Antiangiogenic resistance: Novel angiogenesis axes uncovered by antiangiogenic therapies research

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Summary. The mechanisms of tumor growth and progression involve the activation of different processes such as neovascularization and angiogenesis. These processes involve tumoral cells and stromal cells. Hence, inhibiting angiogenesis affects tumor growth and proliferation in patients with different types of cancer.
Nevertheless, tumoral cells and stromal components are responsible for the resistance to antiangiogenic therapies. The majority of tumors respond to this type of therapy; however, some tumors may be indifferent to antiangiogenic therapies (intrinsic resistance) and other tumors become resistant during treatment (acquired resistance). Different strategies have been proposed to prevent resistance. Preclinical studies and clinical trials are focused to fight this therapeutic approach in order to prevent or delay tumor resistance to antiangiogenic therapies.

Keywords: Anti-angiogenic therapy · tumor cells · stromal cells · intrinsic resistance · acquired resistance

Angiogenesis in tumor development

The main characteristics of cancer cells is the lack of controlling cellular divisionand are able to grow as neoplastic lesion composed of tumor cells and stroma. Both types of cells structurally and functionally contribute to tumor development. Nevertheless, the neoplastic lesion cannot form a tumor mass beyond a certain limiting size, generally 1–2 mm^3, due to a lack of proper diffusion of oxygen and other essential nutrients. Then, tumors induce blood vessel growth, angiogenesis, by up-regulating the expression and secretion of various pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), angiopoietins (Ang), placental growth factor (PlGF), and some integrins, and concomitantly down-regulating several anti-angiogenic factors [1]. Furthermore, there are evidences that the angiogenic process precedes the formation of the tumor, suggesting that angiogenesis may represent the rate-limiting step not only for tumor growth, but also to the occurrence of malignant tumors [2]. In addition, angiogenesis coincides with increased circulating tumor cells facilitating metastatic spread.

Thus, tumor cells cooperate with other cell types of the tumor microenvironment to achieve the essential feature of angiogenesis. Immune cells, inflammatory cells, hematopoietic cells and stromal fibroblasts contribute to activate endothelial cells of tumor angiogenesis by secretion of various types of inducers [3]. Interestingly, tumors often show an inflammatory phenotype, described by Dvorak in 1986 as “wounds that never heal,” which could tip the balance in favor of angiogenesis and thus promote the formation of new vasculature able to oxygenate and nourish the growing tumor mass.

The imbalance in producing sustained pro-angiogenic factors, together with the persistent lack of vasculature stabilizing factors, leads to the formation of immature and dysfunctional vascular system that cannot keep pace with the rapid growth of the tumor mass. Therefore, the vascular tree in a tumor is typically chaotic with dead-end vascular branches and areas of inverted and intermittent blow flow, which some impairs the vascular function and leads to regions of lowered perfusion and hypoxia. Nevertheless, different types of tumors have low oxygenates areas (hypoxic regions) and present up-regulation of different transcriptions factors such as HIFs and hypoxia-depend genes (Carbonic anhydrase, glucose transporters...) [4].

Different processes such as glycolytic metabolism, oxygen consumption, survival, angiogenesis, migration and invasion could be modulated through HIF-1, nevertheless

their stabilization has an important repercussion in the behavior of the cells and in their gene expression profile [5, 6].

Moreover, hypoxia actively participates in the activation of tumor angiogenesis, this being responsible for regulating the inducers and inhibitors factors that contribute to angiogenesis. It is in fact capable of regulating the expression of molecules that disrupt endothelium and pericyte coverage, as angiopoietin-2, which further contributesto the start of sprouting (developing vascular branches). Furthermore, multiple types of mobilizing stem cells from the bone marrow and the recruitment of immune cells to the tumor microenvironment are positively modulated by tumor hypoxia [7].

Interestingly, recent advances in molecular biology techniques and the study of families with hereditary renal cancer (Syndromes 'Von Hippel-Lindau,' 'Hereditary papillary,' 'Birt-Hogg-Dube' and 'hereditary leiomyomatosis and renal cancer') have permitted the recognition of genes and proteins involved in the pathogenesis of some tumor entities, giving the ability to select the most appropriate therapy for a given disease[8, 9]. In particular, inactivation of the VHL gene (tumor suppressor gene) in patients with RCC involves hyper activation of HIF1α signaling due to lack of degradation even under normoxia, resulting in an accumulation of HIF which promotes transcription its down-stream effectors such as VEGF, GLUT1, TGF-α and PDGF [10, 11]. Therefore, the therapy against VEGF and the usage of inhibiting molecules of the receptor that binds ligand have been used in many types of tumors [12, 13]

Anti-angiogenic strategies

The neoplastic dependence on tumor angiogenesis and the stromal contribution to the formation of new vessels suggested new therapeutic targets to control tumor growth. Recent approaches in cancer therapy are targeting endothelial cells that help tumor growth. These cells are genetically more stable and accumulate less mutations; allowing them to prevent drug resistance. Since in 1971 Judah Folkman proposed the inhibition of angiogenesis as a target for cancer treatment, several anti-angiogenic drugs have been developed mainly targeting endothelial cells. More recently, other cell types in the tumor microenvironment different or together with endothelial cells have been propose as therapeutic target, such as pericytes, which contribute to the maturation of the vasculature sending signals of survival to endothelial cells and structurally supporting the vessel walls [14] (Fig. 1).

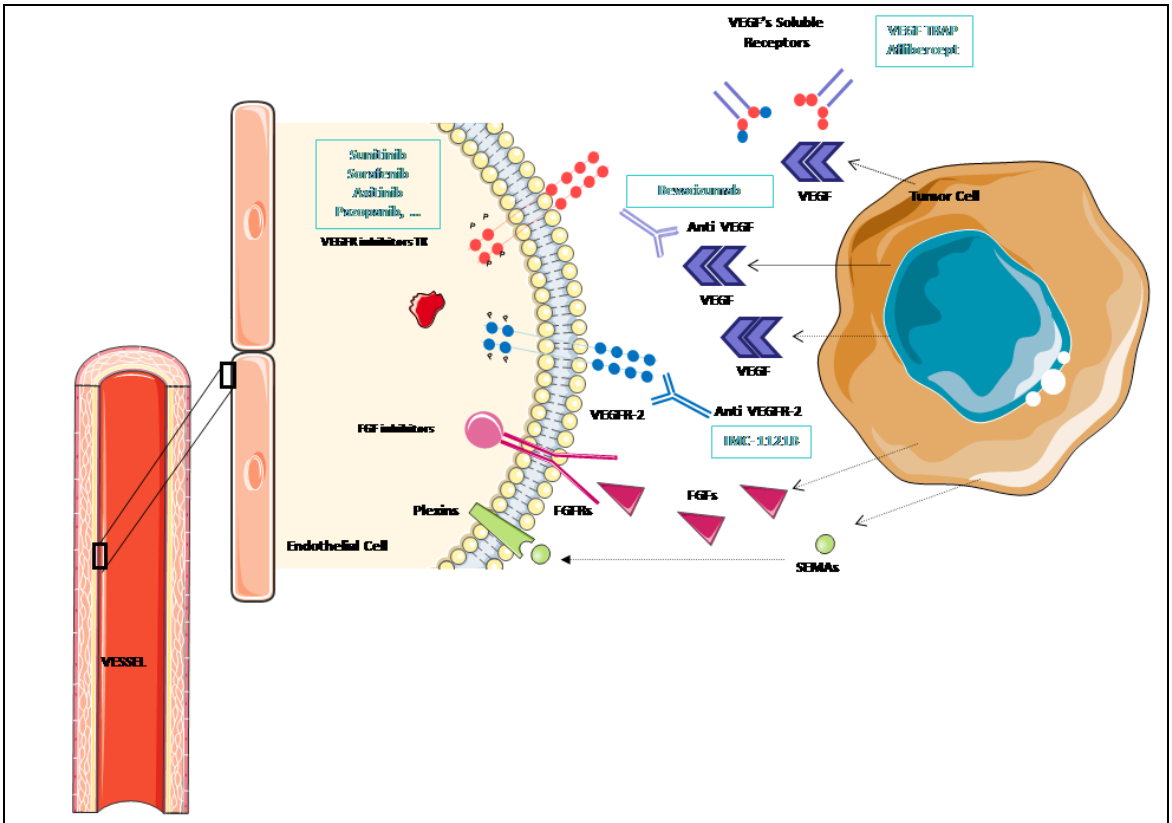

Fig. 1. Angiogenesis as a therapeutic target. The use of angiogenesis as a therapeutic target is the basis of current clinical treatments in several type of tumors. Initially FDA approved the use of blocking VEGF antibody (bevacizumab) in combination with chemotherapy in metastatic colorectal cancer, metastatic breast cancer and other cancers. Later on, chemical inhibitors of VEGF receptor 2 (VEGFR2) as sunitinib or sorafenib have been approved in the first line used as monotherapy for metastatic kidney cancer. In addition, new recombinant molecules such as VEGF TRAP, receptor-blocking antibodies have been developed, and other pro-angiogenic factors (i.e. FGF) or endogenous anti-angiogenic molecules (i.e. SEMAs) have been proposed as possible future anti-angiogenic targets for therapy.

Based on their mechanism of action we classify anti-angiogenic drugs in two groups:

Direct Anti-angiogenic drugs. those that prevent vascular endothelial cells from proliferating, migrating or avoiding cell death in response to a spectrum of pro-angiogenic proteins, including VEGF, FGF, IL-8, platelet-derived growth factor (PDGF) among others.

Indirect Anti-angiogenic drugs. those that secondarily prevent the expression or block the activity of tumor proteins that activate angiogenesis. Their target is a signaling pathway in the tumor cells responsible for synthesis or secretion of pro-angiogenic molecules. The typical example being mTOR inhibitors that target a tumor cell survival pathway and secondarily decrease VEGF expression thus secondarily indirectly exert an anti-angiogenic effect.

In this review we will only cover the direct anti-angiogenic drugs, which are typically directed to inhibit pro-angiogenic signaling pathways. For its role as the main promoter of angiogenesis, vascular endothelial growth factor (VEGF) is the main target of the anti-angiogenic drugs currently approved [1].

VEGF as a prototypical angiogenesis target

Monoclonal Antibodies: they have a direct and indirect action. The direct action is to block the ligand (VEGF) or its receptors (VEGFRs), which blocks its signaling function. The indirect action is mediated by the immune system (complement system activation, cytotoxic lymphocytes and macrophages) and contributes to the destruction of the tumor cell. This class is the first anti-angiogenic drug that demonstrated a clear clinical effect increasing survival in metastatic colorectal cancer. The most well-known example is Bevacizumab, an antibody against human VEGF ligand [15].

Selective inhibitors of kinase activity: compete with ATP for binding to the catalytic domain of the protein, thereby blocking the kinase activity of VEGFRs. These drugs also were initially tested as anti-proliferative agents for tumor endothelial cells, which started the development of a large number of inhibitors that act at different pathways and cell types apart from VEGFRs (promiscuous tyrosine-kinase inhibition profile). Currently sunitinib and sorafenib are the most widely used drugs of this class since they demonstrate the best anti-angiogenic activity [16].

Other novel targets recently proposed

FGF-FGFR inhibitors: the fibroblast growth factor/fibroblast growth factor receptor (FGF/FGFR) signaling axis plays an important role in normal organ, vascular, and skeletal development. Deregulation of FGFR signaling through genetic modification or overexpression of the receptors (or their ligands) has been observed in numerous tumor

as the FGF/FGFR axis also plays a key role in driving tumor angiogenesis. Pre-clinical data shows that inhibition of FGFR signaling can result in anti-proliferative and/or pro-apoptotic effects, both *in vitro* and *in vivo*, thus confirming the validity of the FGF/FGFR axis as a potential therapeutic target [17]. Several drugs agains different pro-angiogenic targets have been developed for their anti-angiogenic effect in pre-clinical and clinical studies [1]

Sema-like ligand: semaphorins (SEMAs) are a superfamily of secreted or membrane-associated glycoproteins implicated in the control of axonal wiring and involved in angiogenesis and cancer progression. The proliferation, cell survival, alteration in cell adhesion and tumor invasiveness can be positively or negatively modulated in tumoral cells by SEMAs [18]. These can also alter cell migration and proliferation in stromal components [19, 20]. Thus, increased expression in Sema3E produced a decrease in tumor burden, neutralizing tumor angiogenesis and moreover increasing the metastatic capacity of tumors.
Casazza et al deeply explored the pleiotropic therapeutic activities associated to an uncleavable Sema3E isoform (Uncl-Sema3E) [18, 21]. SEMAs have antiangiogenic activity and also anti-invasiveness and anti-metastatic effect on the tumor [18]
Endogenous p61-Sema3E isoform binds to PlxnD1 in endothelial cells inducing SEMA-driven antiangiogenic collapsing response.
Furthermore, in tumor cells, the Uncl-Sema3E-PlxnD1complex fails to elicit the ErbB2-mediated pro-invasive and pro-metastatic pathway [18]
With these results they proposed Uncl-Sema3E as a novel anti-angiogenic and anti-metastatic therapeutic approach.

Angiopoietin2 inhibition*:* the angiopoietins are proteingrowth factors that promote angiogenesis and help stabilize the development of blood vessels from pre-existing blood vessels. Ang1 and Ang2 are required for the formation of mature blood vessels, as demonstrated by mouse knock out studies [22]. Moreover, Ang2 is critically associated tumor angiogenesis and progression. It has been described that Ang2 regulates tumor angiogenesis in cooperation with VEGF as well as Ang1 through the Tie2-dependent pathways. On the other hand, Ang2 stimulates tumor angiogenesis, invasion, and metastasis through Tie2-independent pathways involving integrin-

mediated signaling.Therefore, Ang2 is currently an attractive therapeutic target, as it has been corroborated by recent studies using a neutralizing anti-Ang2 antibody [23].

CLINICAL CONTROVERSIAL RESULTS

Pre-clinical studies often report positive results about the benefit of anti-angiogenic treatment, but clinical trials' results vary depending on the cancer type and anti-angiogenic therapy used. Phase III studies have indeed shown the benefits of Bevacizumab or Sunitinib as well as other VEGF-targeted therapies, either as single agents or in combination with chemotherapy. Blocking the formation of new blood vessels with anti-angiogenic therapy is currently used to treat certain types of cancers, including metastatic renal cancer [12, 13]. Therefore, the metastatic renal cell carcinoma which is characterized by being dependent on VEGF growth is controlled by the antiangiogenic therapy, confirming the positive effect of this kind of therapy and supported by various clinical trials [24-27]. Nevertheless, many authors coincide in the observation that anti-angiogenic treatments are more effective in the increase of theprogression free survival (PFS) than on the prolongation of overall survival (OS). However, based on obvious clinical benefits with a remarkable increase in PFS although in the absence of robust statistically significant increase in OS, VEGF pathway inhibitors are the mainstay of therapy in RCC approved by FDA [28-30]. This discrepancy between PFS and OS feeds the controversy of how to best measure clinical benefit of treatment, because anti-angiogenic therapies typically exert an effect in terms of increased necrosis as observed by imaging studies. As mentioned before, antiangiogenic treatments have an effect on cavitation and loss of viable tumor burden, causing an impact in tumor growth with no alteration in the parameters of RECIST (Response Evaluation Criteria in Solid Tumors) [31, 32].

Tumor development is deeply affected by tumor type and induces modifications on the formation of tumors; in particular by their own angiogenic characteristics and their pro-angiogenic capacity coming from tumor-stroma specific interaction.

The inactivation of VHL tumor suppressor is highly frequent in RCC [33], for that reason angiogenesis is presumably highly dependent on VEGF. Similar to hepatocellular carcinoma (HCC) that are particularly angiogenic when growing in liver displacing the normal parenchyma, moreover the dependence on angiogenesis is presumed to be the key for the efficacy of antiangiogenic therapy. On the contrary, colon-rectal cancer (CRC) shows considerably less clinical benefits and VEGF-targeted

therapy is therefore administered in combination with chemotherapy. On the other hand metastatic foci of CRC, typically growing in the liver, often replaces the liver parenchyma, rather than displacing it, by the FAS ligand-induced death in the hepatocytes. This leads to the co-option of existing blood vessels instead of dependence on sprouting angiogenesis [30, 31, 34].

The adaptability of the tumors to classical chemo-therapy and radiation emerges also for anti-angiogenic therapy [35, 36]. Thus, anti-angiogenic therapies have proven to be beneficial in many patients, but these clinical benefits are overshadowed by apparent acquired resistance to anti-angiogenic therapies. Moreover, some patients don´t respond to these therapies at all demonstrating upfront refractoriness to therapy or intrinsic resistance.

Resistance to antiangiogenic therapy

The initial assumption was that antiangiogenic therapy does not cause resistance, because it was specific against endothelial cells that showed no genetic instability [37]. However, experimental and clinical evidence has shown that the benefit of this therapy have been mild and transitory [13].

The majority of tumors respond to therapy but it is important to differentiate between refractoriness, intrinsic or acquired resistance [31].

Intrinsic resistance (IR) to anti-angiogenic therapy. In this type of resistance the tumor becomes indifferent to antiangiogenic therapy and there is no response to treatment (Figure 2). Some patients treated with Bevacizumab, Sorafenib, and Sunitinib developed this type of resistance [38, 39].

It has been shown that tumors are capable of expressing from the beginning of its progression multiple pro-angiogenic factors, so that anti-VEGF therapy is not fully effective, as it is only able to partially block the process of angiogenesis [40]. Another molecular mechanism that may be involved in the intrinsic resistance is the de-regulation of the HIF pathway. In the tumors with activation of HIF, such as renal tumors, are consistently found high levels of genes of pro-angiogenic molecules controlled by this factor, thereby reducing the effect of anti-angiogenic therapy [12, 13]. Other mechanisms could be independence from angiogenesis process that have a role in tumor revascularization, including sprouting, co-option of pre-existing vessels, vasculogenic mimicry, mosaic vessels, and mobilization of latent vessels [41].

FIGURE 2

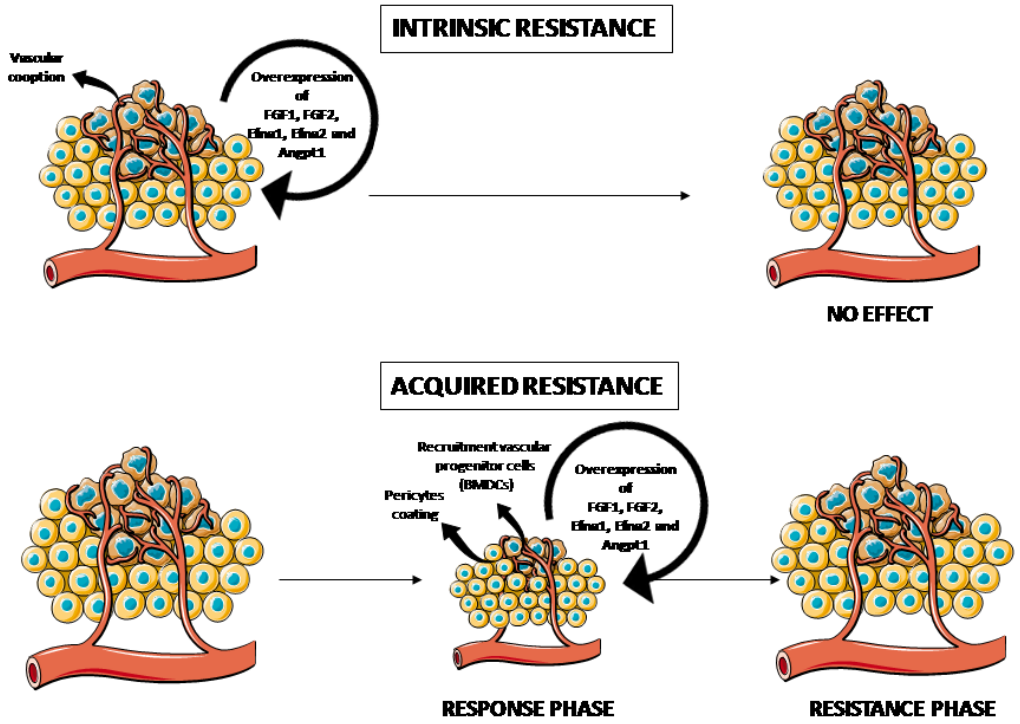

Fig. 2. Modes of resistance to anti-angiogenic therapy. Two types of resistance to anti-angiogenic therapies have been described: Intrinsic Resistance or refractoriness defined as total lack of response to antiangiogenic therapy. The specific mechanisms of resistance of this type include the multiplicity of pro-angiogenic factors produced by tumor or stromal cells within tumor mass or vascular co-option. The therapy is unable to reduce or stabilize tumors and there is no beneficial effect of anti-angiogenic therapy. Acquired Resistance refers to adaptive capacity presented by tumors leading to evade the therapeutic blockage after a phase of effectiveness. Induced adaptive mechanisms, including overexpression of pro-angiogenic factors, recruitment of vascular progenitor cells (BMDCs) and increase pericytes coverage. Altogether, they allow for revascularization despite therapeutic inhibition, allowing for tumor regrowth and the progression of the disease.

Could the differential angiogenic features of each tumor have a repercussion in their upfront sensitivity or resistance to anti-angiogenic therapy? Interestingly in astrocytomas, a class of highly oxygen dependent brain tumors, their development is mediated by changes in the way of tumors acquires their supply through blood vessels. .

Thus low-grade astrocytomas grow coopting pre-existing normal brain vessels whereas progressing from grade III to grade IV, so called glioblastoma multiforme (GBM), an enhanced request of oxygen and nutrients activates an angiogenic program [42].

Bevacizumab was approved by the United States Food and Drug Administration (FDA) for the treatment of recurrent GBM based on several studies demonstrating efficacy in terms of increased PFS and OS in combination with conventional chemotherapy [30].

Unfortunately, tumor resistance occurs with new distant foci of progression or diffuse in-situ infiltration associated or not with local tumor recurrence as shown by fluid attenuated inversion recovery (FLAIR) and magnetic resonance imaging (MRI) analysis [30, 43, 44].

Acquired resistance (AR) to anti-angiogenic therapy. In addition to the traditional resistance of some drugs, which is acquired by mutations that affect the target of drugs or alterations of entry mechanisms of the compound [45] the AR resistance to anti-angiogenic therapies is more indirect and evasive. Typically, alternative mechanisms are created that lead to activation of angiogenesis even when the target of the drug remains inhibited [46]. Tumors have long been shown to have remarkable plasticity and adaptability to classical chemotherapy and radiation, which contributes to resistance to anti-angiogenic therapy [3, 47, 48]. However, the specific mechanisms of acquired resistance to anti-angiogenic therapies are unique, and many of these mechanisms show reversibility after anti-angiogenic therapy has been stopped (Paez-Ribes and Casanovas, unpublished observations). Indicating that these types of resistance could reflect the adaptations to therapy instead of mutations or gene amplifications characterizing acquired resistance to other therapeutic strategies. In fact, clinical evidence of this reversibility has been described in metastatic renal cell carcinoma treated repeatedly with VEGFR inhibitors [13, 18].

Several different mechanisms of acquired resistance to anti-angiogenic therapy have been described among which are (Fig. 2):

- Overexpression of alternative pro-angiogenic factors*:* initially be described in pre-clinical a transgenic mouse model of neuroendocrine tumors (RIP-Tag2). After receiving anti-VEGFR2 therapy there is a reduction of angiogenesis followed by initial tumor regrowth reinduction induced angiogenesis. This reinduction is promoted by overexpression of pro-angiogenic factors VEGF-

independent, as fibroblast growth factor 1 (FGF1), FGF2, ephrin A1 (EFNA1), EFNA2, and angiopoietin1 (ANGPT1). [36, 49].

- Recruitment of stromal pro-angiogenic cells: hypoxic conditions induced by anti-angiogenic treatment promote the recruitment of large numbers of cells derived from bone marrow (BMDCs) at the boundaries of the tumor. These cells have the ability to promote tumor revascularization [50]
- Vessel coverage by pericytes: preexisting tumor vessels that have a high number of pericytes surface coverage remain functional and exhibit no regression [2, 51-53]. This suggests that endothelial cells have the ability to recruit pericytes, which are able to secrete VEGF and other factors promoting their survival [2, 54, 55].
- Vascular mimicry: defined as the formation of microvascular channels by the aggressive tumor cells themselves, which would allow the transport of oxygen and nutrients [41].

Interestingly, there are some parallelism among the mechanisms that lead to IR and AR. The difference lies in the intrinsic characteristics of each tumor as tumors with AR require some time in order to generate these molecular changes and become resistant to this therapy, whereas tumors with IR are immune to this therapy since from the beginning have over expression of these factors. Furthermore, resistance to anti-angiogenic therapies for cancer implicates tumor cells and stromal components, but its contribution is relatively different in each cancer subtype.

One crucial step for the development of the neoplastic lesion is the interaction between tumor cells and tumor microenvironments; moreover the tumor-stromal cell collaboration is also involved in tumor responses to therapeutic inhibition of VEGF-pathway [30].

However, tumor and stroma cells contribute to the inefficacy of the therapy in tumors that present intrinsic resistance similar to acquired resistance tumors

Most of the tumors present different mechanisms of resistance that depend on cells which involve the modification of the stroma components across the modification of the stroma as the recruitment of infiltrating cells, such as cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs), or the production of alternative pro-angiogenic factors [30]. One of the main modifications induced by anti-angiogenic treatment in tumors is the increase of hypoxia and HIF-1 stabilization. Interestingly,

neoplastic cells could react to hypoxia becoming tolerant and modifying the metabolic characteristics to resist to low levels of oxygen. Alternatively, tumor cells could engage in an escape from hypoxic environment alone or sustained by their stromal neighbors [30].

A perspective

Hence, approaching antiangiogenic resistance is a key step in the generation of novel antiangiogenic drugs. A number of strategies have been postulated to prevent resistance, targeting multi-pathway inhibitors or multi-combination of anti-angiogenic therapies that inhibit different pathways that could avoid resistance. Moreover, the plasticity to the treatments observed in pre-clinical studies suggest a new therapeutic hypothesis that sequential treatment with an anti-angiogenic drug followed by a non-anti-angiogenic drug (i.e. another targeted therapy or chemotherapy) could resensitize patients to another anti-angiogenic drug as a third line of treatment. Obviously, many studies are warranted to unravel the pre-clinical basis and clinical potential of these strategies to finally determine its clinical benefit for patients.

Furthermore, the implication of stroma in the emergence of resistance to anti-angiogenic therapies definitively has important clinical implications. First of all it reveals another relevant culprit causing the short-lasting effects of anti-angiogenic in neoplastic patients, thus alleviating the exclusive fault of tumor cells. Secondarily, it opens innovative perspectives for the prevention of resistance and pro-invasive effects of anti-angiogenic therapy, such as the modulation of the emerging pro-metastatic stroma. Therefore, as both tumor cells, stroma and their interactions initiate tumorigenesis, sustain neoplastic growth, and allow for metastatization and therapeutic resistance, these two neoplastic partners should be considered in the development of new therapeutic approaches. In this sense, clinical studies that investigate and address these approaches in the coming years are warranted.

Acknowledgement. The authors would like to thank Lidia Moserle for critical reading of the manuscript and helpful suggestions. The authors' work is supported by research grants from EU-FP7 (ERC-StG-281830), and MinEco-Spain (SAF2012-36575) and AGAUR-Generalitat (SGR681). The authors declare that no conflict of interest exists.

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