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Molecular Mechanisms of Tumor Angiogenesis

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1. Introduction

It is well established that progression from a pre-malignant to malignant invasive tumor phenotype is dependent on angiogenesis[1-7]. As such, a hallmark of all solid cancers is their ability to induce the formation of their own blood supply thereby sustaining their growth and is characterized by increases in endothelial cell (EC) proliferation and blood vessel heterogeneity[8-10]. The 'angiogenic switch', is a complex balance of multiple pro- and anti-angiogenic factors secreted by both host and tumor cells, which when balanced in favor of pro-angiogenic factors will trigger new vessel formation (**Figure 1)**[1-7]. The expression of these pro- and anti- angiogenic regulators is dependent on various physiological and pathological factors in addition to the tumor type, stage and microenvironment[2,8]. It has been shown previously that although tumor angiogenesis to some extent recapitulates the normal process of angiogenesis, it is not well organized and leads to the majority of solid cancers having tortuous and dilated vessels that have abnormal physiological function. This commonly leads to insufficient blood flow, poor delivery of oxygen and nutrients, inadequate removal of waste, increased vessel permeability and tumor edema due to alteration in EC tight junctions and contacts[2,11-15]. These inefficiencies in blood flow result in changes within tumor microenvironment that can trigger further expression of a battery of angiogenic factors, setting up a continuous cycle of dysfunctional vessel formation[1,2,8,9]. The precise mechanisms governing expression of pro- or anti-angiogenic factors are still not fully understood. In response to oncogene activation and/or metabolic stress, such as that seen in solid tumors, tumor cells can directly secrete growth factors including vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1) stimulating the angiogenic switch to enhance vessel formation[17-23] (**Figure 1**), or attracting macrophages that can indirectly promote release of angiogenic factors[10]. Multiple candidate factors that signal tumor cells to initiate this cascade have been proposed[1,2,11-14]. The main contributors include hypoxia and increased physical forces, both generated in rapidly growing tumors, that disrupt the EC connections within the extracellular matrix (ECM), and the products of oncogenes and mutated tumor suppressor genes[1,2,11-14]. The relative contribution of various angiogenic pathways, their interactions and combinatorial impact on tumor angiogenesis is not precisely known and requires further characterization in order to establish a comprehensive picture of tumor angiogenesis.

Principles of anti-angiogenic therapy

Originally, the knowledge that tumor angiogenesis was vital for solid tumor growth held much promise for designing efficacious treatments for cancer, with the hope of arresting tumor growth and progression, therefore maintaining a patients health in a stable

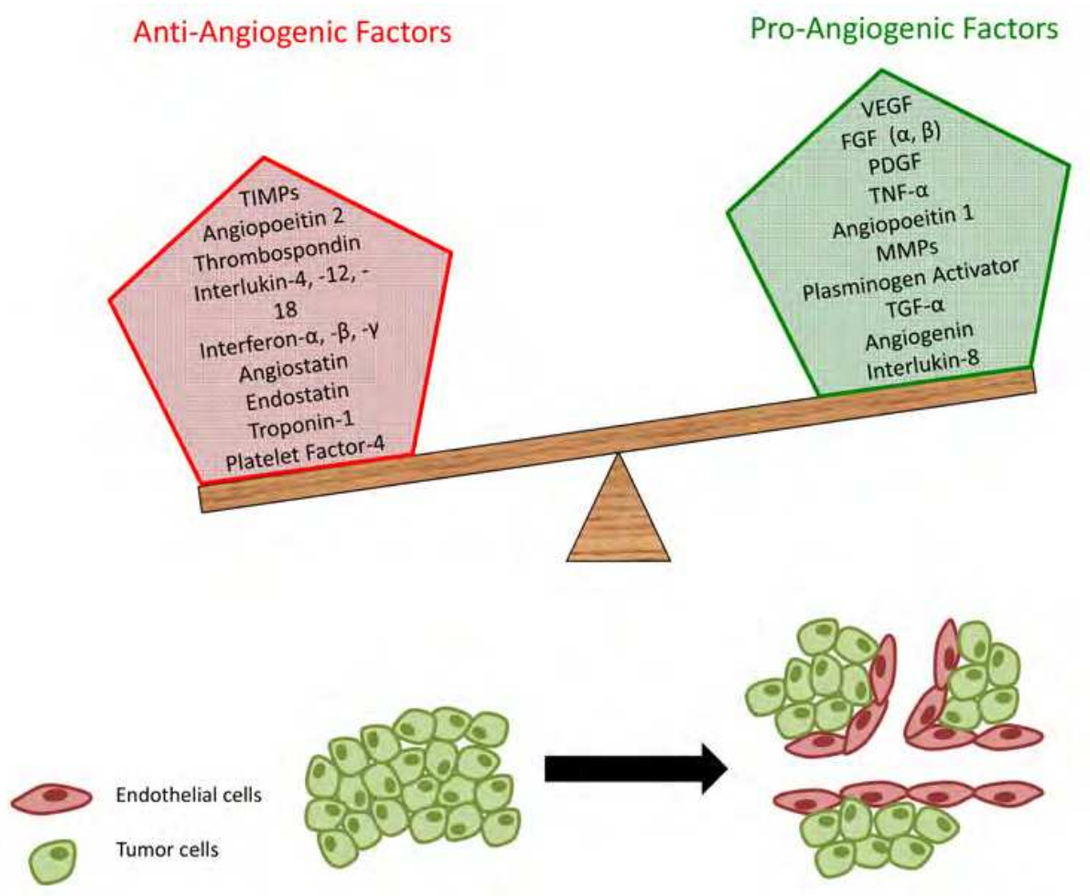

Fig. 1. The Angiogenic Switch
A complex balance between pro- and anti- angiogenic factors exists in all microenvironments and is instrumental in the enhancement or decrease in vessel formation. The main angiogenic factors that have so far been elucidated are listed, not all of their mechanisms are fully understood.

asymptomatic state. Anti-angiogenic cytostatic agents were thought to have several advantages over the traditional cytotoxic chemotherapeutic agents[7,13,15-18]. First, it was hypothesized that regardless of the extent of tumor heterogeneity, tumor angiogenesis is a non-neoplastic homogenous process; hence anti-angiogenic strategies would be efficacious against a variety of human solid cancers. Second, the issue of resistance to chemo- or radiation therapy (CT or RT) of tumor cells would not apply to the angiogenic component of a solid cancer. Third, the vascular compartment is readily accessible and no interstitial pressure would be required to reach the targeted ECs. Fourth, the presence of up-regulated and altered EC receptors in tumor vasculature, would permit specific targeting of therapeutic molecules to tumor vasculature, while normal blood vessels would not be targeted.

Overall the results of anti-angiogenic based clinical trials, however, have been somewhat disappointing[12,19]. With increased understanding and experience using anti-angiogenic treatment, certain limitations and causes for failure of anti-angiogenic therapy have come to light [8,12,19]. One of the principle realizations being that the process of tumor angiogenesis is as heterogeneous as tumor cells, and the dynamics of tumor vessel biology alters with

tumor type, tumor stage and phase of tumor growth. Other reasons are that these trials have either targeted only one angiogenic pathway, or in several instances the exact anti-angiogenic mechanism is not known. We therefore need to expand our knowledge of the qualitative differences in tumor vessel formation that are specific to each tumor and individualize the therapeutic approach. We also need a more detailed understanding of the relevant angiogenic regulators in specific tumors, which differ according to tumor microenvironment, in order to generate target specific agents for testing in clinical trials

Additional reasons why there has been some disappointment in the efficacy of translation of pre-clinical results to clinical trials include: (1) a lack of appropriate pre-clinical tumor models. To date, primarily xenograft models have been used where tumors are grown in an ectopic microenvironment in an immune deficient mouse. (2) There has been a lack of end-points of treatment and surrogate markers of response to anti-angiogenic therapy. To date, the primary method for establishing response has been measurement of tumor size in xenograft models, extent of EC apoptosis, number of EC progenitor cell (EPC) circulators and changes in EC signaling. Dynamic imaging of tumor characteristics in response to treatment would be of significant clinical and translational value. The future direction of tumor angiogenesis and anti-angiogenic therapy will focus on designing small animal imaging modalities that will best identify the extent of effective and functional blood flow within a tumor vascular network and the impact of treatment on the dynamic blood supply of a tumor. By establishing these methodologies we can then translate end-points of therapeutic response to drug more accurately that can be applied to clinical therapy.

An important evolution in anti-angiogenic therapy is the use of combinatorial therapy. Combinatorial therapy takes advantage of using anti-angiogenic strategies together with RT in order to improve the clinical efficacy of both treatment modalities. The principle of combinatorial therapy in large part relies on the concept of 'vascular normalization', which was introduced and popularized primarily by Rakesh Jain over the past decade. Vascular normalization states that abnormal pathological tumor vasculature, in response to anti-angiogenic therapy, becomes normalized and results in a window of opportunity during which more efficient tumor blood flow can be delivered to the tumor, improving delivery of tumor oxygenation and therapeutics, and ultimately response to RT [20-22]. Improvement in tissue oxygenation in particular the centre of tumor increases the functionality of RT ad CT in the tumor.

The precise timing of this window of opportunity and extent by which 'vascular normalization' improves response to RT however remains unknown. There are some pre-clinical studies and early clinical trials that have explored the therapeutic benefits of combining anti-angiogenesis with RT, but results have been inconsistent[34,37-48] and the optimal scheduling of these adjuvant treatments has to be established. Moreover, individual tumor type plays a significant role in determining the correct combinatorial scheduling and very few studies have focused on brain tumors.

1.1 Normal vessel formation: Vasculogenesis and angiogenesis

Our understanding of the molecular mechanisms of tumor angiogenesis heavily relies and derives from studies of the mechanisms of normal vessel development. Vascularization is critical for embryonal development and normal physiological functions in large multi-cellular organisms. It is also pivotal for the progression of a multitude of pathological processes.[6,7,23-27]. The primitive vascular network is modified by the process of angiogenesis, leading to maturation, branching and formation of a complex vascular network[6,7,9,23,28].

During physiological angiogenesis new vessels are formed from pre-existing ones via sprouting and non-sprouting mechanisms[6,7,9,23,28] concomitant with an increased interaction between EC and the pericytes (PC) and smooth muscle cells (SMC) of the ECM, creating a stabilized vascular network (**Figure 2 and 3**)[9,28]. The main organs that are vascularized primarily by angiogenesis are mesodermal organs such as the kidney and brain that do not contain angioblasts[6,7,9,23,28]. In addition to maturation of the vasculature into a complex network, determination of vessel fate is also a crucial aspect of normal vessel development[29,30]. Vessel fate was originally thought to be regulated by hemodynamic factors such as blood flow, extent of blood oxygenation, blood pressure and alteration in other blood and microenvironmental characteristics [29,30]. However, recent evidence suggests that it is primarily governed by genetic regulation and cytokines such as Ephrins and the Notch signaling pathway [29,30]. Normal angiogenesis is vital for wound healing and for the development of the endometrium during the uterine cycle. Angiogenesis is prevelant in pathological conditions such as tumor growth, ischemia, vascular malformations and inflammatory reactions however, the process is particularly abberant leading to the

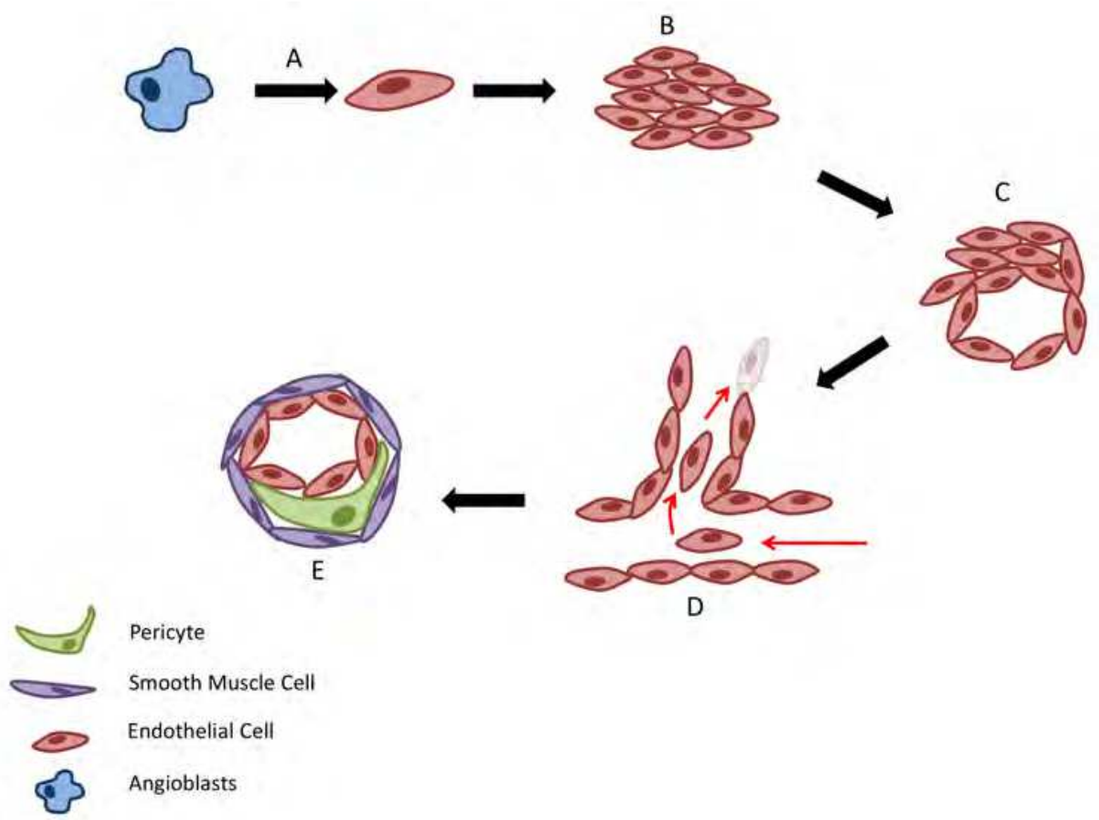

Fig. 2. The process of Physiological New Vessel Formation
A. Angioblasts differentiate to form endothelial cells ***B.*** Endothelial cells proliferate to form blood islands ***C.*** Blood islands coalesce to form hollow lumen vessels or primitive vascular tubes that subsequently form a primary vascular network ***D.*** Maturation of the vessel leads to new vessels sprouting through migration and proliferation of ECs with reassembly into new lumens ***E.*** The last step in the process of angiogenesis is stabilization of the vessels through recruitment of PC and SMC surrounding the vessel.

torturous heterogeneic vessels that have come to be associated with tumor vascular networks[19,31]. In some disease processes such as cerebral ischemia the angiogenic response is deficient, while in other disease processes, such as tumor angiogenesis or vascular malformation, there is excessive angiogenesis resulting in vessels that are abnormal in structure and function[19,31].

An alternative pathway for vessel formation is vasculogenesis, a process whereby mesenchymal progenitors migrate from the bone marrow (BM) and differentiate into ECs[6]. In turn, mesenchymal derived ECs then proliferate to form a *de-novo* primitive vascular network in an a vascular tissue in the process of in-situ angiogenesis (**Figure 3 and 4)**[6,7,23]. The main organs that are vascularized by means of vasculogenesis include the heart, great vessels, spleen and other endodermal organs[6,7,23]. Vasculogenesis has long been thought of as a pre-natal stage vessel formation occurring exclusively in the developing embryo. However, recent data has speculated that neovascularization in adult life, both in pathological and physiological conditions, can also occur by vasculogenesis[58-61], and is particularly prevalent in large-solid cancers[62].

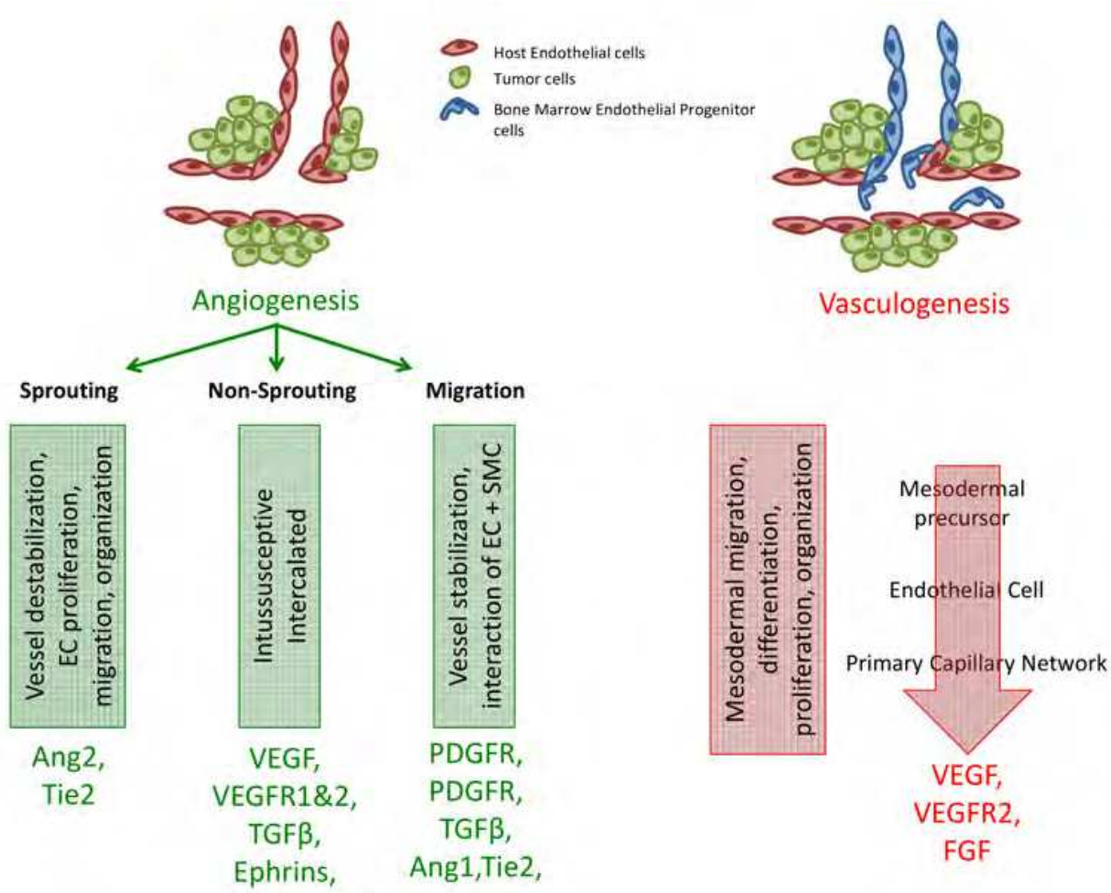

Fig. 3. Regulators of neo-angiogenesis

Although not fully understood the various Pro- and Anti- angiogenic factors can be broadly linked to specific mechanisms underlying neo-angiogenesis. As there is a high level of redundancy within this system many of the factors have multiple functions and multiple factors can be involved in each step.

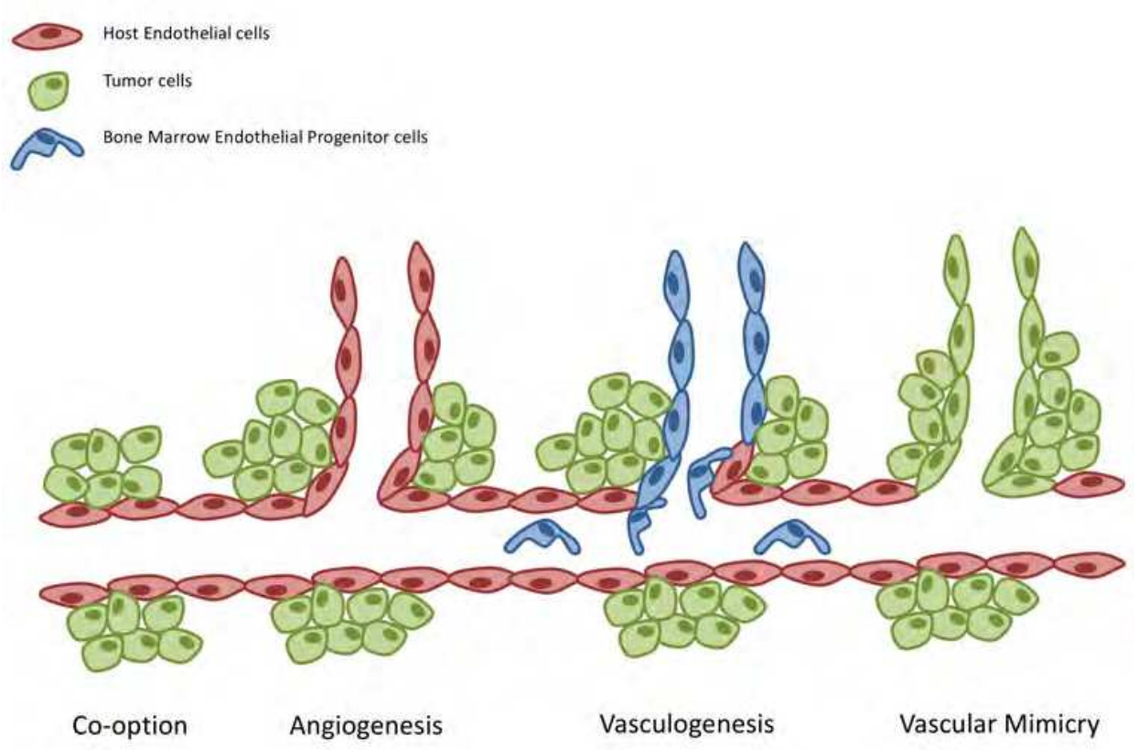

Fig. 4. Mechanisms of Tumor Neo-angiogenesis
Tumor angiogenesis is thought to arise from 4 mechanisms:
Co-option – the colonization of existing vessels
Angiogenesis – the branching and colonization of existing vessels
Vasculogenesis – the formation of new vessels *de novo* from circulating EC progenitors migrating from the bone marrow
Vascular Mimicry - the newest suggested source of vessels whereby the tumor cells directly transdifferentiate into EC forming their own vessels

Circulating BM-derived endothelial progenitor cells (EPC), the adult counterpart of embryonic angioblasts, were first isolated over 15 years ago from the peripheral blood of patients with vascular trauma, septic shock, sickle cell anemia and cancer[63,64]. Accumulating evidence indicates that these EPCs can be mobilized from the BM to initiate *de novo* vessel formation in response to oncogenic mediators in solid cancers[65-67 6,7,23]. When isolated from circulation and exposed to angiogenic factors, they formed highly proliferative endothelial-like colonies[63,64]. However, there is controversy as to whether these BM derived cells (BMDCs) contribute directly to vessel endothelium as ECs or as perivascular cells (PVC) and whether this varies with tumor type, growth stage and potentially in response to treatment[58,63,68-72]. A variety of BMDCs have so far been linked to vasculogenesis, however conflicting results exist over the degree of these BMDCs influence on the vascular endothelium. In particular EPCs[73-78], CD11b+ myeloid cells[79], Tie2+ monocytes[80,81], VEGFR1+ hemangiocytes, and tumor associated macrophages (TAM) [82,83] have all been seen to come from the BM and incorporate into tumors and their vasculature both directly, through generation of endothelium and indirectly, by stabilizing the tumor vasculature through localizing around the perivascular regions[62-64,84].

On the basis of recent studies, it appears that some, but definitely not all, experimental tumors and types of ischemia utilize vasculogenesis in generating their blood vessel endothelium, emphasizing the need to identify factors that may promote vasculogenesis. Once these factors are identified, possible therapeutic applications are numerous, including: (1) enhancing the pharmacologic ability to stimulate neovascularization after ischemia or disrupt tumor vasculature by targeting all types of vessel formation and (2) a gene therapy approach in which BM or EPCs are utilized as cellular delivery vehicles to deliver therapeutic genes to ischemic areas or tumors [63,64].

Alternate mechanisms of tumor neovasculogenesis exist including Co-option, whereby the tumor cells will accumulate around existing vessels and colonize the already efficient vascular networks. Although less supporting evidence exists, it has also been suggested that tumor cells themselves transdifferentiate into EC which are then able to form functional vessels in the tumor **(Figure 4).**

1.2 Molecular regulators of angiogenesis

A large number of endogenous pro- and anti-angiogenic factors have been identified (**Figure 1)**[1,14,18,19,31] whose signaling pathways interact in a highly complex and coordinated manner in order to produce functional vessels **(Figure 3).** Each angiogenic factor can play multiple roles depending on the context in which it is expressed. The regulatory role of each molecule is dependent on the microenvironment, the temporal and spatial expression profile, and the combinatorial effect of other angiogenic factors[32]. Additionally, angiogenesis is indirectly regulated by many transcriptional factors, oncogenes and tumor suppressor genes, which regulate other aspects of cellular function such as proliferation, apoptosis and motility[1]. Among the angiogenic factors identified, there are three groups that are thought to have an endothelial specific role, because their receptors are found exclusively on ECs[32]. These three groups are: VEGF, which binds to its receptors VEGFR1/Flt-1 and VEGFR2/Flk-1/KDR, Angiopoetins (Ang1 and Ang2), which bind to their receptors Tie2/TEK, and the more recently identified EphrinA and EphrinB, which bind to EphA and EphB receptors[32]. All three classes make a critical contribution to mature vessel formation (**Figure 3 and 5)**.

1.2.1 Pleotropic angiogenic factors

Several cytokines are direct regulators of angiogenesis in addition to being indirect modulators of EC specific factors such as VEGF[26,33]. Acidic and Basic Fibroblastic Growth Factor (aFGF, bFGF), were amongst the first cytokines implicated closely in angiogenesis[26,34]. FGFs (aFGF and bFGF) induce EC proliferation, migration and tubule formation, in addition to providing a mitogenic signal to many other cell types[2,26]. However, in transgenic knock-out mice for FGFs vessel development is normal, suggesting a level of redundancy in the pathway. Platelet Derived Growth Factor (PDGF) activate their receptors, homo- or hetero-dimeric complexes of PDGF-α or PDGF–β (αα,αβ,ββ) subunits[88-90] (**Figure 5**). Increased expression of PDGF-β is noted in these PVC both physiologically and in many solid tumors, and activation of PDGF receptors results in proliferation of many cell types including astrocytes. Transforming growth factor-β (TGF-β) is able to regulate EC biology directly, affecting proliferation, differentiation, adhesion and apoptosis, and indirectly by upregulating VEGF[26]. There is speculation that at low doses TGF-β stimulates, while at high doses it inhibits growth of ECs, with a similar effect on EC tubule formation[35,36].

Additionally, TGF-β is thought to regulate angiogenesis by acting as a chemotactic agent for monocytes, fibroblasts and other inflammatory agents important in angiogenesis [26].

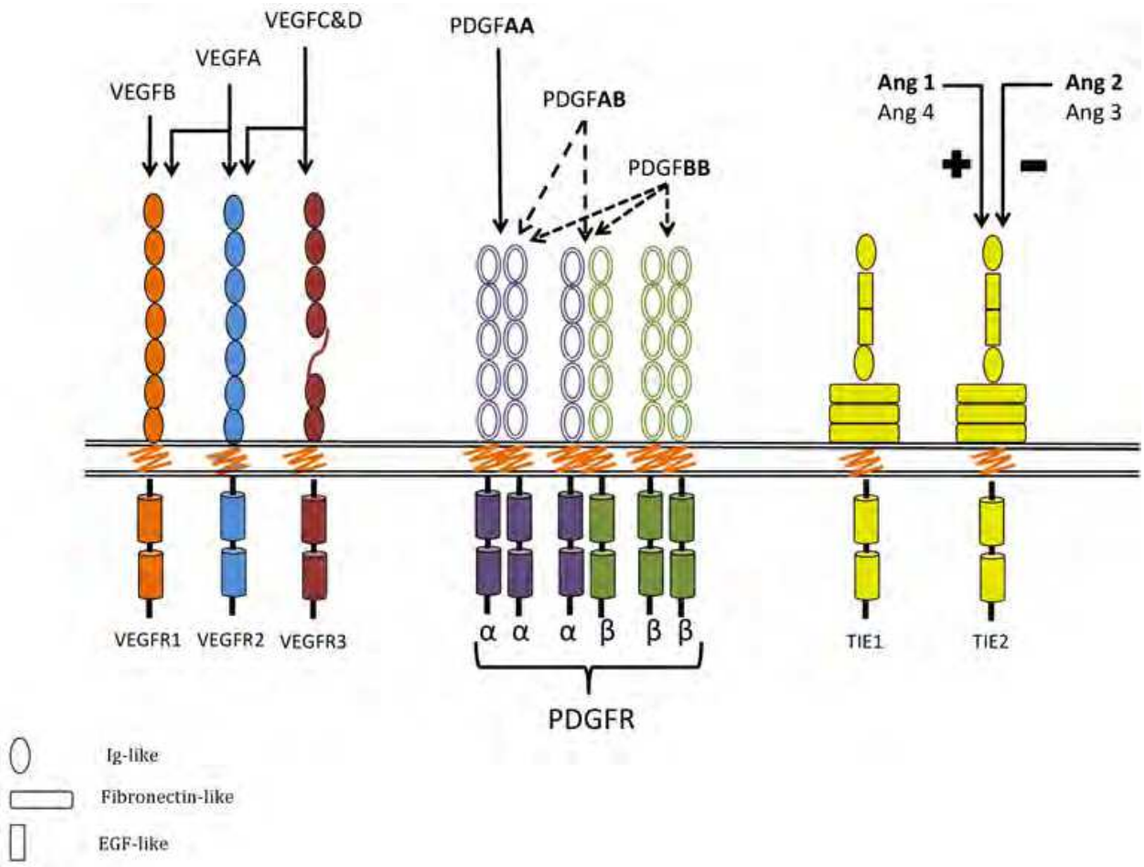

Fig. 5. Diagrammatic representation of VEGF, PDGF and Ang signaling
Schematic demonstrates the numerous interactions feasible amongst each signaling family, suggestive of the level of redundancy in these pathways determined by the microenvironment and so presence of signals and receptors.

1.2.2 Role of the extracellular matrix in tumor angiogenesis

Angiogenesis takes place in a complex ECM, which is critical for modulation of EC behavior. The ECM is composed of highly organized proteins and proteoglycans, and its composition effects EC shape, structure and proliferation, together with EC growth factor expression and interactions with blood vessels[37]. Remodeling of the ECM is a pre-requisite for formation of new blood vessels. The basement membrane provides structural support to the vasculature and is composed of type IV collagen, laminin and fibronectin[38]. Proteases such as Matrix Metalloproteinases (MMPs), and their tissue inhibitors, (TIMPs), regulate the breakdown of ECM and are associated with tissue destruction in many pathologic settings[38-40]. The relative contribution of individual MMPs to vessel formation is not precisely understood, however, certain MMPs are thought to have a more specific angiogenic regulatory role, in particular MMP-2 (gelatinaseA) and MMP-9 (gelatinaseB) both cleave collagen type IV, fibronectin and laminin, the major components of blood vessel basement membrane[41]. Various microenvironmental factors contribute to the regulation of MMPs including direct interactions

of angiogenic factors. For instance, bFGF upregulates the expression of the gelatinases (MMP-2 and MMP-9), whereas VEGF markedly increases only the expression of MMP-2[42]. TIMPs tightly regulate the extent of MMPs activity in addition to directly inhibiting endothelial cell invasion, proliferation and migration. Therefore, identification of TIMPs holds great potential for designing effective anti-angiogenic treatment.

1.2.3 Role of Endothelial cell specific angiogenic factors

Vascular Endothelial Growth Factor

The best-characterized and most extensively studied EC-specific angiogenic factor is Vascular Endothelial Growth Factor-A, commonly referred to as VEGF, with the isoform VEGF-165 being the major secreted isoform and most abundant in human brains[27,43]. The various isoforms, which exist have distinct angiogenic roles, demonstrating organ specificity and making unique contributions to vessel development. VEGF through paracrine activation of its receptors VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR) that are predominantly expressed by ECs, triggers differentiation of EC precursors, promotes EC survival, mitogenesis and migration leading to the formation of tubule structures and neoangiogenesis[31] (**Figures 3 and 5**).

The critical role of VEGF in vessel development is demonstrated in knock-out mice, where deletions of a single allele of the VEGF gene is is embryonic lethal, due to lack of vessel formation in addition to deficient blood island formation in the yolk sac[44]. The biological response to VEGF is highly dose dependent, requiring strict control of *in vivo* VEGF expression for normal vessel formation[45,46]. Over-expression of VEGF in a dose dependent manner is also detrimental, leading to abnormal vessels characterized by an attenuated compact layer of myocardium, remodeling of the large vessels and tortuous and dilated epicardial vessels[100,102,103,] .

During adult life, VEGF expression is low to absent in most organs, including the brain, other than at sites of physiological neo-angiogenesis[33,47,48] however, at sites where new vessel formation is required, VEGF is up-regulated. Various regulators of VEGF have been identified, the most prominent being hypoxia, which acts to regulate VEGF expression at both the transcriptional and post-transcriptional levels[49].

Although hypoxia is the most important physiological regulator of VEGF, other growth factors also play a role. Specific cytokines and receptors and their major downstream signaling pathways, are usually considered to be important mitogenic regulators, but also indirectly regulate expression of VEGF and VEGF receptors. In gliomas, some of these cytokine/receptors pathways include activated Epidermal Growth Factor Receptors (EGFR) or PDGFR and their signaling pathways such as those mediated by activation of p21-Ras or PI3-Kinase[33,49,50]. In specific, relating to tumor-mediated angiogenesis, VEGF is regulated by mutations in oncogenes and tumor suppressor genes, suggesting they act to initially trigger the signalling events involved in new vessel formation, thereby supporting further proliferation of tumor cells. It has been previously demonstrated that P53 and *ras* activating mutations result in a marked upregulation of VEGF expression. The cross talks between mitogenic and angiogenic signals, mediated by VEGF, are of high relevance, particularly in solid tumor vascularization.

Angiopoietins and Tie2/TEK Receptor

Angiopoietins, (Ang1/2) are important modulators of angiogenesis and are a second class of angiogenic factors to have their receptor expressed exclusively on ECs[31]. The two main

family members are Ang1 and Ang2, with their cognate EC specific receptor being Tie2/TEK[51-53]. Ang1 is the activating ligand to Tie2/TEK[53], while Ang2 is the naturally occurring antagonist (**Figure 5**). The expression pattern for Ang1 and Ang2, and their isoforms, among variable tumor cell lines has been examined and suggests an as of yet undetermined role for the different isoforms in pathological scenarios [111,112].

Early in mouse development, Ang1 is found prominently in the myocardium and endocardium whilst later in development, it is primarily seen in the mesenchyme surrounding developing vessels, in close in close proximity to ECs[53,54]. This expression pattern suggested that Ang1 plays a role in the development of the heart and vascular structures[53,54]; however, a direct role in angiogenesis was not easily evident, since unlike the EC mitogenic signals of VEGF, Ang1 has no direct effect on EC proliferation[53,54]. Deletion of Tie2/TEK caused embryonic lethality due to defects in vascular development, characterized by a reduction in EC number, defect in the morphogenesis of microvessels, compromised heart development and internal hemorrhage[51].

Ang2 expression was primarily observed in the major vessels such as the dorsal aorta, but not in all vascular structures[55]. In adult life, Ang2 is present at sites of vascular remodeling such as in the ovary, placenta, uterus and other areas of active neo-vascularization[55]. Interestingly, transgenic mice over-expressing Ang2 died embryonically with a similar phenotype as the Ang1 and Tie2/TEK knock-out mice, suggesting that Ang2 is a natural antagonist for Ang1-mediated Tie2/TEK activation in EC, making Tie2/TEK the first known naturally occurring receptor tyrosine kinase (RTK) that is so precisely regulated *in vivo* [55].

Tie2/TEK expression persists throughout life in the quiescent endothelial cells including those in the normal brain, providing continuous stabilizing force to the mature adult vasculature through Ang1 activation[56,57]. Tie2/TEK is critical for normal embryonic vessel development and knock-out of Tie2/TEK or ablation with a dominant negative mutant causes embryonic lethality[58-60]. The abnormal vessels are characterized by a reduction in EC number and lack of recruitment of PVC[58-60]. There is a decrease in sprouting and remodeling of the primitive vascular network, leading to restricted growth of the head and heart, together with a loss of heart trabeculation[58-60]. During normal physiological processes such as wound healing and pathological angiogenic states such as tumor vascularization, Tie2/TEK expression and activation are increased[56,57,61,62], indicating a requisite role for this pathway in neo-angiogenesis.

A paradigm incorporating interactions of VEGF and Ang1/2 in the development of normal embryonal and adult vasculature has been proposed[63]. VEGF and VEGFRs are essential for the formation of the primitive vascular network, while Ang1/2 and Tie2/TEK interactions signal maturation of the primitive vessels[63]**(Figure 3).** During normal and pathological angiogenesis, a relative increase in Ang2 expression by the ECs inhibits Tie2/TEK activation, thereby destabilizing the vessels and sensitizing ECs to VEGF, which results in EC proliferation, sprouting and neo-angiogenesis [63]. However, this paradigm is an over-simplification as we are gradually deciphering the multiple areas of EC biology that Angs and Tie2/TEK play a role in[64-71]. Activation of Tie2/TEK by Ang1 modulates EC adhesion, motility and survival[67-71]. Additionally, Ang1/2 play a highly variable role in angiogenesis depending on the levels of VEGF expression, and the microenvironmental and tissue context in which they are expressed[64-69].

An important factor governing the functional role of Ang1/2 is their interactions with other angiogenic factors, with data suggesting that Ang1/2 can have a dual role in angiogenesis depending on the context in which they are expressed[32]. Recent biochemical research has

focused on the collaborative functions that exist between Ang1, Ang2 and VEGF[72-75]. Transgenic mice over-expressing Ang1 in cardiac cells demonstrate no increase in angiogenesis, which is in contradiction to the findings of increased angiogenesis with Ang1 over-expression in skin[54]. Double transgenic mice over-expressing VEGF and Ang1 showed restricted angiogenesis and dampening of the potent angiogenic response Seen following VEGF signaling [65]. These findings highlight a very context specific role for Ang1 and how it can play both a positive or negative regulatory roles in normal physiological angiogenesis. Similarly, the role of Angs in tumor angiogenesis is proving to be context dependent, as demonstrated by our work in astrocytomas, described below.

Our current understanding of the Tie2/TEK-Ang pathway indicates that these cytokines have a multi-faceted role in promoting and maintaining normal vessels. The regulatory effects of Ang1/2 and Tie2/TEK can vary according to the organ and context in which they are expressed. Therefore, we postulate that this pathway potentially contributes in a similar context-dependent manner to the formation of the abnormal vasculature.

Ephrins

Eph receptors together with their EphrinA and EphrinB ligands were first identified in the nervous system for their role in neuronal patterning and axonal guidance [76,77]. Ephrins and Eph receptors are also postulated to play important functions in arterio-venous differentiation and maintenance. EphrinB2 is an early marker of arterial EC and the receptor EphB4 an early marker of venous EC[30,76-78]. Mice with a knock-out of EphrinB2 die embryonally due to lack of appropriate orchestration of arterial and venous ECs. EphB4 knock-out mice show similar phenotypic alterations such embryonal lethality with vascular changes that are identical to the EphrinB2 knock-out mice [30,76-78]. This suggests that EphB4 is the most important receptor interacting with EphrinB2 that regulates normal differentiation of the arterial and venous system. Furthermore, most recent evidence indicates that EphB4 along with Angiopoietin pathway are important not only in physiological but tumor vascular patterning [79,80]. Specifically it is thought that Ang1 and EphB4 interaction reduces the permeability of the tumor vasculature, potentially by altering EC and SMC interaction, in other words influencing vessel maturation [80].

1.3 Role of progenitor cells in tumor angiogenesis

The paradox of the sensitivity of the vasculature to irradiation and the resistance to RT could be resolved if, as postulated, circulating cells outside of the radiation field can re-colonize and/or stabilize the tumor vasculature after irradiation, thereby supporting any remaining viable tumor cells. This restoration of the vasculature facilitated by BMDCs is observed in ischemic normal tissues and in malignant tissues[73-75]. These BMDCs are also thought to create an environment for tumor growth and invasion, and metastasis [77, **(Figure 6)**.

Whether EPCs are a true subpopulation of BMDC that are mobilized from the BM versus being present mainly in the circulation remains controversial and data supports that there may not be a clear distinction between angiogenesis and vasculogensis as traditionally proposed. One of the major limitations in the study of EPCs is the lack of exclusive cell-surface markers for these cells.

EPCs that are derived from BM can be mobilized during adult life to sites where new vessel formation is required[81,82]. It is also conceivable that they can reside in organs dormantly, become activated and differentiate into ECs in response to certain physiological and pathological triggers, thereby contributing to angiogenesis. EPCs express markers similar to

ECs, such as VEGFR2, Tie2/TEK, CD34, CD146, von Willebrand factor and PECAM[83,84]. Therefore, making a distinction between EC lining the vessel lumen and EPCs present within a vessel lumen can be difficult. Though their relative contribution to angiogenesis is not clear[85] very recent data suggests that EPCs within a specific vessel wall region, localized between smooth muscle and adventitial layer, act as a source of EPCs that will then trigger neo-vascularization in adult tumors[86]. The stem cell marker, CD133, with a yet to be determined function, stains EPCs[83,84]. Stem cells are $CD133^{+}$ /$VEGFR2^{-}$, but *in vitro* can be induced to become $CD133^{-}$/$VEGFR2^{+}$, and behave as a mature EC[83,84]. Until these recent observations it was thought that adult vessel formation relied solely on neo-angiogenesis however these recent results suggest that *de novo* vessel formation by EPCs to be potentially possible [86-88]. Recent studies using Id1&3 knock-out mice demonstrate an impaired VEGF induced EPCs mobilization and tumor vascularisation, suggesting bone marrow derived EPCs are incorporated to the vascular structures in both B6RV2 lymphoma and Lewis lung carcinoma tumors. The recruitment of EPCs occurs concurrently with myeloid cells, which are believed to stabilize the structure of newly formed vessels.

There is evidence that chemotactic factors expressed by injured tissue recruits hematopoetic stem cells and progenitor cells to aid in neoangiogenesis[85,89], however, their role and mechanism of activation in tumor angiogenesis is not known. The molecular mechanisms of

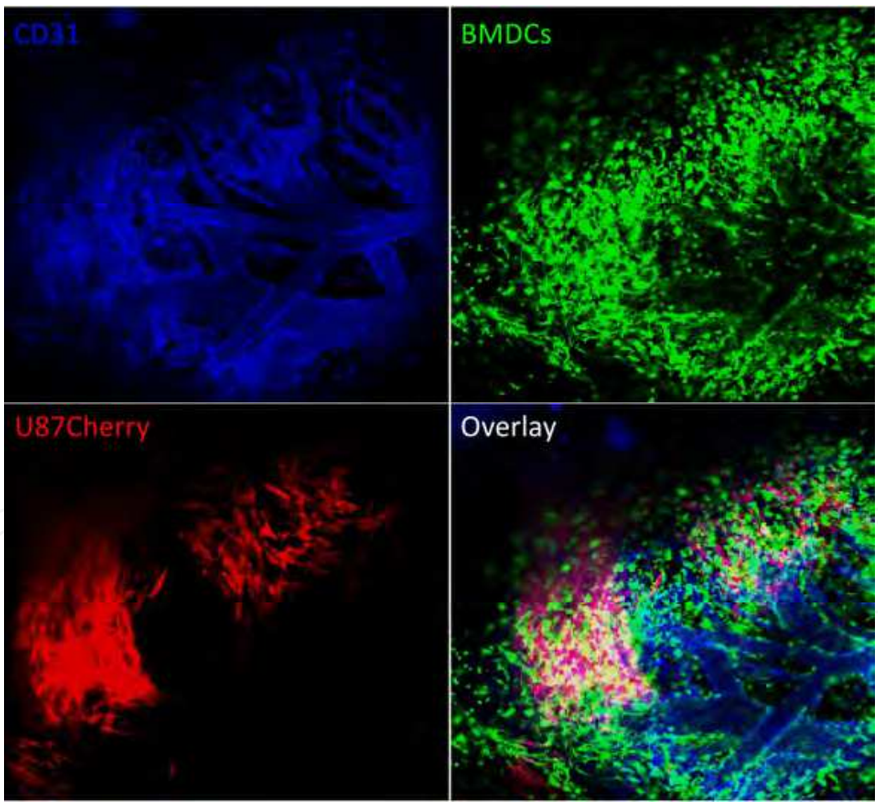

Fig. 6. BMDC recruitment to Tumor

Using *in vivo* 2-photon imaging it is possible to watch with time the recruitment of BMDCs to the site of tumor growth and ultimately their dynamic integration into the vasculature network. BMDCs distribution can be confirmed following end point histology looking at sections through the tumor.

activation, recruitment and differentiation of EPCs to tumor angiogenesis is beyond the scope of this chapter, but this is an exciting new direction for angiogenesis research, which will most likely result in significant advances and potentially yield therapeutic strategies for tumors.

1.4 Role of immuno-modulatory cells in tumor angiogenesis

In addition to EPCs, other subpopulations of BMDCs are thought to contribute to tumor neo-vascularization. It is well established that the role of macrophages extends beyond their originally recognized role as scavenger cells and as part of inflammatory cells they are proposed to play a crucial role in tumor neo-vascularization, though a specific role has not been definitively established. Recent studies demonstrate that monocytes derived from the bone marrow are recruited from the circulation and migrate into the tumor stroma where they differentiate to form macrophages. Several studies have shown tumor-associated macrophages (TAM) to promote tumor angiogenesis and metastases[90] and extent of TAM correlates with increased tumor angiogenesis. These TAMs can in turn promote neo-vascularization[61]. To date only a few studies have focused on this area in solid tumors, and it has been suggested that a small subpopulation of monocytes expressing an angiogenic specific receptor tyrosine kinase, TIE2 expressing Monocyte (TEM), also play a very specific role in neo-vascularization of tumors[91]. TEMs can be distinguished from TAMs by their surface markers (CD45,CD11b,TIE2,F4/80) and lack of mature EC cell surface markers (VEGF-R2/CD133/CD34)[92]. Depletion of the TEM population in mice results in altered endothelial staining, which indicates a dramatic reduction in vascular structures, suggesting that TEMs are important in the process of glioma tumor angiogenesis[93]. Another key subpopulation of BMDCs are BM derived myeloid cells or myeloid-derived suppressor cells (CD45/CD11b/Gr-1), which are thought to eventually differentiate into TAM, TEM and granuloctyes. The role of myeloid-cells in cancer progression, in particular vascular dependent growth, is becoming more recognized, and interest in using them as therapeutic targets is increasing as their depletion can prevent tumor recurrence post-RT[94].

Colony-stimulating factor (CSF-1) gene plays a critical role in macrophage growth and development. Inhbition of CSF-1 decreases tumor growth[95]. The paracrine loop signaling between macrophages and tumor cells is essential to the ability of tumor cells to invade within the primary tumor. TAMs are found to express a range of angiogenic regulating factors, most notable of which are epidermal growth factor (EGF), FGF-2, TGF-a and b and CSF-1[96]. A few studies have shown macrophages to be a source of EGF, which acts as a chemotactic for tumor cells in mammary tumors *in vivo* and *in vitro*. Another chemokine thought to be involved in macrophage recruitment is CCL2/monocyte chemotactic protein-1 (MCP-1), synthesized by ECs MCP-1 is seen to increase in both ischemia and malignancy.

1.5 Role of hypoxia in tumor angiogenesis

The responses to hypoxic condition are mostly mediated through activation of hypoxia inducible factor (HIF-1) and influenced by tumor microenvironment and underlying vessel density[97]**.** HIF-1 consists of a constituently active subunit, HIF-1β, and a secondary subunit, HIF-1α, which is not present under normoxia due to ubiquitin-mediated proteolysis. Hypoxia inhibits proteolytic degradation of HIF-1α, resulting in dimerization with HIF-1β and activation of many downstream targets through interaction with a 28-base sequence in the 5′ promoter region of the gene, termed the Hypoxia Response Element (HRE)[98,99]. The

critical regulation of HIF-1α by hypoxia is demonstrated during embryonal development, because whereby HIF-1α knock-out embryos die due to vascular defects.
Hypoxia induces the transcription of many key angiogenic signaling pathways including VEGF [100-105], through increasing the VEGF mRNA stability through binding of several RNA binding proteins [100,102,106-109].
Similarly it has been shown that in a tumor hypoxic environment, the activation of HIF1α induces Stromal Derived Factor-1 (SDF-1), a mobilizer of BMDCs, in turn recruiting EPCs, pericyte progenitor cells (PPCs) and CD45+ myeloid cells to the tumor contributing to neovascularisation. EPCs and PPCs directly contribute in vascular structure whilst myeloid cells have been shown to induce the bioavailability of VEGF, thus promoting the angiogenic potential of the tumor[63].
In addition to HIF-1α, a very recent study has identified the distinct upregulation of HIF-2α gene expression by glioma stem cells (GSCs) in response to hypoxia, potentially explaining the increased VEGF expression and highly angiogenic activity of tumors originating from GSC[110]. Therapeutic intervention such as RT will increase the hypoxic level of tumor and therefore contribute to the HIF-1 enhancement of the formation of new blood vessels. Kioi *et al* have shown that irradiation increases tumor hypoxic condition, which in turn enhances the number of BMDCs, such as myelomonocytes (CD11b+), recruited to the tumor environment, implying that vasculogenesis rather than angiogenesis contributes in RT induced tumor progression. Through the blockade of SDF-1, a downstream target of HIF1, the recruitment of BMDCs EPCs was completely inhibited demonstrating the specificity of the recruitment following RT[62].

1.6 Ionizing radiation and its affect on neo-vascularisation

A more comprehensive knowledge of the molecular mechanisms of angiogenesis in response to RT is required to allow more efficient targeting of angiogenic pathways. There is experimental evidence demonstrating that RT regulates tumor angiogenesis, both direct and indirectly. RT directly modulates EC biology, by inhibiting EC survival and proliferation, preventing EC invasion and tube formation and inducing EC apoptosis[111,112]. Some studies have shown a dose dependent response to RT, where at lower doses, RT can promote EC proliferation (<10 Gy)[113] while at higher doses (> 10 Gy) induce EC apoptosis[114,115]. EC apoptosis is promoted through inhibiting the main signaling pathway regulating EC survival, the PI3K/Akt pathway. Therefore, PI3K/Akt has been proposed as a strong candidate target for combinatorial therapy with RT[116,117]. In addition to regulating EC biology, RT also directly regulates expression of angiogenic factors and hence tumor angiogenesis. RT upregulates VEGF and VEGFR-2, in turn promoting EC proliferation and tumor angiogenesis, and overall tumor growth[45,46,111,118,119]. Levels of VEGF expression by ECs is suggested to be dependent on RT dose and fractionation schedule[120,121]. Other candidate angiogenic factors also found to modulate RT include Ang-1 and bFGF which protect ECs against RT damage[115,122], while bFGF enhance anti-tumor effect of RT[114,123]. Similarly, PECAM-1 elevation following RT increases anti-tumor effect of IR by upregulating vessel thrombosis and promoting anti-angiogenesis[124]. RT can regulate tumor angiogenesis indirectly as well, by generating hypoxia. Hypoxia in turn induces VEGF and VEGFR2 upregulation[125-127] and tumor angiogenesis. The tumor angiogenic response elicited post-RT is considered as a potential 'escape mechanism' that might provide an opportunity for tumor cells to avoid radiation cell kill and facilitates cancer recurrence. It is conceivable that inhibition of VEGF and other relevant angiogenic cytokines will eliminate the

angiogenic survival response post-RT, radiosensitizing ECs and potentiating the benefits of RT. The above results are supported to an extent by recent *in vivo* studies[120,121,123,128], however, unfortunately clinical trials involving combination of RT and anti-VEGF therapy have not proven beneficial, suggesting other mechanisms regulating tumor neo-vascularization in response to RT may be involved [34,37,39,48].

Recent evidence has demonstrated the specific migration and recruitment of BMDCs to the site of radiation specifically, although negates the process of differentiation following integration. The BMDCs instead are retained at the site of RT until a secondary signal, such as an oncogenic signal initiates their differentiation, possibly providing a vascular escape mechanism for the RT. The molecular mechanisms that regulate BMDC to site of cranial irradiation remains to be identified and their potential to be harnessed towards a therapeutic benefit further elucidated.

2. Abbreviations

EC – Endothelial Cell
VEGF – Vascular Endothelial Growth Factor
Ang 1&2 – Angiopoietin 1 & 2
ECM – Extracellular Matrix
CT – Chemotherapy
RT - Radiation Therapy
EPC – Endothelial Progenitor Cell
PC – Pericyte
SMC – Smooth Muscle Cell
BM – Bone Marrow
BMDC - Bone Marrow Derived Cell
PVC – Perivascular Cell
TAM - Tumor Associated Macrophage
GSC – Glioma stem Cells
PPC – Pericyte Progenitor Cells
HIF1 – Hypoxia Induced Factor 1
SDF-1 – Stromal Derived Factor 1
PDGF – Platelet Derived Growth Factor

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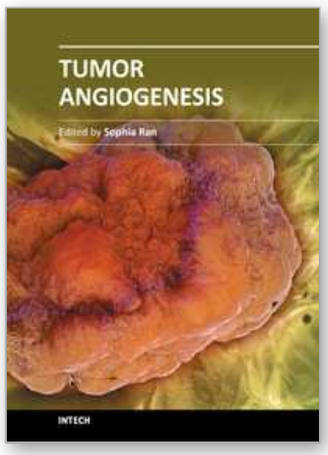

Tumor Angiogenesis
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Tumor angiogenesis is the main process responsible for the formation of new blood vessels that promote tumor growth and metastasis. This process is driven by potent pro-angiogenic factors that are predominant in the tumor environment and are produced by both malignant cells and the host cells recruited to the tumor site. Tumor environment is characterized by the imbalance between pro-angiogenic and anti-angiogenic factors, which drives the construction of numerous but structurally defective vessels. These poorly perfused and abnormal vessels significantly contribute to the tumor pathology not only by supporting the expansion of the tumor mass but also by promoting chronic inflammation, enhancing thrombosis, impeding drug delivery, and disseminating tumor cells. These problems associated with tumor vasculature continue to attract great attention of scientists and clinicians interested in advancing the understanding of tumor biology and development of new drugs. This book complies a series of reviews that cover a broad spectrum of current topics related to the pathology of tumor blood vessels including mechanisms inducing new vessels, identification of new targets for inhibition of tumor angiogenesis, and potential clinical use of known and novel anti-angiogenic therapies. The book provides an update on tumor angiogenesis that could be useful for oncologists, cancer researchers and biologists with interests in vascular and endothelial cell behavior in the context of cancer.

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