase in tumor microvessel pericyte coverage (Stoeltzing et al., 2003). Although ANG1 is known as a growth factor activating TIE2, thereby providing EC with survival signals, it was found that introduction of ANG1 in colorectal tumor cells leads to smaller tumors with less vasculature, suggesting it to work as antiangiogenic therapy. Controversially, this was accompanied by increased vascular pericyte coverage, resulting in protection of EC from this very antiangiogenic therapy (Stoeltzing et al., 2003). A similar observation was described in a more recent study after treatment of tumor bearing mice with antibodies

against ANG2. Although inhibition of tumor growth was achieved—supposedly by antiangiogenic activity judging by the decreased amount of microvessels in these tumors—the tumor vessels were found to be more heavily covered by pericytes (Thomas et al., 2013), suggesting a decreased sensitivity for angiogenesis inhibitors. Furthermore, in combination studies with chemotherapy similar responses were observed. Metronomic topotecan in combination with pazopanib resulted in significant tumor growth inhibition and vascular density reduction in neuroblastoma tumors (Kumar et al., 2013). At the same time an increased number of vessels were covered with pericytes. Similar results were found in a preclinical malignant glioma model after treatment with temozolomide in combination with sunitinib (Czabanka et al., 2013). Interestingly, when used as monotherapy, preoperative sunitinib treatment also caused a reduction in nonpericyte covered tumor blood vessels in renal cell cancer patients (Griffioen et al., 2012), suggesting ongoing resistance induction.

2. Cancer-Associated Fibroblasts. Activated fibroblasts present in the tumor are referred to as CAF (Mueller and Fusenig, 2004; Kalluri and Zeisberg, 2006). CAF are activated by growth factors released from tumor cells and inflammatory cells, including TGF β , PDGF, and FGF (Löhr et al., 2001; De Wever and Mareel, 2003; Mueller and Fusenig, 2004; Turner and Grose, 2010). CAF also secrete several growth factors themselves, such as EGF, HGF, insulin-like growth factor, and FGF, which can influence cancer cell function (reviewed by Bhowmick et al., 2004) or regulate angiogenesis. Indeed, Dong et al. (2004) showed that recruited VEGF-producing CAF can maintain tumor angiogenesis in VEGF-deficient tumor cells. This recruitment was dependent on PDGFR- α signaling. Interestingly, Crawford and Ferrara (2009) found that only CAF isolated from anti-VEGF therapy-resistant EL4 tumors mixed with TIB6 tumor cells (sensitive to anti-VEGF therapy) were able to promote tumor growth when VEGF-signaling was blocked, whereas those isolated from anti-VEGF treatment-sensitive TIB6 tumors were not. In CAF derived from therapy resistant tumors, the expression of various proangiogenesis genes including PDGF-C, angiopoietin-like protein 2 and cyclooxygenase-2 was found to be elevated. Furthermore, treatment of anti-VEGF refractory tumors with a PDGF-C neutralizing antibody could reduce tumor growth (Crawford and Ferrara, 2009), supporting a role for CAF in resistance to antiangiogenic therapy. Another means by which CAF promote tumor growth and angiogenesis is the production of the chemokine SDF1. This factor directly stimulates carcinoma cells but also recruits EPC (Orimo et al., 2005) and other bone marrow-derived cells into the tumor tissue, where they are captured in close proximity to angiogenic blood vessels (Grunewald

et al., 2006). Other than production of growth factors, CAF also produce proteases, including MMPs (Stetler-Stevenson et al., 1993; Sternlicht et al., 1999; Boire et al., 2005), which stimulate the release of matrix-bound proangiogenic growth factors, thereby promoting angiogenesis and possibly resistance to angiogenic therapy.

D. Vessel Co-Option and Vasculogenic Mimicry

Apart from growth factor redundancy and recruitment of different cells that facilitate resistance to therapy, it has also been recognized that tumor cells may escape the activity of angiogenesis inhibitors by adopting different growth patterns (Hillen and Griffioen, 2007). Next to sprouting angiogenesis, new vasculature can be generated by attraction of EPC (Asahara et al., 1997), intussusceptive angiogenesis (Djonov et al., 2000), vessel co-option, and vasculogenic mimicry. The latter two can directly be involved in the induction of drug-induced resistance and will be discussed in more detail here.

1. Vessel Co-Option. It is clear that tumors evolve different strategies to provide in the need for oxygen for efficient outgrowth. One of these is independent of the classic angiogenic switch, occurs in the absence of angiogenic growth factors, and is called vessel co-option. In this process, tumor cells grow along the existing vasculature. It was first described in brain tumors, originating in the exceptionally well vascularized brain parenchyma, but several other cancer types have also shown the capacity for vessel co-option (for an overview of different studies, see Donnem et al., 2013). It is well conceivable that vessel co-opting tumors are not sensitive to angiogenesis inhibitors. A major question is whether this represents intrinsic resistance or whether aggressive angiogenic tumors can revert to vessel co-option in response to antiangiogenic treatment. To study this, it has to be known which molecular regulators underlie the process of vessel co-option and if these regulators are different from the ones inducing angiogenesis. It seems that the major factors regulating vessel co-option are the survival factors VEGF and the angiopoietins. Indeed, several studies report on increased vessel co-option after angiogenesis inhibition, such as the study showing that treatment with the antiangiogenic compound ZD6474 results in sustained cerebral melanoma metastasis growth via vessel co-option (Leenders et al., 2004). Also, anti-VEGF antibody treatment demonstrated increased vessel co-option (Rubenstein et al., 2000). It remains to be seen how general this phenomenon is among the different tumor types and how large the impact is on clinical results of patient survival.

2. Vasculogenic Mimicry. At the end of the previous millennium, a report was published on a phenomenon in uveal melanoma, describing certain tumors to be nonangiogenic while inducing a circulatory system

formed by dedifferentiating tumor cells. These tumor cells were suggested to form vascular-like structures that can transport blood and contribute to oxygenation of the tumor (Maniotis et al., 1999). This phenotype, although rather rare, was described to be heavily associated with poor patient survival. In later years, this process, termed vasculogenic mimicry, was also described in other sarcoma type tumors (van der Schaft et al., 2005; Hillen et al., 2008) as well as in epithelial tumors (Sood et al., 2001; Shirakawa et al., 2002). An emerging observation regarding vasculogenic mimicry is the fact that tumor cells need to dedifferentiate to gain features of EC, such as expression of the endothelial markers VE-cadherin, TIE1, ephrin A2, and the tissue factor pathway inhibitors (Maniotis et al., 1999).

The dedifferentiation of tumor cells into endothelial-like cells may suggest that these tumor cells can be inhibited by antiangiogenic drugs. An early study in the field showed that tumor cells that embark on the mimicry phenomenon do not codevelop sensitivity for angiogenesis inhibitors (van der Schaft et al., 2004). This result was seen as bad news, because this directly suggested that vasculogenic mimicry is a way for tumor cells to escape inhibition of angiogenesis.

Although there is a large body of evidence now on the presence of vasculogenic mimicry and the molecular mechanisms that regulate the process, there is not a lot of research that directly demonstrates vasculogenic mimicry in clinical samples as an escape mechanism from antiangiogenic therapy. In preclinical studies there is evidence for increase in vasculogenic mimicry after antiangiogenic treatment with bevacizumab and indirectly by induction of hypoxia (Sun et al., 2007; Xu et al., 2012), validating the need for anticancer therapy in combination with targeting of vasculogenic tumor cells.

E. Increased Aggressiveness and Metastasis

Paradoxically, therapeutic inhibition of angiogenesis has been associated with increased local invasiveness and distant metastasis despite overall inhibition of tumor growth. The landmark papers by Ebos et al. (2009) and Paez-Ribes et al. (2009) were the first to describe this phenomenon and others followed more recently.

Although increased aggressiveness and spread of tumors has been reported in different preclinical models, the effects seem to vary with treatment type, dosing, and scheduling. Short-term, high-dosage sunitinib treatment seems to have the most deleterious effects. Ebos et al. (2009) showed that treatment with 120 mg/kg per day just before (or after) intravenous breast tumor cell inoculation into severe combined immunodeficient mice increased tumor growth and reduced survival. This was accompanied by pronounced colonization of lungs and livers. Comparable observations were made using sorafenib

and SU10944, and the results were consistent among both xenogenic and syngeneic models (Ebos et al., 2009; Paez-Ribes et al., 2009). However, others reported contrasting results in different additional studies. High-dose sunitinib (120 mg/kg per day) treatment before intravenous inoculation of tumor cells enhanced metastasis of lung tumor cells (4T1) but not of renal tumor cells, despite similar sensitivity *in vitro*. In contrast, 30 and 60 mg/kg per day had no stimulating effects on metastasis formation (Welti et al., 2012).

Apart from increased metastatic potential, treatment with angiogenesis inhibitors has also been found to enhance tumor invasiveness. For example, treatment of spontaneous RIP1-Tag2 insulinomas with the anti-VEGFR2 antibody DC101 resulted in more invasive tumors (Paez-Ribes et al., 2009), suggesting that increased aggressiveness might be a general feature of VEGF signaling blockade. Supporting this notion, tumor-specific VEGFA deletion in the RIP1-Tag2 model mimicked the invasive behavior induced by DC101. However, Singh et al. (2012) observed different effects of sunitinib and anti-VEGF antibody therapy in a panel of mouse tumor models, where sunitinib increased aggressiveness whereas the antibody did not. Similar effects were observed by Chung et al. (2012), who compared different receptor TKIs with antibody therapeutics in a pretreatment model. Only pretreatment with TKIs (sunitinib, sorafenib, imatinib) increased the number of lung nodules after injection of 66c14 cells. Interestingly, anti-VEGFR2 antibody inhibited the number of lung metastasis, whereas anti-VEGF antibody had no effect (Chung et al., 2012). All these findings show that increased metastasis and enhanced invasiveness in response to antiangiogenesis therapy is variable and depends on the tumor model, the type of agent, and its dosing.

Different molecular mechanisms have been associated with the promotion of tumor aggressiveness and metastatic spread. In the RIP1-Tag2 model, in which spontaneous tumors form in the pancreata of mice, defined lesions of different invasiveness can be observed. Injection of pimonidazole to reveal hypoxic regions indicated that hypoxia is increased during antiangiogenic treatment of primary tumors, with a concomitant increase in HIF-1 α expression (Paez-Ribes et al., 2009; Cooke et al., 2012; Maione et al., 2012; Sennino et al., 2012; Rovida et al., 2013). Hypoxia and HIF-1 α are known drivers of epithelial to mesenchymal transition (EMT), a process profoundly implicated in promoting tumor metastasis (Jung et al., 2014). Notably, the expression of several EMT-related genes, such as the master regulators Twist and Snail, as well as the loss of the epithelial marker E-cadherin and the induction of the mesenchymal marker vimentin, have been observed after antiangiogenic treatment (Cooke et al., 2012; Maione

et al., 2012; Sennino et al., 2012). Several studies report on the involvement of c-Met in promoting invasiveness and metastasis in response to antiangiogenic therapy. Although both sunitinib and a VEGF antibody reduced tumor growth in a RIP1-Tag2 model, invasiveness, hypoxia, and EMT markers were increased (Paez-Ribes et al., 2009; Sennino et al., 2012). c-Met and phospho-c-Met expression increased markedly upon treatment; however, its ligand HGF remained constant. In addition, in vitro hypoxia studies revealed a direct effect on c-Met and phospho-c-Met expression (Sennino et al., 2012). A similar regulation was observed in vivo after genetic or pharmacological ablation of NG2⁺ cells (pericytes) (Cooke et al., 2012).

The observed increased invasiveness of glioblastoma multiforme (GBM) after bevacizumab treatment was recently linked to inhibitory actions of VEGF directly (Lu et al., 2012). In a xenograft model of GBM with and without VEGF expression, it was observed that VEGF-deficient tumors progressed more invasively than VEGF expressing lesions, which was accompanied by increased phospho-c-Met levels. Treatment of GBM cells with HGF stimulates phosphorylation of c-Met. Strikingly, this was inhibited by VEGF and inhibition was attenuated using anti-VEGF antibody. It was demonstrated that VEGFR2 and c-Met physically interact and that VEGF induces dephosphorylation of c-Met through the attraction of the phosphatase PTP1B to the complex (Lu et al., 2012). These data provide an alternative explanation for increased invasiveness after antiangiogenic therapy.

In addition to the above-described tumor-intrinsic changes, antiangiogenic treatment can also affect or condition the host to be more permissive for metastatic spread. Vascular changes in sunitinib-treated mice were comprised of reduced basement membrane and pericyte coverage, reduced perfusion, increased leakiness, and decreased adherens junction protein expression (Chung et al., 2012; Maione et al., 2012; Singh et al., 2012; Welti et al., 2012). Because these phenotypic changes occurred both in tumor vessels and in normal organ vessels, systemic action of antiangiogenic treatment can facilitate local intravasation of invasive tumor cells as well as create permissive niches for extravasation of tumor cells in target organs for metastatic colonization distant of the tumor (Welti et al., 2012). Finally, altered cytokine expression in the vasculature as a consequence of antiangiogenic treatment is posed to contribute to facilitate metastasis. By creating a proinflammatory environment and hence attraction of diverse bone marrow-derived cells, a more receptive niche for tumor extravasation is thought to occur (Ebos et al., 2009; Shojaei et al., 2012). Taken together, antiangiogenic agents, most notably those that also target pericytes, cause excessive vascular disruption, leading to hypoxia

with concomitant reprogramming of the tumor cells to a more aggressive phenotype, facilitating blood-borne metastasis and enhancing invasiveness. Consequently, vascular normalization appears to be key to prevent increased invasiveness and metastasis as a consequence of hypoxia.

It is important to note that despite the evidence in preclinical studies, to date no solid evidence is present to substantiate any adverse effects of antiangiogenic treatment on metastasis control in patients (Vasudev and Reynolds, 2014). In fact, this will be difficult to prove and document in clinical practice. One notable exception is a study by de Groot et al. (2010), who described 3 GBM patients who developed more diffuse infiltrative tumors after treatment with bevacizumab.

One important aspect to consider is the resemblance (or lack thereof) in the "natural" course of cancer progression and treatment in a clinical setting versus that in experimental investigations. Most dramatic inductions of metastatic spread were obtained after pretreatment of animals, followed by intravenous injection of tumor cells. However, in human clinical care, antiangiogenic treatment is frequently administered in established metastatic disease. This may affect not only the efficacy of the therapy but also the putative development of resistance. Relating to the studies by Paez-Ribes et al. (2009) and Ebos et al. (2009), neoadjuvant antiangiogenic treatment of solid tumors, or adjuvant therapy after surgery, might increase invasiveness and metastasis, thereby contributing to resistance to therapy.

F. Emerging Mechanisms of Resistance

Apart from the more common and well studied mechanisms described above, several alternative mechanisms of resistance to antiangiogenic therapy have been reported (Fig. 1B).

1. Endothelial Cell Heterogeneity. Endothelial cells in different organs fulfill specialized functions and therefore differ in morphology, gene expression, and function. Tumor EC differ from normal EC in phenotype, gene expression profile, as well as drug response (van Beijnum and Griffioen, 2005; Hida et al., 2013). It was recently shown that tumor EC from tumors with different metastatic capacity also differ in their angiogenic capacity (Matsuda et al., 2010; Ohga et al., 2012). This heterogeneity might be a mere consequence of local tumor growth characteristics and tumor cell–endothelial cell cross-talk through physical interactions and soluble mediators, which may impact therapeutic efficacy. For example, increased expression of multidrug-resistant protein-1 renders tumor EC more refractory to a diverse array of chemotherapeutic drugs (Akiyama et al., 2012). However, although common for tumors, acquired drug resistance as a consequence of cytogenetic aberrations in tumor EC is not fully established (Hida et al., 2004; Akino et al.,

2009). Depending on organ or tumor characteristics, EC may display more mesenchymal properties, referred to as endothelial to mesenchymal transition, which contributes to enhanced angiogenic and invasive capacities (Dudley et al., 2008; Anderberg et al., 2013; Hida et al., 2013). All this could determine the sensitivity of EC to antiangiogenic treatment.

2. Antiangiogenic Vascular Endothelial Growth Factor. VEGF is considered as the major proangiogenic factor driving tumor angiogenesis and is subject to diverse approaches of therapeutic inhibition. Alternative splicing accounts for the generation of different isoforms of VEGFA, such as VEGF121 and VEGF165 (Harper and Bates, 2008). Interestingly, alternative splicing of VEGFA has also been described to generate an antiangiogenic isoform, i.e., VEGF165b, characterized by incorporation of exon 8b instead of 8a (Woolard et al., 2004). The expression of the different isoforms is regulated by growth factors, where insulin-like growth factor 1 and tumor necrosis factor- α favor the 8a isoform and TGF β the 8b isoform (Nowak et al., 2008, 2010). VEGF165b binds VEGFR2 with the same affinity as VEGF165 but does not activate downstream signaling. By binding to bevacizumab, antiangiogenic VEGF isoforms can scavenge the antibody and reduce binding to proangiogenic VEGF. However, the abundance of expression and more so the detection is subject of controversy (Harris et al., 2012; Bates et al., 2013), and further research is required to unravel the role and contribution of alternative VEGF splicing in resistance to antiangiogenic therapy.

3. Extracellular Vesicles. Extracellular vesicles (EV) secreted by tumors have been shown to contain a variety of molecules that can be taken up by stromal cells and vice versa. The content of these vesicles is dictated by the nature of the secreting cell. Apart from containing proteins such as cytokines, EV can also contain mRNA and miRNA. Interestingly, a defined sorting process determined the presence of specific species of mRNA and miRNA, because the RNA content of EV is not a mere reflection of the RNA content of the parental cell (Finn and Searles, 2012). Indeed, selective proangiogenic miRNAs can be present in circulating EV (Skog et al., 2008; Wurdinger et al., 2008; Kosaka et al., 2010), thereby facilitating (distant) outgrowth and counteracting (antiangiogenic) therapy. In a recent study, it was demonstrated that EV isolated from tumor cells with a mesenchymal or EMT phenotype activated recipient EC to a much larger extent than EV from epithelial tumor cells. In addition, activated EC-derived EV were more tumorigenic than normal EC-derived EV, suggesting a positive enforcement of tumor growth and angiogenesis through EV (Pasquier et al., 2014). This way, tumors would have the ability to systemically condition the endothelium to create premetastatic niches by activating multiple signaling pathways through EV secretion. In addition, cytotoxic stress of tumor cells

induced by treatment may enhance the secretion of EV (Lv et al., 2012), thereby further stimulating angiogenesis and metastasis.

4. Lysosomal Sequestration. Vesicles that reside intracellularly have also been implicated in resistance to antiangiogenic therapy. This relates to the sequestration and accumulation of therapeutic compounds in the lysosomes and endocytic vesicles, a phenomenon that has already been described for chemotherapeutics (Selbo et al., 2010; Adar et al., 2012) and photosensitizing compounds (Berg et al., 2010). We recently showed that this also applies to one of the antiangiogenic TKIs, i.e., sunitinib. Sunitinib preferentially accumulates in lysosomes of tumor cells (Gotink et al., 2011) or tumor EC (Nowak-Sliwinska et al., 2015). This process was described to be involved in acquired sunitinib resistance in renal cell cancer patients. Because the sequestration of sunitinib is a transient process, i.e., the drug is gradually removed from the lysosomes and excreted from the cell, resistance to sunitinib disappears over time and patients can become sensitive for therapy again. Although there are still many questions regarding the mechanisms behind this resistance induction, it can be hypothesized that strategies that release the drug from the lysosomes may reinstate sensitivity to sunitinib.

5. Glycosylation-Dependent Resistance. Recent evidence suggests that activation of angiogenic receptor signaling can also occur independent of ligand binding. Croci et al. (2014) reported that galectin-1, a glycan-binding protein, could activate VEGFR2 signaling in the absence of VEGF. This was dependent on altered receptor glycosylation that allowed binding of galectin-1, resulting in increased VEGFR2 clustering and delayed receptor internalization (Croci et al., 2014). This extends the known role of glycosylation in regulating growth factor binding to its cognate receptors (Yayon et al., 1991; Gitay-Goren et al., 1992; Ferreras et al., 2012) and can be exploited to inhibit angiogenesis (van Wijk, 2013). In fact, the galectin-1 permissive glycosylation was associated with resistance to anti-VEGF therapy, and blocking galectin-1 could restore sensitivity to therapy (Croci et al., 2014). This corroborates previous findings by us and others showing that galectin-1 promotes tumor angiogenesis and is a target for antiangiogenic cancer therapy (Rabinovich et al., 2006; Thijssen et al., 2006, 2010; Ito et al., 2011; Croci et al., 2012). Moreover, other members of the galectin protein family are expressed by EC (Thijssen et al., 2008) and have been associated with angiogenesis, including galectin-3 (Nangia-Makker et al., 2000, 2010; Markowska et al., 2010), galectin-8 (Delgado et al., 2011), and galectin-9 (Heusschen et al., 2014; Thijssen and Griffioen, 2014). Similarly, as described by Croci et al. (2014), their angioregulatory activity appears to involve glycan-dependent homo- and heterotypic receptor clustering

as well as increasing the surface retention of receptors (Hsieh et al., 2008; Markowska et al., 2011; D'Haene et al., 2013). Although this has identified galectins as promising targets for antiangiogenic therapy (Thijssen et al., 2007, 2013), the exact role of galectins and altered receptor glycosylation in resistance to antiangiogenic therapy still requires further investigation.

6. Genetic Polymorphisms. Intrinsic sensitivity of tumors to VEGF(R) inhibiting therapeutics may be mediated by genetic variability in genes in the VEGF pathway. Single nucleotide polymorphisms (SNPs) are commonly occurring DNA sequence variations. SNPs can be present in both coding and noncoding (intronic, promoter) regions, and—within coding regions—can be synonymous or nonsynonymous, the latter resulting in an altered protein sequence. Although SNPs in the VEGF gene are in the noncoding region, SNPs in the promoter and 3' and 5' untranslated regions may differentially regulate hypoxia sensitivity; contributing to variable VEGF levels (Scartozzi et al., 2013). An example of a proangiogenic SNP is rs7993418 in the VEGFR1 gene, which results in a shift in codon usage from TAT to TAC. Although both coding for tyrosine, it was demonstrated that this resulted in more efficient protein translation and hence higher VEGFR1 and sVEGFR1 levels (Lambrechts et al., 2012). This way, SNPs can functionally contribute to more angiogenic tumors and hence less efficient therapy.

Although mechanistic explanations of the contribution of polymorphisms to disease progression and/or treatment response are not always available, they are a useful tool to select patients that will maximally benefit from therapy (de Haas et al., 2014) or predict adverse side effects (Lambrechts et al., 2014). Van der Veldt et al. (2011) studied polymorphisms of genes involved in sunitinib pharmacokinetics, and variations in the drug-converting enzyme CYP3A5 and efflux transporter ABCB1 were associated with enhanced progression-free survival in sunitinib-treated patients with metastatic renal cell cancer. In this study, no effects of SNPs in genes involved in pharmacodynamics of sunitinib, including its different targets, were found, whereas others do report a predictive value of VEGF and VEGFR3 (Beuselink et al., 2013; Scartozzi et al., 2013). These studies show the complexity of linking SNPs to resistance to therapy, because multiple SNPs can be involved, exerting their effects on different levels of disease progression. Further research is warranted to get a more comprehensive picture of the role of SNPs in therapy resistance.

III. Overcoming Resistance

It is clear from the above that multiple mechanisms of drug-induced resistance against angiogenesis inhibitors exist. It is also clear that some antiangiogenic treatment strategies are more vulnerable to induction

of resistance than others. It is likely that improvement of antiangiogenic therapy will therefore come from the selection of drugs with a low resistance profile and combination with strategies that prevent or overcome the development of resistance.

A. Counteracting Growth Factor Redundancy

The most obvious way to counteract the resistance due to growth factor redundancy is to target multiple growth factors simultaneously or sequentially. Indeed, Fischer et al. (2007) showed that anti-PIGF treatment effectively inhibited tumor growth in murine tumor models that were resistant to anti-VEGF therapy. It was further suggested that anti-PIGF therapy did not induce an evasive proangiogenic phenotype and showed fewer side effects compared with anti-VEGF therapy. All this identifies PIGF as a growth factor that contributes to evasive resistance and that combination therapy targeted at both VEGF and PIGF signaling might improve therapeutic benefit. Similar beneficial effects have been reported after combining bevacizumab with anti-FGF therapy. For example, bevacizumab combined with the FGFR inhibitor PD173074 completely abolished tumor growth in xenograft HNSCC tumors in mice (Gyanchandani et al., 2013a). Comparable results were found after combining VEGFR2 inhibition with an FGF blockade using a soluble FGF receptor (FGF-trap) (Casanovas et al., 2005). On the other hand, in the latter study this approach still did not completely reduce the tumor vasculature, indicating the involvement of other angiogenic factors as well (Casanovas et al., 2005). Another combination therapy involved blocking of IL-8, which was found to resensitize the resistant xenograft RCC tumors to sunitinib treatment (Huang et al., 2010). Finally, Cascone et al. (2011) showed that acquired as well as intrinsic resistant tumors showed improved progression free survival when bevacizumab was combined with erlotinib (EGFR inhibitor). However, it was also recognized that this might apply to only lung cancer, because a clinical trial combining both treatments in colorectal cancer showed worse outcome (Hecht et al., 2009).

Thus, although combining antiangiogenic agents might improve treatment benefit, it can be anticipated that the numerous alternative pathways will eventually result in acquired resistance. To optimize the selection of the most effective drugs, extensive and patient-specific profiling of angiogenesis signaling pathways is required. Furthermore, combining different antiangiogenic drugs might require adjustment of dosing to increase efficacy while avoiding overt toxicity. Resolving these issues provides the major challenges for future research.

B. Targeting Bone Marrow-Derived Cells

As described previously, the cytokine SDF1 (CXCL12) is the major bone marrow-derived cell (BMDC) recruiting

factor. Targeting the SDF1 pathway could therefore potentially reduce BMDC infiltration and overcome resistance to antiangiogenic therapy, not only in cancer but also in eye diseases. Treatment with a SDF1 neutralizing antibody in a transgenic mouse model of breast cancer showed that infiltration of MDSC and angiogenesis could be inhibited (Liu et al., 2010). In a mouse xenograft model of rhabdomyosarcoma, the anti-human CXCR4 monoclonal antibody (CF172) inhibited metastasis formation (Kashima et al., 2014). Currently, the anti-CXCR4 drug plerixafor (Mozobil; Sanofi, Bridgewater, NJ) and the CXCR4 inhibitor CTCE-9908 are approved for clinical use in patients with leukemia and osteosarcoma, respectively (Burger and Stewart, 2009; Sun et al., 2010). Several other drugs targeting SDF1 and its receptors are in the pipeline (Duda et al., 2011). Other drugs, shown to reduce expression of CXCR4 and responsiveness to SDF1 in MDSC, derived from ascites isolates of ovarian cancer patients, are the selective cyclooxygenase-2 inhibitor celecoxib and agents blocking prostaglandin E2 receptors (Obermajer et al., 2011), leaving perhaps a treatment possibility for good old aspirin. Inhibition of Bv8 (prokinectin 2), which is induced in the bone marrow in response to VEGF blockade and leads to recruitment of MDSC into the tumor tissue, could possibly improve the effect of antiangiogenic therapy as well. A recent study showed that combination therapy of an anti-Bv8 monoclonal antibody and weekly gemcitabine therapy could reduce tumor regrowth, angiogenesis, and metastasis in mice with adenocarcinoma (Hasnis et al., 2014). In addition, in the RIP1-Tag2 insulinoma model of pancreatic cancer it was shown that anti-Bv8 antibodies could block MDSC recruitment and tumor angiogenesis (Shojaei et al., 2008).

Blocking the recruitment of monocytes/macrophages would also be a means to overcome resistance to antiangiogenic therapy. Inhibition of CCL2 (monocyte chemoattractant protein-1, MCP1) has been tried in a phase I clinical study, using a human anti-CCL2 monoclonal antibody (carlumab, CNTO 888) in patients with solid tumors. Targeting of CCL2 led to a transient decrease in free CCL2 and preliminary antitumor activity (Sandhu et al., 2013). Furthermore, dual blockade of ANG2 and VEGFR2 in RIP1-Tag2 pancreatic neuroendocrine tumors resulted in decreased infiltration of TIE2 expressing monocytes and suppressed revascularization and tumor progression (Rigamonti et al., 2014). Macrophages express colony stimulating factor-1 receptor and targeting colony stimulating factor-1 receptor is currently tested in several phase I clinical trials (NCT01346358; NCT01004861; NCT01596751). Treatment of patients with the anti-colony-stimulating factor-1 receptor antibody RG7155 was shown to result in a reduction of macrophage infiltration into tumor tissue and clinical objective responses in diffuse-type giant cell tumor patients (Ries et al., 2014).

Another therapeutic opportunity would be to target MMPs released by BMDC to prevent release of matrix-sequestered growth factors. Unfortunately, most MMP inhibitors have failed in the clinic (Bauvois, 2012). However, a few are still in development, one of which is currently in a phase II clinical trial for Kaposi's sarcoma (Cianfrocca et al., 2011). Another MMP inhibitor has shown some effect in a phase I clinical trial in patients with advanced and refractory solid tumors (Chiappori et al., 2007).

C. Targeting Pericytes and Cancer-Associated Fibroblasts

Targeting both angiogenic endothelium and pericytes seems to be a promising strategy for improved treatment efficacy. Reducing the pericyte coverage of the tumor vasculature has been suggested to be a therapeutic approach in breaking the resistance to and increasing the efficacy of antiangiogenic therapies. Hence, targeting blood vessel maturation may sensitize tumors to VEGF pathway inhibition and prevent or delay the occurrence of resistance. EC secrete PDGF-B that mediates migration and proliferation of pericytes that express platelet-derived growth factor receptor β (PDGFR- β) (Reinmuth et al., 2001). It was already shown that combinatorial targeting of receptor tyrosine kinase selectivity for PDGFRs shows promise for treating multiple stages of tumorigenesis, most notably the often intractable late-stage solid tumor. In the RIP1-Tag2 model it was shown that PDGFRs were expressed only in perivascular cells, suggesting that PDGFR(+) pericytes in tumors present a complementary target to EC for efficacious antiangiogenic therapy (Bergers et al., 2003). However, blocking the PDGF pathway alone is not sufficient to prevent pericyte coverage. Stem cell factor, SDF1 (Stratman et al., 2011), and heparin-binding EGF-like growth factor were shown to have a major role in pericyte behavior as well.

Many studies confirmed that targeting pericytes and EC leads to impaired tumor growth (Bergers et al., 2003), whereas others suggested that such targeting combination does not potentiate treatment outcome. An example of the latter was published by Nisancioglu et al. (2010). In this study, the treatment of Lewis lung carcinoma in the pericyte-deficient PDGF-B (ret/ret) mouse with a specific anti-VEGFA antibody (G6-31; neutralizes both murine and human VEGFA), did not increase the antitumor effect already generated by anti-VEGF drugs. There are also studies showing that pericytes are the gatekeepers against cancer progression and metastasis and that depletion of pericytes, although suppressing tumor growth, can lead to enhanced metastasis formation (Cooke et al., 2012). Similar results were found earlier by Xian et al. (2006). This suggests that an antipericyte strategy should always be combined with other therapies. Combination

with chemotherapy is such an option. In a transgenic mouse model, two TKIs (imatinib and SU11248) were used to block PDGFR-mediated pericyte support of tumor EC in combination with cyclophosphamide treatment (administered at maximum-tolerated or metronomic dose) and/or VEGFR inhibition (Pietras and Hanahan, 2005). Combinations of these therapies were significantly better than the monotherapies, whereas combination of all three approaches resulted in complete responses. On the other hand, as it was demonstrated in neuroblastoma mouse xenograft models, prolonged combination therapy with metronomic topotecan and pazopanib led to sustained antiangiogenic activity but also induced resistance, potentially mediated by elevated glycolysis (Kumar et al., 2013).

Clinical data suggest that low pericyte coverage correlates with a low survival in patients (Stefansson et al., 2006). It is important to realize that other pathways than the VEGF or PDGF axes may contribute to therapy resistance. VEGFR2 blockage may lead to upregulation of ANG1 that is known to increase pericyte coverage of the vasculature (Winkler et al., 2004). ANG1 is expressed by pericytes and delivers a paracrine signal to the endothelium and binds to TIE2, expressed on the EC. This signaling stabilizes mature vasculature and mediates cell–matrix interactions. Other pathways such as sphingosine-1-phosphate (S1P)/edg-1, TGF- β 1/Alk5, or MMPs (Chantrain et al., 2006) should be also be considered while trying to overcome possible resistance associated with pericyte coverage.

Targeting of cancer-associated fibroblasts might further contribute to overcoming resistance to antiangiogenic therapy. A monoclonal antibody against FGF2 (GAL-F2) inhibited tumor growth and angiogenesis of human hepatocellular carcinoma xenografts in nude mice. Furthermore, an additive treatment effect was observed together with an anti-VEGF antibody or the TKI sorafenib (Wang et al., 2012). Addition of a PDGF-C inhibitor to anti-VEGF treatment might also reduce resistance to therapy, as was demonstrated by a study from Crawford and Ferrara (2009). Combined inhibition of VEGFR and FGFR with the TKI brivanib extended progression-free survival in patients with recurrent and persistent endometrial cancer (Powell et al., 2014). Inhibition of the PDGF signaling pathway might also overcome resistance to antiangiogenic therapy, because a study performed by Pietras et al. (2008) showed that blockade of PDGFR- α and - β by imatinib reduced expression of the proangiogenic factors FGF2 and FGF7 in CAF.

D. Antagonizing Vessel Co-Option and Vasculogenic Mimicry

Currently, it is not completely clear whether vessel co-option is a true feature of resistance to antiangiogenic therapy or a mere characteristic of certain types

of tumors. However, in either case, diffusely infiltrating and migrating cells, especially in brain tumors, are responsible for enhanced aggressiveness. Hence, targeting such cells with antimigratory agents might aid in halting the invasive phenotype.

The emergence of vasculogenic mimicry as an alternative vascular system in tumors made researchers realize that angiogenesis inhibition should always be combined with an antitumor cell strategy. However, such transition of tumor cells into a more tumor stem cell–like phenotype is associated with less sensitivity to chemo- and radiation therapy as well, making the design of efficient anticancer strategies a challenge. It was therefore realized that an antitumor strategy based on targeting the vasculogenic mimicry performing tumor cells was required. Several studies have tried to identify the molecular players of vasculogenic mimicry to find ways to specifically intervene in the process. It was found that many of these molecular players are involved in prevention of coagulation, such as the tissue factor pathway inhibitors (TFPI)-1 and -2. Other markers of vasculogenic structures are involved in the plasticity and stem cell-like phenotype of tumor cells. A good example of this is the overexpression of NODAL, a marker of brain development (Hendrix et al., 2001; Topczewska et al., 2006; Paulis et al., 2010; Chen et al., 2014). Recently, CD44 was found to be an overexpressed molecule on vasculogenic tumor cells (unpublished observation). Results of a clinical study with an anti-CD44 antibody for the treatment of solid tumors (NCT01358903), not yet published at this writing, will likely shed light onto the importance of CD44 in the process of vasculogenic mimicry. Targeting CD44 may also benefit cancer therapy through an independent mechanism recently described in renal cell carcinoma (Mikami et al., 2015). Direct targeting of said molecules might provide a therapeutic option as would be the circumvention of hypoxia that contributes to induction of vasculogenic mimicry (Sun et al., 2007; Xu et al., 2012).

E. Overcoming Increased Invasiveness and Metastasis

Increased invasiveness and metastasis as a consequence of angiogenesis inhibition, although currently not proven to have significant implications for patient care, may be a point of concern in designing new (combination) treatments. Diverse preclinical studies implicated the induction of hypoxia and a subsequent reprogramming of tumor cells to more invasive ones (EMT). HIF-1 α , Twist, and c-Met are key molecular players in this process (reviewed by Jung et al., 2014). As such, combination of targeting the VEGF/VEGFR axis and induced compensatory pathways may prove to limit evasive resistance.

Different inhibitors of c-Met have been used in preclinical studies and demonstrated promising effects. Crizotinib, a dual c-Met and ALK inhibitor, was

effective in reverting sunitinib and anti-VEGF antibody induced invasion and metastasis in different preclinical models (Cooke et al., 2012; Sennino et al., 2012; Shojaei et al., 2012). A similar c-Met inhibitor, PF7903, reversed invasiveness and metastatic spread, whereas an inhibitor of both c-Met and VEGFR2, XL184, mimicked the effects of combining anti-VEGF antibody or sunitinib with PF7903 (Sennino et al., 2012). Interestingly, hypoxia was not always inhibited; however, the expression of EMT markers downstream of c-Met, Vimentin, Snail, and *N*-cadherin, was markedly reduced (Cooke et al., 2012; Sennino et al., 2012).

By silencing Twist, the master regulator of EMT (Yang et al., 2004a), as well as c-Met inhibition, metastasis formation in both wild-type and NG2-depleted (pericytes deleted) tumors was almost fully abrogated (Cooke et al., 2012). Thus, although depletion of pericytes creates a metastasis-permissive vasculature, tumor cells must acquire the invasive phenotype associated with EMT to initiate distant colonization. Other molecular players in antiangiogenic treatment-induced invasiveness may be subject of therapeutic interference. Semaphorin 3A (Sema3A) is an endogenous antiangiogenic molecule, although its expression is frequently lost in tumors. Adenoviral Sema3A expression in sunitinib-treated RIP1-Tag2 tumors increased median survival by an impressive 10 weeks and reduced metastasis and hypoxia. Normalization of the tumor vasculature was evident, and the expression of markers of EMT, including c-Met, were reduced. Similar effects of Sema3A were seen in combination with anti-VEGF antibody and in an independent tumor model (Maione et al., 2012). Expression and phosphorylation of Pyk2, a promigratory kinase, is induced in bevacizumab-treated gliomas. Knockdown of Pyk2 or inhibition of its activation by protein phosphatase 1, suppressed bevacizumab-induced glioma invasion (Xu et al., 2014). Other molecular players facilitating (hypoxia-driven) invasion, such as cyclin G2 (Fujimura et al., 2013), Axl, and MMPs (Sennino et al., 2012), may also prove valuable additional therapeutic targets.

Combining chemotherapy with bevacizumab but not with TKIs is common clinical practice. As different cytotoxic agents have different modes of action, their effects in combinations may vary. Rovida et al. (2013) investigated the use of conventional chemotherapeutics to counteract metastasis formation by sunitinib. Gemcitabine and topotecan, but not paclitaxel, cisplatin, and doxorubicin, were effective in reverting sunitinib-induced metastasis formation as well as in reducing primary tumor growth (Rovida et al., 2013). Mechanistically, topotecan was shown to inhibit HIF-1 α accumulation, thereby preventing hypoxia-driven invasiveness. Gemcitabine was moderately effective in combination with anti-VEGF antibody therapy in an

established pancreatic ductal adenocarcinoma model but had no effect in a preventive setting (Singh et al., 2012).

F. Targeting Emerging Mechanisms of Resistance

As described previously, emerging mechanisms of resistance can be considered at an intrinsic level, i.e., the characteristics of the tumor and its vasculature, as well as at the extrinsic level, i.e., the therapeutic activity of a specific agent. To counteract the intrinsic resistance, insight in the molecular make-up of the tumor cells as well as of the cells in the tumor microenvironment can be of assistance. For example, the presence of certain markers on the tumor EC might determine sensitivity to particular agents, analogous to Herceptin, which is only active in Her-2-positive breast tumors. Likewise, efficacy of antiangiogenic agents is likely influenced by endothelial cell heterogeneity as well as by genetic polymorphisms. Furthermore, the cellular phenotype is not static, as exemplified by the change in VEGF receptor glycosylation in response to antiangiogenesis therapy, which alters VEGF sensitivity. A better insight in the mechanisms underlying this intrinsic resistance is thus pivotal. Moreover, regular monitoring of biomarkers in patients to identify these changes is warranted. In this regard, the resistance mechanism involving extracellular vesicles might actually provide therapeutic opportunities as these vesicles—besides secreted molecules—appear representative for the crosstalk between tumor cells and EC. Consequently, such vesicles, which can be retrieved from body fluids such as serum, may help to determine the direction of additional treatment.

Considering resistance at the extrinsic level, the sequestration of drugs in the lysosomal compartment as described above can decrease the sensitivity to drugs. This route of resistance induction was already demonstrated for sunitinib (Gotink et al., 2011). It can be hypothesized that strategies to target lysosomal vesicles after or during drug exposure could revert this resistance. In the case of sunitinib, an approach would be by taking advantage of the fluorescent features of sunitinib, which endow it with a photosensitizer-like activity (Nowak-Sliwinska et al., 2015). Exposure of sunitinib-loaded lysosomes to light of an appropriate wavelength may cause the disruption of the lysosomal membrane and release of active sunitinib into the cytoplasm. We have postulated that the combination of classic sunitinib-induced angiostasis with the re-exposure of tumor cells to sunitinib after the destruction of lysosomes may lead to a clinically applicable strategy (Adar et al., 2012; Nowak-Sliwinska et al., 2015).

Apart from sequestration, drugs can also lose their activity due to variations in drug-converting enzymes, as described for, e.g., sunitinib (van der Veldt et al., 2011). In such cases, patient screening before therapy

and individual dose adjustments might increase effectiveness of the treatment. In addition, development of drugs that are less sensitive to metabolic conversion, or drugs that inhibit the enzymes responsible for the conversion, could be considered to counteract this type of resistance.

IV. Resistance to Antiangiogenic Therapy in Eye Diseases

Pathologic angiogenesis is not restricted to cancer. The field of ophthalmology is extremely interesting in this regard, as angiogenesis inhibition in this area has been more successful than in the oncological arena. Excessive ocular angiogenesis is observed in age-related macular degeneration (AMD) and polypoidal choroidal vasculopathy (PCV) and is the cause of loss of eyesight. Hence, antiangiogenic treatment is successfully applied in these pathologies, in particular with anti-VEGF agents. Nevertheless, responses obtained from therapeutic injections in the eye, e.g., in treatment of exudative AMD with anti-VEGF agents (ranibizumab/Lucentis [Novartis, Basel, Switzerland], bevacizumab/Avastin [Genentech, San Francisco, CA], or aflibercept/Eylea [Bayer AG, Leverkusen, Germany]), are not always durable. Ranibizumab and bevacizumab are both antibody moieties that bind VEGFA selectively. Interestingly, different studies showed that when patients became less sensitive to these agents, switching to an alternative inhibitor, aflibercept, which is a fusion of the VEGF binding domains of VEGFR1 and VEGFR2 with an Fc domain, restored responses in these patients (Bakall et al., 2013). The capacity of aflibercept to not only neutralize VEGFA, but also other VEGF isoforms and PlGF, may explain the improved patient response. More indications of resistance to anti-VEGF treatment in eye diseases came from the results of the SEVEN UP trial summarizing the long term outcomes of ranibizumab-treated patients after initial improvement. At long-term therapy a significant number of patients had regressed with poor visual outcomes (Boyer, 2013). This was the case after monthly treatments (SEVEN UP trial), as well as in the "as needed" treatment in the SECURE trial.

It seems that PDGF inhibitors can also reduce anti-VEGF resistance in noncancerous neovascularization-based disorders like AMD or PCV (reviewed in Nowak-Sliwinska, 2012; Nowak-Sliwinska et al., 2013). The rationale for this combination therapy is, on the one hand, that anti-VEGF therapy increases PDGF expression. On the other hand, anti-PDGF is believed to "strip" pericytes away from the choroidal neovasculature, causing the vasculature to become more susceptible to anti-VEGF therapy and inducing neovascular regression (Jo et al., 2006). A phase II clinical trial with ranibizumab versus ranibizumab

combined with PDGF inhibitor Fovista (Ophthotech Corporation, New York, NY) showed statistically significant responses in the combination group compared with the ranibizumab only group (www.ophtotech.com). Fovista prevents PDGF from binding to its natural receptor on pericytes, thus causing pericytes to be stripped from abnormal neovasculature. When unprotected, the EC are highly exposed to the effects of anti-VEGF treatment. A phase III clinical trial is now underway to prove if gains in visual acuity continue to increase over time.

Growing evidence suggests that S1P modulates exudative-AMD-associated neovascularization, inflammation, and fibrosis. S1P's effects on protection from cell death have been observed in multiple cell types, including fibroblasts, EC, pericytes, and inflammatory cells, all implicated in the pathogenesis of exudative AMD. S1P is also implicated in the activation and production of VEGF, FGF, PDGF, and other growth factors that play a major role in the pathogenesis of choroidal neovascularization and are targets of other choroidal neovascularization therapeutics. iSONEP, the ocular formulation of a humanized mAb against S1P, sonpepcizumab, could deprive fibroblasts, pericytes, endothelial, and immune cells of important growth factors. The ability of sonpepcizumab/iSONEP to neutralize S1P-mediated activation of VEGF and PDGF could prove effective in mitigating macular edema associated with these growth factors (Vinores et al., 2000). Due to the pleiotropic nature of S1P's actions in inflammation, angiogenesis, and fibrosis, it is possible that anti-S1P treatment in wet AMD could have beneficial long-term outcomes, including lesion regression and prevention of pigmented epithelial detachments secondary to exudative AMD or PCV (NCT01334255).

V. Conclusions and Future Perspectives

It is apparent from the above that many tumor and host cell mechanisms can be identified that act individually or in concert to avoid the activity of angiogenesis inhibitors. Variations in the extent that tumors depend on either angiogenesis or alternative vascularization processes have been described. In addition, an increasingly complex picture can be sketched on the interplay of different growth factors, receptors, and cell types, as well as on cellular genetic and proteomic heterogeneity. It is therefore not surprising that resistance to antiangiogenic therapy, most notably to anti-VEGF therapy, has emerged as a clinical burden. The complexity of tumors and their inter- and intrapatient heterogeneity makes it impossible to provide an instant solution to overcome resistance. It is indisputable that improved therapeutic outcome can be reached by carefully designing combinations of therapies. These treatment strategies could involve a combination of multiple antiangiogenic

compounds (Griffioen et al., 2014) or a combination of antiangiogenesis drugs together with other treatment regimens. In particular, care must be taken to not induce additional resistance, such as resulting from excessive hypoxia. Any treatment should also involve a careful patient selection, followed by optimal, intermittent, or sequential dosing and monitoring of treatment efficacy through biomarkers.

It can be foreseen that future therapy will be based on a first step of diagnostic profiling, making use of genomic, transcriptomic, and proteomic techniques, after which the clinician has the disposal of a Swiss army knife-like array of different therapeutic approaches. These different therapeutic entities will be combined

after analysis of the options by system-based models involving various data modeling and algorithm-based strategies (Ding et al., 2014) or computational protocols for dynamic integration of multiple molecular pathway models (Ayyadurai and Dewey, 2011). Such therapeutic combinations should be personalized and matched to the current stage of tumor progression (Fig. 2). Considering the rapid genetic drift of the tumor mass and development of therapy-induced resistance, there is no doubt that it would be highly beneficial to repeat the diagnostic profiling during the course of treatment and adapt the therapy decision-making if necessary.

A new generation of genetically engineered animal models providing a relevant tumor microenvironment

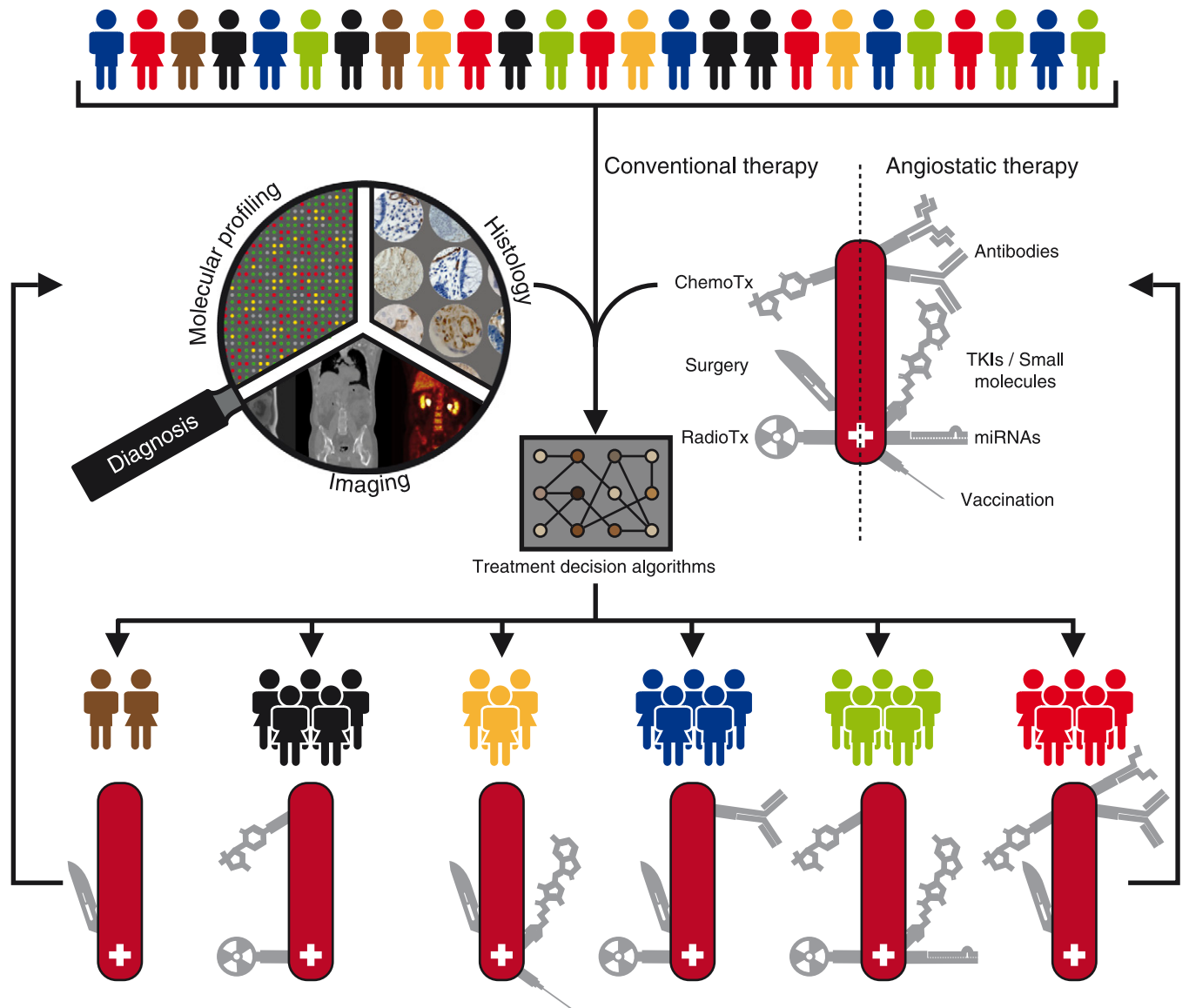


Fig. 2. Schematic representation of how future therapeutic strategies aim to increase clinical benefit and overcome resistance to antiangiogenesis treatment. This strategy relies on combining classic and state-of-the-art diagnostic information with a Swiss army knife of classic and novel antiangiogenic treatment modalities. Matching the diagnostic information with the appropriate combination therapy will involve decision algorithms that are based on insights from preclinical studies as well as clinical trials. Effectiveness of the combination therapy should be monitored during disease progression providing continuous feedback that can be used to adapt and optimize therapy, thereby counteracting or preventing the development of therapy resistance.

and better mimicking human tumor progression will eventually facilitate the design and development of reliable treatment strategies. Such basic and pre-clinical mechanistic studies, in combination with systematic clinical trials and the collection and analysis of patient data, hopefully allows future precision medicine and effective combinations of antiangiogenic and other therapies. This may prevent the early onset of resistance mechanisms or even impede its development.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: van Beijnum, Nowak-Sliwinska, Huijbers, Thijssen, Griffioen.

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