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Redundant Angiogenic Signaling and Tumor Drug Resistance

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Abbreviations: ALK1, activin receptor-like kinase1; ANG/Ang, angiopoietin; Bcl-xL, b-cell lymphoma-extra large; BM, bone marrow; BMDCs, bone marrow derived cells; BMP, bone morphogenetic protein; Bv8, bombina variagata peptide 8; CAFs, cancer/carcinoma associated fibroblasts; CCL, C-C motif ligand; CD, cluster differentiation; CEPs, circulating endothelial progenitors; c-MET, cellular MET, also HGF receptor; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; COX, cyclooxygenase; CSCs, cancer stem cells; CSF-1, colony stimulating factor-1; CXCL, C-X-C motif ligand; CXCR4, C-X-C chemokine receptor 4; Dll4, delta like 4 ligand; DSL, Delta, Serrate, Lag2; ECM, extra cellular matrix; ECs, endothelial cells; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMA, European medicines agency; EMT, epithelial-mesenchymal transition; ENG, endoglin; EPCs, endothelial progenitor cells; Eph, epherin; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FDA, food and drug administration; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Fzd, Frizzled; GBM, glioblastoma multiforme; G-CSF, granulocyte-colony stimulating factor; GSK3β, glycogen synthase 3 beta; HETE, hydroxyeicosatetraenoic acid; HGF, hepatocyte growth factor; HIF-1α, hypoxia inducible factor 1α; HUVEC, human umbilical vein endothelial cells; IL, interleukin; IMG, intussusceptive microvascular growth; JAG1, Jagged-1; JAK, janus kinase; KLEIP, kelch-like Ect2-interacting protein; MAPK, mitogen-activated protein kinase; M-CSF. macrophage- colony stimulating factor; MDR, multidrug resistance; MMPs, matrix metalloproteinases; MMTV, mouse mammary tumor virus; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; NICD, notch intracellular domain; NRP, neuropilin; NSCLC, non-small cell lung cancer; PCs, pericytes; PDGF, platelet derived growth factor; PDGFR, platelet derived growth factor receptor; Phd2, prolyl hydroxylase 2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLCγ-PKC, phospholipase Cγ-protein kinase; PLGF, placental growth factor; PNETs, pancreatic neuroendocrine tumors; PyMT, polyomavirus middle T antigen; RAF, rapidly accelerated fibrosarcoma; RAS, ra(t) s(arcoma); RCC, renal cell carcinoma; RTK, receptor tyrosine kinase; SCF, stem cell factor; SCID, severe combined immunodeficiency; SDF-1α, stromal-derived factor 1α; STAT, signal transducer and

activator of transcription; TAFs, tumor-associated fibroblasts; TAMs, tumor associated macrophages; TCF, T-cell factor; TEMs, Tie2-expressing macrophages/monocytes; TGFβ, transforming growth factor-β; Tie2, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains; TNBC, triple-negative breast cancer; TNFα, tumor necrosis factor α; VDA, vascular disrupting agents; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VHL, von hippel-lindau; VM, vasculogenic mimicry; Wnt, wingless-type mouse mammary tumor virus integration site family; ZEB2, zinc finger E-box binding homeobox 2; αSMA, α-smooth muscle actin;

Abstract

Angiogenesis research in the past two decades has contributed significantly towards understanding the molecular pathophysiology of cancer progression and inspired target-oriented research and pharma industry for the development of novel anti-angiogenic agents. Currently, over eleven drugs targeting angiogenesis have been approved by the FDA for the treatment of various malignancies. Of the registered anti-angiogenic clinical trials until the end of 2017 (ClinicalTrials.gov), over 47% were completed, 10% were terminated, 3% withdrawn, over 0.5% were suspended and only 4 trials have culminated in FDA approval for marketing. On the one hand, the clinical benefits of anti-angiogenic drugs prompted the development of novel anti-angiogenic agents. On the other hand, however, a plethora of recent studies demonstrated the emergence of tumor drug resistance towards currently used anti-angiogenic therapeutics. Series of preclinical and clinical studies have highlighted the enigma of drug resistance with functional bypass pathways, and identified compensatory or alternative angiogenic mechanisms assuring tumor growth in the midst of an anti-angiogenic stress environment. In the present review the classical literature of such redundant angiogenic pathways in concert with the key angiogenic factors and specialized cells involved in anti-angiogenic escape mechanisms is described. A strategic discourse regarding increasing tumor drug resistance and future modalities for anti-angiogenic therapy is also discussed in view of recent advances.

Key words: Angiogenesis, Redundant angiogenic pathways, Anti-angiogenic drugs, Tumor drug resistance.

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Introduction

Vasculogenesis is the fundamental *de novo* process required for the development of blood vessels during embryonic development, wherein the angioblasts (endothelial progenitor cells) differentiate into endothelial cells in response to local cues and finally assemble to form the initial vascular plexus. Angiogenesis is the extension of vasculogenesis, which involves the development of new capillaries on pre-existing vessels. A vast body of literature has accumulated which describes the dynamics and complexities of new vessel formation (Chappell et al., 2016). From a physiological perspective, angiogenesis appears to be indispensable for tissue homeostasis, as the growth and development of an organism requires a well-developed network of blood vessels to ensure a continuous supply of oxygen and nutrients. Nevertheless, important physiological processes such as embryogenesis, organogenesis, wound healing, tissue repair, etc., cannot be achieved, unless there is a well-developed, mature orchestration of vascular network. Dissolution of vascular basal membrane, degradation of extracellular matrix (ECM), increase in vascular permeability, migration, invasion and proliferation of endothelial cells (ECs) and tube formation are the hallmarks of the angiogenic process. In brief, the process of neovascularization relies on a complex network and cross talks of proangiogenic factors, stromal cell interactions and remodeling of ECM (Gacche and Meshram, 2014). Under normal physiological conditions, the process of angiogenesis is regulated by maintaining a balance between activators and inhibitors of angiogenesis. The vessels developed through physiological angiogenesis are normal, stable and rarely proliferate under physiological conditions. However, in pathological conditions like cancer, the balance is more in favor of proangiogenic factors; as a result, there is excessive remodeling of vasculature. In contrast, the complex, abnormal, leaky and torturous vessels of tumors is the hallmark of pathological angiogenesis (Goel et al., 2011).

The credit of establishing angiogenesis as a therapeutic target goes to Judah Folkman, who highlighted the importance of vascular network for the growth, proliferation and progression of solid tumors in 1971. In a preclinical experimental setting, Folkman and colleagues demonstrated that in the absence of proper vasculature, the growth of tumors may not attain more than 2 mm in size (Folkman, 1971). These pioneer experiments opened a new research avenue with the aim of disrupting tumor vasculature. The area of angiogenesis research got a strong impetus and became more vibrant with the discovery of VEGF by Senger and his colleagues (Senger et al., 1983), followed by numerous studies uncovering the central role of VEGF as a key player in physiological and pathological artefacts of angiogenesis. Ten years of research on VEGF from a therapeutic

point of view have been recently reviewed (Ferrara and Admis, 2016). Thus, the VEGF signaling pathway especially driven by VEGF-A, has remained a focal target for designing novel anti-angiogenic agents. There are over forty pro-angiogenic factors reported to play a proactive role in angiogenesis, either directly or indirectly in different cells/tissues/organs and several new pro-angiogenic molecules are consistently reported in the mainstream of current angiogenesis research. Previously, we have reviewed the structural peculiarities of major angiogenic cytokines that can be further maneuvered for the design and development of novel anti-tumor agents targeting angiogenesis (Gacche and Meshram, 2013). Currently, over 11 anti-angiogenic drugs have been approved by the U.S. Food and Drug Administration (FDA). Out of which, bevacizumab (Avastin), Aflibercept (Eylea: a VEGF-trap recombinant fusion protein of VEGF-binding domains from VEGFR) and Ramucirumab (Cyrazma) are antibodies, while Sorafenib, Sunitinib, Pazopanib, Axitinib, Vandetanib, Lenvatinib and Regorafenib are approved as small molecule receptor tyrosine kinase (RTK) inhibitors. Apart from FDA, the European Medicines Agency (EMA) has approved Nintedanib in 2014 for the treatment of locally advanced, metastatic or second line non-small-cell lung cancer (Jayson et al., 2016). The FDA guidelines for the therapeutic applications of the approved drugs are summarized in Table 1. Series of clinical trials are currently in progress to evaluate the therapeutic concerns of anti-angiogenic agents in a variety of cancers (http://www.cancer.gov/clinicaltrials/developments/anti-angio-table/); at the same time, series of small molecule targeting VEGF signaling pathway, especially RTK inhibitors are at various stages of clinical development:
(http://www.cancer.gov/cancertopics/factsheet/Therapy/angiogenesis inhibitors)

From a pharmacological perspective, targeted therapies are more impressive, have marginal side effects and enhanced therapeutic index. Undoubtedly, anti-angiogenic therapy has emerged as an impressive targeted therapeutic regimen with equal excitement and vibrancy in related research. Moreover, series of anti-angiogenic agents are currently used in combination with other chemotherapeutic agents for the treatment of a variety of malignant cancers. Welti et al., (2013), have extensively and systematically reviewed the treatment success and limitations of the 9 anti-VEGF agents in relation to different types of cancers (Welti et al., 2013). On the one hand, anti-angiogenic therapy has undoubtedly proved to be helpful for the management of various cancers and largely improved the overall prognosis of cancer patients. However, on the other hand, the therapy is questioned on the grounds of sustainable efficacy, side effects, off target toxicities

and emerging drug resistance (Gacche, 2015; van Beijnum et al., 2015; Mitamura et al., 2016; Jayson et al., 2016). Amongst the notable negative concerns of anti-angiogenic therapy, the most formidable is the recurrent aggressive invasion and metastasis after the "drug holidays" (Griffioen et al., 2012). Series of preclinical and clinical studies have linked the poor performance of, and drug resistance to, anti-angiogenic drugs, with activation of series of compensatory angiogenic pathways and a variety of angiogenic factors supporting angiogenic bypass mechanisms (Gacche, 2015; Ribatti, 2016; Al-Abd et al., 2017).

2. Redundant Angiogenic Signaling: A Potential Cause of Evolving Tumor Drug Resistance

From a mechanistic point of view, angiogenesis is a highly modeled and degenerate process. Moreover, the heterogeneity and genomic plasticity of tumors allow them to evolve and employ multiple compensatory pathways in the prevalence of negative growth environment. Recent epidemiological, clinical and experimental studies have demonstrated that tumors employ compensatory/alternative/bypass angiogenic pathways and other adaptive mechanisms for their sustained growth and metastasis, after experiencing a treatment episode(s) with anti-angiogenic agents. Therefore, compensatory signaling pathways driving tumor growth and metastasis, invariably become a potential cause of tumor refractoriness.

The clinical and experimental data accumulated in the recent years have unequivocally proved that revascularization occurs even after blocking VEGF signaling pathways due to upregulation of redundant angiogenic pathways. A plethora of evidence has accumulated in the recent years, linking the role of various compensatory/alternative/bypass angiogenic canonical mechanisms sustaining the growth and progression of tumors when treated with anti-angiogenic agents (Crawford and Ferrara, 2009; Gacche, 2015; Jayson et al., 2016; Ribatti, 2016; Mitamura et al., 2016). In fact, the first clues for the existence of alternate angiogenic pathways emerged from mouse model studies, designed to evaluate the therapeutic potential of monoclonal antibody DC101, designed against the VEGF receptor (VEGFR) in pancreatic neuroendocrine cancer. The results of this experimental model were surprising in demonstrating the recurrence of tumor growth and vascularization after the initial exposure to the test antibody. Further analysis revealed that there were upregulated levels of mRNAs encoding for pro-angiogenic factors such as fibroblast growth factor 1 and 2 (FGF 1 and FGF2), Angiopoietin 1 (ANG 1) and Ephrin A1 and A2 (Casanovas et al., 2005; Ronca et al., 2017). In clinical studies involving human subjects harboring

glioblastoma, detectable levels of VEGF, FGF-2, placenta growth factor (PlGF) and other pro-angiogenic factors were observed after treatment with anti-angiogenic therapy (Lu-Emerson, et al., 2015). The current epidemiological, clinical and experimental evidence has clearly outlined that there exist at least four distinct mechanisms, which can be considered for manifestation of evasive resistance to anti-angiogenic therapies. The first mechanism involves the activation and/or up-regulation of compensatory pro-angiogenic signaling pathways within the tumor, especially driven by FGF, ANGs, platelet derived growth factor (PDGF), Ephrins etc. (Vasudev and Reynolds, 2014). The second alternate angiogenic mechanism is mainly driven by myeloid/stromal cells, which compensates for the requirement of the VEGF-mediated pathway, thereby promoting tumor angiogenesis (Crawford and Ferrara, 2009). The third mechanism is attributed to the dual role of pericytes; first, in establishing the increased pericyte coverage of the tumor vasculature and second, their potential angiogenic attributes, both serving as escape mechanisms from VEGF-mediated angiogenesis (Bergers and Song, 2005; Matsuo et al., 2010). The fourth mechanism is related to remodeling and accessing normal vasculature for invasion and metastasis of tumors in lieu of obligate neovascularization. Vessel cooption, intussusceptive microvascular growth and vascular mimicry are the prominent mechanisms of this category (Vasudev and Reynolds, 2014). A vast body of literature has accumulated in recent years clearly demonstrating the prevalence of compensatory anti-VEGF rescue mechanisms recruited by various alternative angiogenic cytokines (VEGF-C, VEGF-D, FGF, PDGF, PlGF, EGF, ANGs, HGF, Interleukins, Ephrins, etc.), Dll4-Notch signaling, c-MET signaling, Tie-2-Angiopoetin, Wnt signaling, ALK1 signaling, myeloid/stromal cells, pericytes, and various new cross-talk pathways emerged as possible mediators of tumor refractoriness (Crawford and Ferrara, 2009; Gacche, 2015; Jayson et al., 2016; Ribatti, 2016; Mitamura et al., 2016).

2.1 Broad Categories of Redundant Angiogenic Signaling

The pathways involved in alternation of the main VEGF pathways, VEGF-independent pathways, pathways driven by interactions between myeloid/stromal/tumor cells and angiogenesis-independent processes like vessel cooption, intussusceptions, vascular mimicry, postnatal, glomeruloid and looping angiogenesis constitute the major categories of redundant angiogenic signaling (Vasudev and Reynolds, 2014). Perhaps the same rationale can be employed for classifying and explaining the modes and mechanisms of tumor refractoriness. The categorization

is not strictly mutually exclusive, but there exists coordination with an involvement of multiple factors, crosstalk and pathways.

2.2 Pathways Centered on the VEGF axis

Angiogenic factors like VEGF-B, VEGF-C, VEGF-D, and PlGF are the key players involved in VEGF axis-dependent alteration pathways. As VEGF-A plays a crucial role in inducing angiogenesis; the majority of research is centered on it as a therapeutic target, anticipating the non-significant role played by its subtypes. Although the molecular mechanisms unraveling the exact roles played by VEGF subtypes are in their infancy, sizable evidences articulate their role as compensatory angiogenic agents promoting angiogenesis when VEGF-A is blocked. Ziv-aflibercept (Zaltrap, VEGF-Trap) antibody possesses VEGFR1 and VEGFR2 as extracellular domains. The antibody binds not only to VEGF-A but it has also affinity to VEGF-B and PlGF (Simon et al., 2017).

In fact, angiogenic factors like VEGF-C and VEGF-D are involved in lymphangiogenesis. While describing the role of VEGF-C in compensatory angiogenic signaling, it has been reported that the VEGF-C fragment generated through proteolytic cleavage has been shown to possess binding affinity with VEGFR-3. Thus the binding of VEGF-C with VEGFR3 act as potential compensatory angiogenic pathways under anti-VEGF environment (Crawford and Ferrara, 2009; Gacche, 2015). Studies have also demonstrated that the expression of VEGFR-3 is not only present in lymphatic vessels but it is also expressed in tumor vasculature. The preclinical model studies strongly support the involvement of VEGF-C in angiogenesis, tumor progression and thereby imparting resistance to anti-VEGF-A-interacting agents (Ye et al., 2013). Neuropilin (NRP)-1 and NRP-2, which were initially discovered in axon guidance and as neuronal receptors, may also play a role in reprogramming angiogenesis by interacting with VEGF family members. NRP-1 not only binds to VEGF-A, but also binds to PlGF. Factors like NRP-2 have been shown to interact with VEGFR-2 and thereby may act as redundant angiogenic pathway. Nevertheless, apart from VEGF-A, NRP-2 has also binding affinity to VEGF-C, and PlGF, and thereby may be playing inscrutable role in angiogenesis (Hu and Jiang, 2016). The evidence for the role of NRP in regulating angiogenesis has been demonstrated by reaction with anti-NRP antibodies. It was observed that NRPs could modulate angiogenic signaling by VEGFR-2. In the same experimental setting, it was also showed that, anti-NRP antibodies display, at least additive activity, when combined with anti-

VEGF antibodies. These observations indicate the adoption of different modes of action by NRPs in driving VEGF-independent angiogenesis (Chenxi and Jiang, 2017). The recent literature describes that NRP-1 drives angiogenesis via the NRP-1-ABL pathway which is independent of VEGF-VEGFR2. The protein RAD51 was identified as a key player in signaling pathways of NRP1-ABL and PDGF(R). This pathway is positively associated with resistance towards cancer chemotherapeutics (Hu and Jiang, 2016). The redundant angiogenic signaling centered around VEGF axis, is summarized in **Figure 1**.

PlGF is also a member of the VEGF family having pleiotropic and multitasking activity. In a variety of pro-angiogenic cells such as ECs, bone marrow progenitors, macrophages and tumor cells (primarily express VEGFR-1), PlGF relays its action directly by binding to VEGFR-1. PlGF was found to stimulate angiogenesis, stromal cell migration, leukocyte infiltration, tumor growth and revascularization of ischemic tissues (Dewerchin and Carmeliet, 2012). In the current discourse of alternate angiogenic signaling, clinically and experimentally, it has been proved that the myeloid, stromal and tumor cells are strongly involved in coordinating the compensatory angiogenic pathways (Gacche, 2015). PlGF secreted by stromal cells in association with tumor cells, promotes angiogenesis in medulloblastoma or leukemic bone marrow and induces NRP1-mediated tumor cell proliferation (Schmidt et al., 2011). The elevated levels of PlGF were detected in cancer patients exposed to anti-angiogenic therapy; these observations suggest the involvement of PlGF in bypass angiogenic mechanisms under anti-VEGFR therapy. Nevertheless, animal model studies have clearly demonstrated that PLGF upregulation is in fact a host response to antiangiogenic therapy (Bagley et al., 2011). The circumstantial literature cited above clearly indicates that the concrete molecular underpinnings are yet to be established in VEGF-axis related angiogenic escape mechanism.

2.3 Redundant Angiogenic Mechanisms that are Independent of VEGF

Multiple molecular mechanisms have been hypothesized and currently being tested in various animal models and in clinical studies for unraveling the secrets of sustained tumor angiogenesis in the absence of VEGF. Amongst the various possible mechanisms described here, the compensatory role of other angiogenic factors in driving angiogenesis is more prominently discussed in the current mainstream of tumor resistance and anti-VEGF treatment (Petrillo et al., 2012). Moreover, the puzzle of crosstalk of multiple angiogenic pathways and the role of various

myeloid/stromal/tumor cells acquiring pro-angiogenic functions under VEGF blockade has not only accelerated the circumstantial research but also given strong impetus for tailoring novel therapeutic strategies to overcome tumor refractoriness to anti-VEGF therapy. Series of VEGF-independent factors and pathways like fibroblast growth factors 1 and 2 (Ronca et al., 2017; Welti et al., 2011), HGF/cMet pathway (Shojaei et al., 2010), angiopoietins (Eklund and Saharinen, 2013), and Delta-Notch signaling pathway (Li et al., 2011). PDGF-C (Crawford et al., 2009; di Tomaso et al., 2009), interleukins (Huang et al., 2010), Ephrins (Salvucci and Tosato, 2012) and epidermal growth factor (Cascone et al., 2011) have been described as inscrutable players in anti-VEGF escape mechanisms. The details of the abovementioned angiogenic factors and their redundant angiogenic signaling pathways are summarized in **Figure 2.**

2.3.1 Fibroblast Growth Factors (FGFs) and Anti-VEGF Escape Mechanisms

In fact, research on FGF and its role in angiogenesis has begun early, because FGFs were among the first proangiogenic factors identified (Folkman 1971). FGFs is a family of 22 structurally-related ligands involved in regulating multiple fundamental pathways related to embryonic development, organogenesis and cellular behavior by interacting with FGF receptors: FGFR (Turner and Grose, 2010; Ornitz and Itoh, 2015). In humans, the FGF family comprises of at least 18 ligands (FGFs) and 4 FGFRs. Like the VEGF-A molecule, FGFRs 1, 2, and 3 undergo alternative splicing resulting in multiple isoforms of FGFR displaying specific binding affinity with set of FGFs. Heparin sulphate proteoglycans (HSPGs) plays a crucial role in maneuvering different isoforms of FGFRs. There are several reports of altered FGFR signaling in different cancers such as breast, bladder, benign skin tumors and prostate. FGFR1, 2, 4 were reported to be upregulated in breast cancer cells with mutations in FGFR2 and 4 (Chae et al., 2017).

Degradation of ECM, EC proliferation, migration and organization of ECs into a tubular assembly are the major concerns of neovascularization, wherein FGF signaling plays a key role. Degradation of the ECM is the basic prerequisite for detaching ECs from the complex network of ECM. The signaling functions of FGFs 1, 2, and 4, are directly or indirectly involved in stimulating ECM degradation and activating matrix metalloproteinases. Once ECs are made free from ECM, their proliferation and migration at the destination is essential for new vascular orchestration. It has been described that FGFs 1, 2, 4, and 8 activate FGFRs 1, 2 and the signaling pathway through the activation of MAPK and PKC leading to proliferation of ECs (Turner and Grose, 2010; Ornitz

and Itoh, 2015). Integrin and cadherin receptors play an important role in cell-cell adhesion: a process needed for EC migration. FGF2 regulates the expression of several integrin and cadherin receptors. In general, the primary concerns of FGF2 is attributed to regulation of cellular motility and orchestration of ECs into capillary-like structures through autocrine signaling. Numerous animal model studies have demonstrated that aberrant FGF signaling mainly promotes tumor growth through increased cell proliferation, survival and angiogenesis. Therefore, FGFs have been revisited for remodeling FGF traps as a new therapeutic approach in the mainstream of targeting tumor angiogenesis (Matkar, et al., 2017).

Amongst many compensatory pro-angiogenic pathways, FGF signaling has been described to be associated with adaptive tumor resistance to anti-angiogenic therapy (Tran et al., 2016). The major manifestation of binding of FGFRs to FGF ligands results in the triggering of several signaling pathways like phosphatidylinositol 3-kinase (PI3K-AKT-mTOR), phospholipase Cγ-protein kinase C (PLCγ-PKC), janus kinase-signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase: (MAPK), RAS/RAF/MAPK and RAS/MAPK/ERK (Turner and Grose, 2010). The activation of FGF-driven pathways and crosstalk of these pathways regulates several proangiogenic physiological processes like cell growth and ultimately leads to angiogenesis under an anti-VEGF environment. Therefore, FGFs have been reported to drive angiogenesis independently of VEGF (96). FGF signaling is also believed to be involved in regulation of epithelial-to-mesenchymal transition (EMT) lymphangiogenesis and tumor metastasis via VEGF-C-mediated pathways (Larrieu-Lahargue et al., 2012). The VEGF-independent role of FGFs has been also demonstrated in a RIP-Tag model, wherein FGF-1 and -2 were overexpressed in tumors that relapsed after treatment with DC101: an anti-VEGFR antibody. In several preclinical models, FGF2 levels were reported to be upregulated upon arresting the VEGF-pathway. In *in vivo* animal model studies, it has been clearly demonstrated that inhibition of FGF reduces the growth of anti-VEGF resistant tumors (Winterhoff and Konecny, 2017). In animal model studies, mice were initially treated with the VEGFR inhibitor alone followed by treatment with FGF-trap (FGFR–Fc fusion protein) at the peak of the response phase of the VEGFR inhibitor. The combination treatment strategy significantly reduced the recurrence of vascularization and tumor growth. The findings of the study clearly indicate the involvement of FGF signaling in regulating restoration of angiogenesis. A clear clinical evidence of FGF-mediated revascularization was observed in patients with recurrent glioblastoma experiencing the treatment

of the potent VEGFR inhibitor Cediranib (Recentin, Astra Zeneca). Nevertheless, upregulation of FGF-2 was also observed in the drug holiday in patients (Lieu et al., 2011). In a xenograft mouse model study, designed for profiling gene expression patterns in relation to resistance to VEGF inhibitors, the FGFR and EGFR pathways were found to be upregulated in the stroma (Cascone et al., 2011). There exists a substantial crosstalk between FGF and VEGF pathways leading to excessive angiogenesis, however in some cases, FGF-induced signaling may confer resistance to VEGFR inhibitors (Casanovas et al., 2005; Winterhoff and Konecny, 2017). Most frequently, these two pathways work in synergy for the promotion of angiogenesis. The action of the two pathways is complementary to each other, wherein FGF-2 mediates the upregulation of VEGF and VEGFR in ECs, on the other hand, VEGF upregulates the expression of FGF2. In xenograft model studies, co-expression of FGF2 and VEGF were observed to be associated with high vessel density in fast growing tumors. In an interesting experimental setting designed to understand the mechanism of synergistic functions of VEGF-A and FGF-2, it was observed that there was enhancement of endogenous PDGFB–PDGFRβ signaling pathway. In a cell type specific expression pattern, VEGF-A was found to enhance endothelial PDGF-B expression, while FGF2 was associated with enhancement of mural PDGF receptor (PDGFR). The results of the study demonstrated the indispensable role of enhancement of endogenous PDGFB signaling pathway in coordinating the synergistic functions of VEGF-A and FGF-2 in neovascularization. FGF2 also functions in synergy with PDGF-BB and promotes murine tumor angiogenesis and metastasis (Yu et al., 2017). Nevertheless, PDGFB signaling pathway has been identified as one of the important compensatory pathways playing crucial role in anti-VEGF rescue mechanism by promoting neovascularization in stroma cells (Xue et al., 2012).

Interestingly, the synergistic functioning of FGF and VEGF also upregulates the expression of Dll4 (delta like 4 ligand in notch signaling) in ECs. The involvement of Dll4 is proved as an important mediator in anti-VEGF escape mechanisms and indispensable for EC proliferation, migration and overall orchestration of vascular network formation (Li et al., 2011). Owing to the synergy of FGF and VEGF functions in neovascularization, the inhibition of one pathway may adversely affect the functioning of the other pathway. However, paradoxically to the role of FGF in the anti-VEGF escape mechanism, there are several reports describing the inhibition of FGF-induced angiogenesis by anti-VEGF agents. In *in vitro* and *in vivo* experimental settings, it was observed that anti-VEGF antibody inhibits FGF2-promoted angiogenesis (Alessi et al., 2009).

2.3.2 Dll4-Notch Signaling: Tip/Stalk Cell Fate Promoting Angiogenesis

The NOTCH signaling pathway was originally discovered in *Drosophila* and was named after the notched wing appearance of the first mutant allele in *Drosophila*. Since its establishment as a pathway, it has been identified in virtually all metazoans and extensively studied in worms, flies and mammals. This pathway is an evolutionary conserved system and is associated with regulation of cell fate specification, tissue patterning and morphogenesis, especially in cell differentiation during embryonal and postnatal development, cell proliferation, apoptosis and survival. Mammalian Notch signaling system comprises of four single-pass transmembrane receptors (Notch 1-4) and five canonical DSL (Delta, Serrate, Lag2) ligands called Delta-like ligand 1, 3 and 4 (Dll 1, 3 and 4) and Jagged 1, 2. Amongst the Notch receptors, Notch1 and 4 are expressed by ECs, while excluding Dll3, all Notch ligands have been expressed by ECs. A plethora of recent research findings has clearly demonstrated that activation of the Notch signaling pathway is associated with several aspects of vascular guiding and development (Li et al., 2011; Nowell and Radtke, 2017). *Per se*, all the Notch receptors and their ligands are involved in modeling tumor vasculature. However, in-depth research findings have identified that the Dll4-Notch 1 signaling axis plays a dominant role in tumor angiogenesis and vascular system (Garcia and Kandel, 2012). More recently, the vascular concerns of Dll4 have been proved to be a critical regulator of tumor angiogenesis and thus emerging as an attractive therapeutic target in the mainstream of anti-tumor agents targeting tumor angiogenesis (Brahmi et al., 2017; Kuhnert and Kirschner, 2011). The emerging circumstantial experimental evidence suggests that blocking the activity of Dll4 in tumors, results in excessive but non-productive vasculature adversely affecting the tumor growth, even in tumors which are resistant to anti-VEGF agents (Guo et al., 2014).

Dll4-Notch1 signaling plays a crucial role in maintaining branching network at neovascular site. The crosstalk between VEGFR2 and Notch is involved in coordinating the kinetics of sprouting angiogenesis. In this process, coordination between migrating tip cells and proliferative stalk cells maintains the tip cell number and thereby branching during sprouting angiogenesis. Activated ECs extend filopodia at the leading edge and migrate towards angiogenic cues. In the forefront of VEGF-rich ECs, tip cell migration is guided through the activation of VEGFR2 by

VEGF. Internalization of VEGFR2 (receptor turnover) and activation of ERK1/2 signaling play a significant role in sprouting angiogenesis. Nrp1 (the co-receptor of VEGF) promotes tip cell activity by enhancing VEGFR2 (and VEGFR3)-mediated signaling. Notch signaling regulates the specification between tip and stalk cells (Geudens and Gerhardt, 2011; Logsdon et al., 2014). ECs having activated VEGFR2 upregulate the expression of Dll4 and struggle for the tip position. Dll4 binds to Notch receptors on adjacent ECs and releases Notch intracellular domain (NICD). as a transcription factor, NCID down-regulates *Vegfr2* and *Nrp1* expression and upregulates the VEGFR1, which binds to VEGF. Thus, NCID coordination deprives stalk cells from binding to VEGF. In brief, the negative feedback loop of VEGF mediated Dll4 activation (VEGF-to-Dll4) in tip cells and Notch-mediated inhibition of VEGFR2 (Notch-to-VEGFR2) in stalk cells, permits ECs to sprout and also balances the heterogeneous response of ECs towards angiogenic cues. It is this mechanism, which explains why Dll4-Notch inhibition results in hyperbranching, while normal function reverses the effect (Geudens and Gerhardt, 2011; Logsdon et al., 2014).

Several new experimental settings have unraveled the insights into the function of Dll4-Notch signaling in tumor angiogenesis (Benedito et al., 2013) and the related molecular mechanism of vascular defects arising from inhibition of Dll4-Notch functions. Dll4 is also upregulated in several cancers and the overexpression of Dll4 is correlated with tumor refractoriness to anti-VEGF agents (Li et al., 2011; Kuhnert and Kirschner, 2011; Brzozowa et al., 2013). Sizable literature has been cited towards the role of Dll4-Notch signaling pathway as one of the culprits in conferring tumor resistance to anti-angiogenic drugs (Bergers and Hanahan, 2008; Li et al., 2011; Benedito et al., 2012; Brzozowa, et al., 2013). In an interesting *in vivo* experimental setting, human glioblastoma cells were transduced with retroviruses encoding Notch-Dll4 followed by growing them as tumor xenografts and then the VEGF-A inhibitor bevacizumab was used to treat murine hosts. Dll4-mediated tumor resistance to bevacizumab was clearly observed *in vivo* (Li et al., 2011). It was observed that the Dll4-Notch-induced large vessels increased tumor blood supply and were found to be resistant to bevacizumab. The large vessel disruption and tumor resistance was abolished with the treatment of Notch-Dll4 inhibitor dibenzazepine (a γ-secretase inhibitor). Multiple molecular mechanisms of resistance such as down-regulation of hypoxia-induced VEGF, upregulation of VEGFR1 in the tumor stroma, down-regulation of VEGFR2 in large blood vessels, and overall reduced levels of VEGFR3. Tumors expressing high levels of Dll4 were found to be resistant to a VEGFR-targeted multikinase inhibitor. Nevertheless, Dll4-Notch

signaling has also activated other compensatory tumor resistant pathways like FGF2-FGFR and EphB4-EprinB2 (Li et al., 2011). The results of these studies clearly demonstrated the anti-VEGF compensatory nature of Dll4-Notch signaling and its role in activating other alternative pathways of tumor resistance.

Several animal model studies involving gain-of-function and loss-of-function experiments have clearly showed that the ECs with disrupted functions of VEGF or Notch die prematurely because of defective embryonic vasculature (Blanco and Gerhardt, 2013). Several recent studies have demonstrated the significance of Notch signaling agents such as Dll4, Jagged-1 (JAG1) and Notch1 in EC guidance and development of a functional vascular network. Recent literature describes that DLL4 and JAG1 modulate tumor angiogenesis via different mechanisms. It is now clear that JAG1 is not antagonistic but utilizes DLL4 in tumor angiogenesis. Therefore, clinical Notch therapies should explore the combination of anti-DLL4 and anti-JAG1 therapy modalities as a novel anti-angiogenic regimen (Oon et al., 2017). An interesting experimental setting was designed to test the hypothesis that Notch inhibition is responsible for switching VEGFA-VEGFR2-dependent angiogenesis to VEGFC/D-VEGFR3-regulated mode of angiogenesis. The outcome of the experiment confirmed that, during VEGFA-VEGFR2-mediated angiogenesis, Notch activation downregulates the expression of VEGFR3 in ECs, however, when Notch signaling is low or inhibited, there is significant increase in VEGFR3 levels, which leads to highly deregulated excessive VEGF-independent angiogenesis (Benedito et al., 2012). Notch inhibition strongly impairs tumor growth, even in tumors showing anti-VEGF resistance (Djokovic et al., 2015 Noguera-Troise et al., 2006). The findings clearly indicate that Notch signaling contributes significantly to compensatory angiogenesis.

Perhaps the role of Dll4-Notch signaling in activating other compensatory angiogenic pathways might play a crucial role in rendering tumors more “arrogant” towards anti-VEGF therapy, as there is clear *in vivo* experimental evidence that DLL4-Notch signaling mediates tumor resistance to anti-VEGF therapy (Li et al., 2011). The Dll4-Notch signaling pathway functions as a regulator of angiogenesis in the downstream domain of VEGF cluster. The expression levels of Dll4 in tumor vessels correlate with those of VEGF, indicating the plausible role of VEGF in regulating Dll4 expression (Noguera-Troise et al., 2006; Djokovic et al., 2015). Nevertheless, various model studies have proved the involvement of the VEGF pathway in regulating Notch signaling components (Thurston and Kitajewski, 2008; Teodorczyk et al., 2015). In clinical

studies, it was observed that patients responding positively to anti-VEGF therapy were found to have low levels of Dll4 than patients with progressive tumorigenesis (Hu et al., 2011). In the process of vessel stabilization, Dll4-Notch signaling also influences ECs and pericytes for proper assembly of vessel plexus (Zhang et al., 2011). In NGP neuroblastoma model studies, the simultaneous blockade of Notch and VEGF resulted in blood vessel regression, disruption of pericyte coverage of ECs and increased apoptosis of ECs. However, the combined blockade did not affect tumor weight, but tumor viability was adversely affected. The findings clearly suggest the distinct but complementary role of Notch and VEGF pathway in tumor angiogenesis (Hernandez et al., 2013). Other angiogenic rescue pathways such as FGF, Angiopoietin-1/Tie2, Wnt, some of the inflammatory cytokines, components of extracellular matrix are reported to induce the expression of Dll4 in ECs (Corada et al., 2010; Zhang et al., 2011; Estrach et al., 2011). In a recent investigation, the consequence of Notch blockade was observed to be associated with VEGF-Ang2-mediated invasion of ECs, tube formation, and expression of MMPs and cytokines. The outcome of the experiment indicates the Notch-driven pro-angiogenic effects of VEGF-Ang2 (Gao et al., 2013). The angiogenic concerns of Dll4-Notch signaling are also attributed to ephrin-mediated pathway. EphrinB2 has been identified as a key Dll4-Notch target gene in cultured ECs, but also acts as a regulator of VEGFR endocytosis and signaling in the upstream part of the Dll4-Notch signaling pathway (Sawamiphak et al., 2010). Inhibition of EphrinB2 signaling using soluble EphrinB2-Fc in a subcutaneous squamous cell carcinoma resulted in reduced tumor growth along with the induction of non-productive angiogenesis. Similar effects are manifested in the case of Dll4 blockade (Kuhnert et al., 2015). The results clearly indicate that EphrinB2 acts as a downstream mediator of Dll4/Notch activity. Several reports report about the cross talk of VEGF, Notch and transforming growth factor-β (TGFβ) in modeling and patterning the proper architecture of vasculature. Perhaps, it is beyond the scope of this review to register the molecular underpinnings, cues and consequences of interactions of these pathways. However, a review compiled by Jin et al., provides the detailed insights of coordinated acting of VEGF, notch, and TGFβ in concert with proper orchestration of the sprouting angiogenesis and vascular patterning (Jin et al., 2014). The functional importance of some of the Dll4-Notch interacting pathways in relation to inhibition of Dll4-Notch in tumors is still not fully understood. However, there is a ray of hope of combining Dll4 inhibitors with anti-angiogenic agents as inhibitors of Dll4 to disturb normal angiogenic switch and inducing generation of excessive but non-functional vasculature,

which can be possibly used as an alternative approach for arresting anti-VEGF resistant tumor growth (Benedito et al., 2012; Crawford and Ferrara, 2009).

2.3.3. HGF/c-Met Pathway: A Hub of Pro-angiogenic Crosstalk

The name hepatocyte growth factor (HGF) was assigned to this factor as it was discovered independently as a mitogen for hepatocytes. It is also known as scatter factor as it induces scattering of polarized epithelial cells. Primarily, mesenchymal cells (or endothelial and stellate cells in the liver) secrete HGF. Apart from its role as a growth factor, it is now identified as an indispensable organotrophic factor in many tissues and there are many other physiological concerns of this 90-kD secreted protein as recently reviewed (Imamura and Matsumoto, 2017). HGF plays a significant role in driving intracellular signal transduction by binding to its receptor known as cellular-Met (c-Met). The interaction of HGF with c-Met is a paracrine signaling loop. The process of signal transduction initiates with dimerization of c-Met, followed by its tyrosine autophosphorylation when ligand binding takes place. The consequence of ligand binding leads to the formation of active docking sites for proteins that are involved in mediating downstream signaling, resulting in the activation of the phosphatidylinositol 3-kinase (PI3K)-AKT, mitogen-activated protein kinase (MAPK), v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC), Ras/Mek/Erk and signal transducer and activator of transcription (STAT) signaling pathways. Series of inflammatory modulators such as tumor necrosis factor α (TNFα), IL-6, IL-1, TGF-β, and VEGF are involved in upregulation of HGF (Blumenschein et al., 2012; Imamura and Matsumoto, 2017).

The HGF-cMET pathway acts as a hub for crosstalk with multiple heterogeneous signaling networks and is regulated by other RTK members such as the human EGFR 2, EGFR, Raf kinase, insulin-like growth factor 1 receptor and VEGF. The major consequence of HGF signaling is enhancement of angiogenesis and activation of cellular proliferation, survival, migration and invasion via modulation of epithelial-mesenchymal interactions (Gherardi et al., 2012; Imamura and Matsumoto, 2017). In tumors, mostly fibroblasts and other interstitial cells are known to express HGF, however, pro-angiogenic cells such as ECs, pericytes and a variety of other cells like epithelial cells, neural cells, hematopoietic cells and even cancer cells themselves produce HGF. The c-MET and/or HGF is also considered an important biomarker, owing to its upregulation, activation and gene amplification in several types of cancers including non-small cell lung carcinoma (NSCLC), liver, gastric, breast, ovarian, pancreatic, head and neck, thyroid,

colon and kidney (Sierra and Tsao, 2011). A comprehensive list of solid and soft tumors having upregulated patterns of c-MET and HGF is currently available (www.vai.org/met). High expression profile of c-MET and HGF are strongly correlated with poor prognosis, increased tumor growth rate, metastasis and resistance to radiotherapy (Sierra and Tsao, 2011).

A large body of preclinical and clinical data described the pro-angiogenic attributes of c-Met/HGF signaling in concert with other pro-angiogenic pathways. HGF induces stromal cells to increasingly express VEGF. Moreover, the synergistic functions of HGF and VEGF lead to the induction of EC proliferation and tube formation. *In vitro* and *in vivo* studies clearly demonstrated the role of HGF in promoting growth of ECs (Goyal et al., 2013). In transgenic mice having induced hepatic adenomas and hepatocellular carcinoma, HGF induced transcription of VEGF and excessive angiogenesis. The synergistic crosstalk between the HGF/c-Met and VEGF signaling are reported to have significant positive effect on cell proliferation and migration of ECs (Sulpice et al., 2009). Similarly, crosstalk between c-Met and EGFR is also implicated in supporting tumor cell survival. The interaction of c-Met-cSrc results in phosphorylation of EGFR and survival of cells even in the presence of EGFR inhibitors (Velpula et al., 2012). It has been shown that hypoxia is one of the important factors for upregulation of HGF in tumor and stromal cells in the tumor microenvironment. The hypoxia-driven upregulation of HGF/c-Met signaling in the tumor acts as an inducer of overexpression of VEGF-VEGFR2 in ECs and down-regulate endogenous inhibitors of angiogenesis. Thus, c-Met is an independent angiogenic factor and it also responds to pro-angiogenic signals generated through VEGF.

Numerous studies have shown that HGF plays an important role in VEGF expression thereby recruiting angiogenesis in a paracrine fashion. Ample scientific evidence has recently accumulated linking the role of c-Met/HGF signaling pathway as an alternate pathway for driving VEGF-independent angiogenesis. Plethora of literature has considered HGF as one of the culprits of drug resistance (Razzak, 2012). However, in one interesting study, it was observed that both HGF and VEGF induce angiogenesis, however the synergistic functioning was not observed between the two growth factors. It was further found that selective inhibition of VEGFR by PTK787, did not affect the HGF-induced neovascularization, but it arrested VEGF-mediated angiogenesis. The finding of this investigation clearly advocates the leading role of HGF/c-Met signaling in inducing neovascularization through a VEGF-independent signaling pathway. Perhaps HGF/c-Met might be driving the VEGF compensatory angiogenesis pathway through activation

of the cascades of PI3K and MAPK. As PI3K- and MAPK-mediated pathways have been implicated in HGF- and VEGF-mediated cell proliferation. Moreover, activation of these two pathways leads to initiation of angiogenesis (Garajová et al., 2015). Preclinical experimental evidence clearly attributed the role of HGF/c-Met signaling to conferring resistance towards sunitinib: a clinically approved drug for metastatic renal cell carcinoma (RCC). While investigating the efficacy of sunitinib in experimental *in vivo* models, it was observed that tumor protein lysates of sunitinib-resistant tumors had elevated levels of HGF as compared to sunitinib-sensitive tumors. Further analysis revealed that c-Met expression was found to be upregulated in ECs than in tumor cells, indicating that HGF might be targeting vascular ECs in sunitinib-resistant tumors. Interestingly, exogenous application of HGF in sensitive tumor models developed resistance towards sunitinib by maintaining tumor angiogenesis (Shojaei et al., 2010). The outcomes of the study clearly outline the role of HGF/c-Met as compensatory angiogenic pathways that might undergo activation as a result of VEGF blockade.

In patients of hepatocellular carcinoma (HCC) undergoing treatment with sorafenib (a VEGFR inhibitor), clinical prognosis was significantly correlated with low levels of serum HGF, as compared to patients having progressive disease with high levels of serum HGF (Miyahara et al., 2011). In another clinical observation it was found that there was a progressive increase in HGF levels and re-enlargement of tumors of metastatic colorectal cancer when treated with bevacizumab (anti-VEGF antibody). These findings underlie the possible relationship between the HGF/c-Met signaling pathway and resistance towards VEGF inhibitors (Kopetz et al., 2010). Besides the role of HGF/ c-Met signaling pathway in tumor refractoriness against anti-VEGF agents, the pathway is also attributed to resistance towards tyrosine kinase inhibitors like sunitinib; for example, in xenograft model studies it was demonstrated that targeted inhibition of c- Met successfully ameliorated sunitinib resistance in metastatic RCC (Zhou et al., 2016). Multiple pathways like HGF/c-Met/Akt and JAK2/STAT3 were observed to be involved in conferring resistance towards sorafenib in HCC (Huh7) cells in co-culture studies (Chen et al., 2014). Recently, it has been shown that the cooperative activity of VEGF and HGF induces tumor angiogenesis in HGF-rich cancer cells, and elevated levels of HGF are strongly associated with resistance to lenvatinib (a VEGFR inhibitor) (Nakagawa et al., 2014). In more recent preclinical studies involving human HGF knock-in in SCID mice, the findings indicated that stroma-derived HGF drives metabolic adaptation of colorectal cancer to angiogenesis inhibitors (Mira et al., 2017).

Similarly, the HGF/c-MET pathway has been clearly shown to be involved in conferring VEGFR inhibitor resistance and vascular remodeling in NSCLC (Cascone et al., 2017)

2.3.4 Angiopoeitins/Tie axis: A Suspicious Driver of Anti-VEGF Rescue Mechanisms

Angiopoeitins (ANGs) are an important class of angiogenic factors playing a regulatory role in angiogenesis. ANGs are primarily more proactive towards monitoring the development of blood vessels and their stability. Their critical role is implicated in progression of tumors and certain cancer types attracted researchers to target their activity for enhancing the efficacy of anti-angiogenic therapy (Eklund and Saharinen, 2013). In humans, the ANGs family consists of three members including ANG1, ANG2 and ANG4, however the overall research in relation to angiogenesis is centered around ANG1 and more focused on ANG2. ANGs exert their activity by binding to receptors termed Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptors 1 and 2 (Tie 1 and 2). ANG1 and ANG2 have been identified as ligands for Tie 2, whereas Tie 1 remains an orphan receptor without a specific ligand but it heterodimerizes with Tie 2 for the manifestation of its biological activity (Gerald et al., 2013). ANG1 has been described as a strong Tie2 agonist, mostly produced by tumors as well as mural cells such as pericytes, vascular smooth muscle cells and fibroblasts which act in a paracrine manner. The main function of ANG1/Tie2 signaling is implicated in blood vessel maturation and stabilization. However, ANG2 is mainly produced by ECs, acts in an autocrine manner and is involved in remodeling of blood vessels. ANG2 largely functions as a Tie2 antagonist to promote tumor angiogenesis and inflammation. A study has reported that ANG2 may act as a context-dependent Tie2 agonist (Daly et al., 2013).

The circumstantial literature accumulated recently clearly outlines the controversies related to anti-VEGF compensatory role of ANG1 and 2 in supporting tumor growth and angiogenesis (Eklund and Saharinen, 2013). However, there are many reports describing the plausible role of ANG/Tie signaling in sustaining tumor angiogenesis during anti-VEGF therapy. It is believed that anti-angiogenic therapy itself may induce angiogenic rescue mechanisms that escape tumor angiogenesis. For instance, there is a clear evidence of upregulation of ANG-1 and other compensatory pro-angiogenic factors in tumors treated with anti-VEGFR2 agents (Mazzieri et al., 2011). Treatment with VEGFR2 blockers has been believed to normalize the vascular architecture by placing proper covering of pericytes over the torturous and leaky vessels. The ANG1/Tie2 signaling pathway has been implicated in vessel normalization. Such normalized vessels might act

as an angiogenic escape mechanism. Vessel cooption has been proved to be one of the anti-VEGF escape mechanisms, wherein angiopoietins and VEGF are reported to play a significant role (Holash et al., 1999).

There are several reports of upregulation of ANG1 and ANG2 levels in tumors (Eklund and Saharinen, 2013; Scholz et al., 2016). The ratio of expression levels of ANG2/ANG1 correlates with tumor angiogenesis and poor prognosis in many cancers. ANG2 has been considered as an attractive therapeutic target owing to its role in increasing pericyte coverage and vessel maturation (Scholz et al., 2016). Tumor ECs produce elevated levels of ANG2, which interacts with Tie2 in autocrine and paracrine manners and promotes neovascularization in concert with other angiogenic factors (Eklund and Saharinen, 2013). Series of experimental and clinical model studies have demonstrated inhibition of tumor angiogenesis and attenuation of the growth rate of tumors owing to ANG2 blockade. The results are often more impressive when combined with VEGFA/VEGFR2 inhibitors, increasing the progression-free survival of ovarian cancer patients (Mazzieri et al., 2011; Daly et al., 2013).

In a focused animal model setting, two mouse models, RIP1-Tag2 pancreatic neuroendocrine tumors (PNETs) and MMTV-PyMT mammary adenocarcinomas were used to ascertain the putative role of ANG2 in adaptive tumor resistance to anti-VEGFR2 agents. The experiments in mouse and cell cultures confirmed that simultaneous blockade of ANG2/VEGFR2 arrests the revascularization and progression of late-stage PNETs in RIP1-Tag2 along with increase in PNET hypoxia, hematopoietic-cell infiltration and decrease in invasion and metastasis in RIP1-Tag2 Mice. Interestingly, blockade of VEGFR2 alone resulted in upregulation of both ANG2 and Tie2 in late-stage PNETs, perhaps to reinforce autocrine/paracrine pericytes ANG2-Tie2 signaling in ECs and Tie expressing macrophages (TEMs). The authors suggested the potential use of ANG2 levels as a predictive biomarker of response to bevacizumab in patients with PNET. Upregulated levels of ANG2 were correlated with poor prognosis with bevacizumab treatment in MMTV-PyMT mammary carcinomas. The overall findings of this interesting investigation have unraveled some of the key issues related to evasive tumor resistance to anti-VEGFA and the adaptive role of ANG2-Tie2 signaling in sustaining tumor growth and angiogenesis (Karlan et al., 2012).

Tie2-expressing macrophages/monocytes (TEMs) have been identified in human as well as murine tumors. TEMs are specifically localized in the close proximity of tumor blood vessels

and proved to play a significant role in enhancing tumor vasculature in anti-VEGF compromised tumors. Nevertheless, TEMs expression by macrophages was observed to be necessary to promote the reconstruction of blood vessels after chemotherapy (Crawford and Ferrara, 2009; Mazzieri et al., 2011; Chen et al., 2016). ANGs/Tie2 signaling was shown to be a key regulator of leukocyte infiltration and tumorigenesis. Upregulation of ANG2 levels in tumor vasculature is implicated in enhancing TEMs recruitment and increasing tumor microvessel density (Coffelt et al., 2010). ANG2 expedites the pro-angiogenic functions of TEMs and coordinates the upregulation of the pro-angiogenic enzymes, thymidine phosphorylase and cathepsin B present in TEMs. Mazzieri et al., (2011) clearly identified a number of unique molecular signatures of regulation of TEMs by ANG2 in tumors (Mazzieri et al., 2011). The experimental outcomes of Mazzieri et al., (2011) holds promising therapeutic importance of targeting the ANG2/Tie2 axis, which perhaps may increase the therapeutic index of anti-angiogenic therapy and may also prohibit the ANG2-sponsored recruitment of insidious myeloid cells, which per *se* play an important role in anti-VEGF compensatory signaling (Crawford and Ferrara, 2009; Mazzieri et al., 2011).

Although the secrets of molecular underpinnings driving the multiple vascular responses mediated by ANG1 and ANG2 are poorly understood, it is hypothesized that ANG1, ANG2 and their different oligomerization states may differentially modulate the subcellular localization of Tie2 or perhaps may interact with distinct cellular or matrix co-receptors for the regulation of vascular development in stressed or pro-angiogenic negative environment (Eklund and Saharinen, 2013). Crosstalk of ANG/Tie2 signaling contributes to activating the other compensatory angiogenic pathways like Dll4/Notch signaling. For instance, ANG1/Tie-2 signaling acts as an inducer of Wnt/β-catenin pathway via the PI3K/Akt-mediated blockade of GSK3β, which ultimately results in upregulation of Dll4/Notch signaling: a pathway implicated in anti-VEGF tumor resistance (Li et al., 2011). Paradoxically to the current understandings of ligand-receptor interaction of ANG2/Tie2 axis, a critical study reports the pro-angiogenic activity of ANG2 in a Tie2 independent manner, intensifying the amplitude of multiple crosstalk horizons of ANG2/Tie2 pathway. Activated integrins-mediated down-regulation of Tie2 and ANG2 signals were observed in angiogenic endothelial tip cells. Activated integrins induce focal adhesion kinase (FAK) signaling which results in angiogenic sprouting (Felcht et al., 2012). In recent animal model studies, it was observed that VEGF was not associated with corneal neovascularization in Kelch-like Ect2-interacting protein (KLEIP) knockout mice; however, ANG1 was implicated in pro-

angiogenic activity (Kather et al., 2014). In a cell culture experimental setting involving transfection of human SK-NEP-1 cells with the ANG1, several key issues have been addressed in light of the role of ANG1/Tie2 signaling during VEGF blockade. The results revealed that induced expression of ANG1 induces vessel remodeling, reduces tumor hypoxia, vascular ablation, but does not affect tumor size during VEGF blockade. Tumor engineered to overexpress an ANG1 construct were found resistant to regression by anti-VEGF agents. In brief, the findings of the study clearly showed that ANG1/Tie2 signaling contributes significantly to vascular survival, tumor growth and act as a plausible anti-VEGF rescue mechanism (Huang et al., 2009). Perhaps, for the first time, the role of ANG1, ANG2 and ANG4 are described as inducers for creating a precancerous microenvironment in favor of progression of ovarian cancer cells by stimulating angiogenesis and more interestingly by promoting the accumulation of cancer-associated fibroblasts (CAFs). The ANGs triggered CAFs might act as a vehicle for promoting compensatory angiogenesis, as CAFs or tumor-associated fibroblasts are described as a ‘‘Trojan horse’’ for mediating resistance to anti-VEGF regimens by expressing pro-angiogenic PDGF-C, which in turn induces sprouting angiogenesis (Crawford et al., 2009; Bruncкhorst et al., 2014). Thus, the current state-of the-art indicates the indispensable role of ANGs/Tie2 in driving pro-angiogenic activities in the mainstream of anti-VEGF compensatory signaling and warrants further investigations to resolve this imbroglio.

2.3.5 PDGF Signaling: A Pathway Delivering Pro-angiogenic Education to Tumor/Stromal cells

Apart from myriad physiological functions performed by platelet-derived growth factor (PDGF), their concerns towards regulation of tumor angiogenesis and vascular remodeling are revisited because of providing growth and developmental safeguards to anti-VEGF compromised tumors. The family of PDGF comprises of four single polypeptide chains having structural similarity and assembles into five functionally active dimers (homo- and heterodimers) such as PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD. Members of the PDGF family manifest their functions by binding to two cell surface RTKs including PDGF receptor- α (PDGFR-α) and PDGFR-β. This ligand-receptor binding leads to homo- or heteroreceptor dimerization. The individual members of PDGF display a distinct pattern of receptor binding and under normal

physiological conditions, their biological actions are delivered in a paracrine manner. Perhaps, the cell-specific transduction of PDGFR-α and PDGFR-β might be a potential cause for divergent functions of PDGFs. The major consequences of PDGF-PDGFR signaling at the cellular level leads to activation of processes like cell proliferation, migration, transformation and recruitment of pericyte to vessels (Ishii et al., 2017).

Of note, members of PDGF family and their receptors are expressed both in tumor as well as stromal cells. The mounting evidence in clinical and preclinical research revealed that PDGF members like PDGF-BB and PDGF-DD, and both the PDGFRα and PDGFRβ receptors are upregulated in many cancers including lung, liver, breast, prostate, ovarian, melanoma, kidney, glioma, sarcoma and bone (Hedin, 2013). The gliomas *per se* are considered drug resistant tumors, however the expression of PDGFs and PDGFRs is observed even in low as well as high grade gliomas, suggesting a critical role for PDGF signaling in tumor resistance. In breast and glioma tumors, PDGFR signaling is primarily concerned with angiogenesis and metastasis (Appelmann et al., 2010). There are several reports describing the role of PDGF-B, -C, and -D in stimulating tumor angiogenesis through VEGF expression (Appelmann et al., 2010; Ahmad et al., 2011; Demoulin and Essaghir, 2014; Ishii, et al., 2017). In tumor transplantation model studies, the overexpression of PDGF-B in cancer cells was strongly correlated with the density and number of perivascular cells. When a PDGF-B secreting tumor was transplanted in the PDGF ret mouse, perivascular cells started detaching from the wall of the vessel. This phenomenon illustrates that perivascular cells are sensitive towards the ectopic sources of PDGF-B and migrate away from the ECs. The finding also reveals that PDGF-B not only acts as a mitogenic factor for precursors of perivascular cells, but is probably also involved in educating them for migration during tumor angiogenesis (Abramsson et al., 2003).

In a focused preclinical study, it was shown that PDGF-BB and FGF2 synergistically promote murine tumor angiogenesis. It was observed that simultaneous upregulation of PDGF-BB and FGF2 factors in murine fibrosarcomas resulted in formation of primitive and highly dense vascular plexuses, which had a poor coverage of pericytes and vascular smooth muscle cells (VSMCs). Interestingly, the sole expression of PDGF-BB in tumor cells leads to detachment of VSMCs from tumor vessels and also reduces pericyte coatings over the vessels. FGF2 activated the expression of PDGFR-α and -β in ECs, however, in the absence of FGF2, there was no response of capillary ECs towards PDGF-BB. The findings clearly identified the VEGF-independent

induction of disorganized neovascularization mediated by synergistic activity of FGF2 and PDGF-BB in a murine tumor model (Nissen et al., 2007). Perhaps, this is a unique example of cooperative angiogenesis by PDGF-BB with FGF2, where the role of FGF2 is well-established in driving compensatory angiogenesis under an anti-VEGF environment. It was reported that the effect of anti-PDGF agents may be context-dependent, as it was demonstrated that no significant added anti-angiogenic effect was observed when a combined treatment of anti-VEGF and anti-PDGF was tested in mouse models of colorectal and pancreatic cancer (McCarty et al., 2007). In recent experimental design, PDGF-BB has been shown to promote tumor angiogenesis and growth by induction of erythropoietin. Under such circumstances, PDGF-BB stimulates EC proliferation, migration and tube formation (Xue et al., 2012). An interesting study has been described recently with the aim to investigate the expression levels of PDGF-BB, PDGF-AA and FGF2 and their significance as prognostic/predictive markers before and during bevacizumab treatment. The results of this study clearly demonstrated the significant upregulation of these factors during treatment with bevacizumab and focused their importance as prognostic but not as predictive biomarkers (Madsen et al., 2012). The findings of the study hold importance in light of anti-VEGF escape mechanisms and reiterate the possibility of crosstalk and synergistic activity of these pro-angiogenic factors for sustaining tumor growth under VEGF blockade conditions (Mamer et al., 2017).

The mounting evidence in preclinical and clinical studies has shown that the PDGF-C can drive VEGF-independent angiogenesis in concert with other pathways through at least three ways. The first is by way of stimulating the vascular cells such as ECs, pericytes, smooth muscle cells and promoting their proliferation, survival and migration for recruiting neovasculature. The second way is through activation of stroma cells such as blood vessels, mesenchymal cells especially fibroblasts, ECM, inflammatory cells, lymphatic vessels, nerve cells, etc. These cells are active in creating a pro-angiogenic microenvironment for promoting new blood vessel formation. Fibroblasts in particular, have been identified as the major source of angiogenic growth factors in driving angiogenesis during anti-VEGF treatment (Kalluri, 2016). The third way of PDGF-C-mediated compensatory pathway is through activation of a variety of inflammatory cells, such as monocytes, neutrophils and macrophages. These cells produce an array of pro-angiogenic growth factors, cytokines and helps recruiting new blood vessels through proteolytic mechanisms (Ferrara, 2010). Macrophages in particular, are more concerned with driving VEGF-independent

angiogenesis. Several groups have described the role of PDGF-C in migration, proliferation and gene expression in macrophages (Li et al., 2010). PDGF-C therefore manifests compensatory angiogenic effects via macrophages.

Overall, the major role of PDGFs in anti-VEGF resistance is centered around activating stromal/perivascular proangiogenic cells implicated in conferring tumor drug resistance (Huijbers et al., 2016). The evidence to this observation can be witnessed from the fact that stromal cells such as fibroblasts, pericytes, ECs of the tumor stroma and even cancer cells have been shown to express PDGFR (Pietras et al., 2008; Taeger et al., 2011; Huijbers et al., 2016). Crawford et al., (2009) used two transplantable mouse lymphomas, one (TIB6) was sensitive and the other (EL4) was refractory to anti-VEGF antibody treatment. Upregulated expression of PDGF-C mRNA was observed in anti-VEGF resistant tumor cells along with activation of adjacent tumor-associated fibroblasts (TAFs), which secrete PDGF-C and trigger tumor angiogenesis. Perhaps this was the first report of its kind to demonstrate that TAFs can drive PDGF-C-mediated tumor angiogenesis (Crawford et al., 2009). Consistent with the same line of research, cancer-associated fibroblasts were also reported to secrete PDGF-CC which in turn stimulated angiogenesis via paracrine signaling (Xing et al., 2010). Although the concrete functions of PDGF-D are not known, evidence is accumulating in favor of its role in regulating angiogenesis, cell proliferation, transformation, invasion and metastasis by activating its cognate receptor PDGFRβ (Wang et al., 2009). In a recent investigation, the involvement of the PDGF-B/PDGFRβ axis in drug resistance to anti-angiogenic and other anti-vascular therapy has been clearly demonstrated in RCC (Cumpanas et al., 2016). Taken together, clinical and preclinical evidence warrants targeting of PDGF and PDGFR signaling axis, which might improve the therapeutic index of anti-VEGF drugs and help in overcoming drug resistance. Perhaps, more focused preclinical and clinical studies might unravel the concrete role of PDGF signaling in tumor resistance in general, and anti-VEGF tumor resistance in particular.

2.3.6 Interleukins in Anti-VEGF Tumor Resistance

The excitement of how inflammatory cues drive cancer progression and tumor-mediated angiogenesis is still a major interest of many cancer biology labs across the world. With the establishment of a link between pro-inflammatory cytokines and cancer progression, the scientific unrest addressing the functions of interleukins (ILs) in tumor angiogenesis has uncovered the

underlying mechanisms of ILs in tumor drug resistance. Members of ILs including IL-1, IL-6 (Dmitrieva et al., 2016), IL-8 (Huang et al., 2010), IL-12 (Del Vecchio et al., 2007) and IL-17 (Chung et al., 2013) have been attributed to tumor drug resistance under anti-VEGF therapy. These ILs promote angiogenesis in concert with other pro-angiogenic factors under different circumstances. Multiple *in vitro* studies have demonstrated the positive effects of IL-1 on ECs for their activation, increased migration, proliferation and ultimately their organization into vessel-like structures (Dmitrieva et al., 2016). In the tumor microenvironment, which happens to be a hub for pro-angiogenic crosstalk, IL-1 plays a key role in recruiting a variety of pro-angiogenic cells. IL-1 mainly exerts its angiogenic response in the tumor microenvironment, especially by interacting with stromal, malignant and inflammatory cells through paracrine/autocrine loops. In cancer patients, overexpression of IL-1 has been reported in several solid tumors such as breast, lung, colon, melanomas, head and neck. Of note, increased levels of IL-1 in the tumor microenvironment reported in several clinical and preclinical models, are associated with more virulent tumor phenotypes (Elaraj et al., 2006). IL-1 has been reported to have dramatic effects on the development of myeloid cells in the bone marrow and their subsequent recruitment to tumor sites and induce them to secrete angiogenic factors in the tumor microenvironment (Dmitrieva et al., 2016). Myeloid cell-driven angiogenesis has been implicated in tumor refractoriness or drug resistance to VEGF inhibitors (Lee et al., 2015). IL-1 possibly mediated activation of myeloid cells in the tumor microenvironment, hence serving as a contributing factor for tumor progression and angiogenesis under VEGF-independent modality.

Although there is limited experimental evidence articulating the role of IL-6 and IL-12 in anti-VEGF tumor drug resistance, certain circumstantial observations focus on the plausible involvement of these pro-inflammatory cytokines in tumor refractoriness. For example, upregulation of IL-6 in glioma cells is associated with cell proliferation, angiogenesis, resistance to apoptosis and the overall progression of malignancy. Consistently, IL-6 knockdown resulted in reduced vascularization of the tumors; however, there was increased invasion of residual tumor cells. The combined knockdown of IL-6/VEGF resulted in significant reduction in angiogenesis, tumor growth and prevented invasion of residual tumor cells. Interestingly, treatment with bevacizumab in IL-6 knockdown mice completely abrogated tumor development (Dmitrieva et al., 2016). In xenograft mouse model studies, which mimicked clinical resistance to sunitinib, high density of microvessels was observed in sunitinib-resistant tumors indicating the anti-VEGF

escape of the tumors. Interestingly, the anti-VEGF escape coincided with secretion of IL-8 by tumor cells into the plasma and the anti-VEGF resistant tumors were resensitized to sunitinib treatment when treated with an IL-8-neutralizing antibody. A similar trend was observed in the clinic wherein RCC patients displaying resistance to sunitinib treatment had elevated levels of IL-8. These observations strengthen the concept of using IL-8 levels as a predictive biomarker for the response to sunitinib treatment (Huang et al. 2010,). In an interesting animal model study designed to address the issue of tumor refractoriness, Mizukami et al., (2005) observed that when HIF-1α knockdown colon cancer cells were transplanted into mice, the densely vascularized tumors were observed along with 50% reduction in VEGF expression. In an effort to unravel the mechanism underlying these paradoxical observations, the authors surprisingly observed a 2.5-fold increase of IL-8 in HIF-1α-knockdown cells but not in cells with normal expression of HIF-1α. Similar overexpression patterns of IL-8 were observed in breast, pancreatic, and lung cancer cells having paralyzed functions of HIF-1α. Furthermore, it was found that under hypoxic conditions, reactive oxygen species (ROS) are generated, which activates NF-κB (nuclear factor-κB), a well-known regulator of IL-8 transcription, thereby leading to overexpression of IL-8 (Mizukami et al., 2005). Altogether, these findings clearly demonstrated the involvement of IL-8 signaling in compensatory angiogenesis in colon cancer.

IL-17 promotes tumor development by inducing tumor supportive microenvironments and myeloid-derived suppressor cells. In recent years, more tailored studies have been carried out to assess the involvement of IL-17 as a driver of VEGF-independent angiogenesis and thereby conferring tumor refractoriness towards anti-VEGF agents (Yang et al., 2014). In an interesting experimental setting, Chung et al., (2013) specifically proved that IL-17 secreted by stromal cells in the tumor microenvironment in response to VEGF treatment, triggered the stromal-derived inflammatory and compensatory angiogenic mechanisms that conferred tumor refractoriness to anti-VEGF agents (Chung et al., 2013). The authors selectively designed the *in vivo* experiments to ascertain whether IL-17 is necessary for tumor resistance to anti-VEGF agents. To address this query, EL4 cells lacking IL-17 receptor were transplanted into mice and were treated with anti-VEGF alone or in combination with an antibody against IL-17. In parallel animal model studies, a drug resistant tumor cell line was transplanted into wild-type mice and in mice lacking the IL-17 receptor. It was observed that the combined treatment of anti-VEGF and IL-17 inhibitor or IL-17R-knockout had a significant negative effect on tumor growth (i.e. 80% reduction of tumor

growth) as compared to the moderate efficacy demonstrated by anti-VEGF treatment alone. The authors also demonstrated that the IL-17 signaling cascade involves the G-CSF-dependent mobilization of $CD11b^{+}Gr1^{+}$ myeloid cells known to secrete angiogenic factors, hence possibly acting as one of the key players in conferring resistance towards anti-angiogenic agents (Chung et al., 2013). Of note, IL-17 also acts as an inducer of release of IL-6 and IL-8 (known players in anti-VEGF tumor resistance) in cervical cancer and in human glioblastoma cell lines (Yang et al., 2014).

2.3.7 Eph/Ephrins: A Pro-angiogenic Pathway of Bidirectional Signaling

Eph receptors are the largest family of RTKs comprising of 16 members. Depending on the specific type of ephrin ligand which is bound, they are divided into two classes EphA (EphA1-10) and EphB (EphB1-6). Eph receptors and their ephrin ligands (Eph/ephrins) constitute an important signaling pathway involved in cell communication with myriad functions in normal physiological and pathological conditions. The most notable functions of this signaling pathway includes embryonic patterning, development of the nervous system and angiogenesis. Interestingly, Eph/ephrin pathway generates bidirectional signaling at cell-cell contact sites and display functional diversity. The bidirectional signaling consists of forward signals that propagate in the receptor-expressing cell and depend on Eph kinase activity, however the reverse signaling propagates in the ephrin-expressing cell and is dependent on Src family kinases (Barquilla and Pasquale, 2015). A sizable amount of literature has accumulated in the recent years describing the significance of Eph/ephrin signaling in cancer progression. A plethora of preclinical and clinical studies has reported on the elevated levels of Ephs and ephrins in different human cancers/tumors such as breast, melanoma, neuroblastoma, colon, glioma, prostate, pancreas, lung, ovary, gastrointestinal tract, esophagus, thyroid and liver. The overexpression profiles of Ephs and ephrins in a variety of human cancer cells and tumor stromal cells is correlated with poor prognosis and dense vascularity (reviewed by Xi et al., 2012), suggesting their plausible involvement in tumor refractoriness. However, the clear role of Eph/ephrin signaling as alternative pathways of angiogenesis and anti-VEGF tumor resistance in context with different cancers is yet to be resolved.

Several reports describe the involvement of Eph receptors and ephrins in tumor angiogenesis by establishing cross-communication between vascular cells and tumor cells. Sizable

evidence accumulated in favor of the tumor-supportive functions of the Eph/ephrin family in angiogenesis. Examination of the tumors transplanted in *Epha2*-knockout mice or the activity of *Epha2* blocked by EphA-Fc fusion proteins in mice, suggests that forward signaling is involved in promoting angiogenesis (Wykosky and Debinski, 2008). Several *in vitro* and *in vivo* experimental settings have demonstrated that EphA2-mediated forward signaling increases vascular permeability, perhaps partly through phosphorylation of claudins (Miao and Wang, 2009). Ephrins are also reported to regulate the functions of VEGFR2 in development and tumor angiogenesis. Collectively, deregulated functions of Eph/ephrin signaling in humans is reported to promote tumorigenesis (Sawamiphak et al., 2010).

The role of EphA2/EphrinA1 signaling in bypass angiogenic mechanisms and tumor drug resistance is well described. Perhaps the first experimental evidence stems from the experimental setting designed to understand the functions of VEGFR1 and VEGFR2 in controlling vascularization in a mouse model of pancreatic islet carcinogenesis. The resistance to antibody-dependent blocking of the functions VEGFR1 and VEGFR2 was observed in the form of recurrent angiogenesis and regrowth of tumors during treatment, but after a lapse of initial period of positive activity of the treatment. This resistance to VEGF blockade was strongly correlated with significant upregulation of Ephrin A1 and other compensatory pro-angiogenic factors such as members of FGF family and Ang1. Of note, the levels of these pro-angiogenic factors were substantially induced ranging from 1.5-fold to almost 3-fold in the anti-VEGFR2-treated tumors. These findings clearly demonstrated the hypoxia-induced overexpression of EphrinA1 and other proangiogenic factors that restored tumor vascularization in a VEGF-independent manner (Casanovas et al., 2005). In tumors, both ECs and tumor cells express Ephrin A1. In an interesting mouse model study with pancreatic tumors, the upregulation of Eph A2 and Ephrin A1 expression observed in VEGF-treated tumors was significantly correlated with tumor drug resistance. It has been suggested that anti-VEGF tumor resistance might be associated with ECs wrapping by smooth muscle cells and pericytes (Casanovas et al., 2005; Okazaki et al., 2009). In a recent investigation involving a murine high-grade glioma model, following treatment with bevacizumab, HIF-1α induced the EMT repressor ZEB2 (zinc finger E-box binding homeobox 2) which significantly downregulates ephrinB2 through promoter binding thereby enhancing tumor invasiveness. The results clearly showed that Ephrin B2 repression via ZEB2 was strongly associated with tumor invasion and resistance towards treatment of bevacizumab (Depner et al.,

2016). Perhaps future preclinical and clinical studies assessing the combined activities of the EphA receptors and EphrinA & B ligands in concert with capillary sprouting, vessel permeability, invasion and pericyte coverage in tumor blood vessels, might create a rationale for designing effective and safe anti-angiogenic agents targeting the Eph/Ephrins signaling axis.

2.3.8 ALK1 Signaling: A potential Pathway in Tumor Refractoriness

Activin receptor-like kinase1 (ALK1) is a type I transforming growth factor-β (TGF-β) transmembrane serine/threonine kinase receptor, which is mostly expressed on proliferating ECs and is reported to be a critical factor in TGF-β-mediated neovascularization. Activation of ALK1 is a consequence of binding of different members of the TGF-β ligand family especially bone morphogenetic protein (BMP) 9, BMP10, and TGF-β. Mounting pharmacologic, genetic and histopathological evidence clearly outline the key functions played by ALK1 signaling in driving both physiological and pathological angiogenesis (Cunha and Pietras, 2011). Being an EC-restricted receptor, ALK1 has a regulatory role in growth and development of ECs. Several *in vitro* experiments have shown that activation of ALK1 by TGF-β leads to proliferation and migration of ECs: a basic prerequisite of neoangiogenesis (Hu-Lowe et al., 2011). Disparate and contextual results have been described for the binding of ALK1 with its alternate ligand BMP9. In an investigation, ALK1/BMP9 signaling has been shown to be anti-angiogenicin function and inhibited the FGF mediated angiogenesis (Scharpfenecker et al., 2007). On the other hand, in a xenograft model of pancreatic cancer ALK1/BMP9 signaling pathway has been shown to play an important role in proliferation of ECs and promotion of tumor angiogenesis (Suzuki et al., 2010).

ALK1 is expressed on circulating ECs, vascular endothelium, tumor blood vessels, and in variety of malignancies such as lung, prostate, renal cell, pancreas, skin, thyroid, liver, kidney, colorectal carcinoma and ovary (Hu-Lowe et al., 2011). Although the concrete molecular mechanism of ALK1 signaling as alternate angiogenic pathways under VEGF blockade is not understood fully, the circumstantial literature describes the potential involvement of ALK1 signaling in sustaining compensatory angiogenesis. For example, a clue for the possible involvement of ALK1 signaling in bevacizumab resistance can be observed from the experimental outcome wherein the combined treatment of bevacizumab with PF-03446962 (an anti-ALK1 monoclonal antibody) significantly improved the anti-angiogenic activity in a melanoma xenograft model designed to evaluate the resistance mechanisms towards VEGF/VEGFR targeting agents

(Hu-Lowe et al., 2011). Similarly, the combination of VEGFR tyrosine kinase inhibitor with ALK1 inhibitor (ALK1-Fc) was also found effective in VHL-deficient murine RCC xenograft model study. However, combination of VEGFR tyrosine kinase and ALK1 inhibitor (using ALK1-Fc peptibody RAP-041 from Acceleron) therapy resulted in improved efficacy of anti-VEGFR agents and adversely affected the progression of the disease (Wang et al., 2012). This finding clearly addresses a clue of potential interventions of ALK1-Fc in blocking a mechanism of angiogenic escape. In a similar type of investigation, targeting ALK1 using the kinase inhibitor K02288 resulted in preventing Notch cooperativity and inhibited functional angiogenesis (Kerr et al., 2015). The advantage of combining this therapy is that VEGFR and ALK1 inhibition affect sequential steps in angiogenesis. The consistent molecular updates on the significant role of ALK1 in tumorigenesis and vascularization, lead to the development of several novel anti-ALK1 agents, some of which are underway and many have entered phase 1 clinical trials with a significant hope of ameliorating anti-VEGF/VEGFR resistance (de Vinuesa et al., 2016). Future preclinical and clinical settings endeavoring the combination/s of anti-VEGF/VEGFR and anti-ALK1 agents in concert with compensatory angiogenesis, tumor refractoriness and toxicity issues might generate an understanding for rationale inclusion of anti-ALK1 agents for enhancing the efficacy of current anti-angiogenic agents exhibiting tumor refractoriness.

2.3.9 Contribution of Wnt Signaling to VEGF-Independent Angiogenesis

The Wnt (wingless-type mouse mammary tumor virus integration site family) signaling pathway is evolutionarily conserved and regulates many fundamental physiological processes including cell fate decisions, cell survival, proliferation, and overall organogenesis. The recent reports have unraveled several molecular underpinnings of Wnt signaling in physiological and pathological angiogenesis. In humans, the Wnt family consists of 19 secreted glycoproteins having affinity to >10 transmembrane Frizzled (Fzd) receptors. Nevertheless, there are several co-receptors for mediating Wnt signaling. The firsthand information regarding the involvement of Wnt signaling in regulation of angiogenesis has emerged from the fact that ECs express a number of Wnt receptors such as Fzd1-2, Fzd4-7, Fzd9-10, Lrp5, Lrp6 and Ryk: the Wnt signaling modulator. Despite the fact that major Wnt pathways have been characterized, the function of Wnt signaling in concert with cancer biology is intriguingly complex and only partially understood (Dejana,

2010; Zhan et al., 2017). Signaling mediated via Wnt glycoproteins such as Wnt1, Wnt3a and Wnt5a has been reported to regulate proliferation of ECs as well as migration in some cases (Newman and Hughes 2012). In the context angiogenesis, it has been reported that the canonical WNT/β-catenin signaling up-regulates the expression of cyclooxygenase-2 (Cox2) and thereby promote tumor angiogenesis and metastasis. It has been also reported that the metastasis-stroma interaction in human breast cancer metastasis was mainly regulated by the HGF/nuclear Met/phospoho-c-Src/β-catenin-TCF/WNT pathway (Yang et al., 2016)

Although there are limited evidences of direct association of Wnt signaling in compensatory angiogenesis, there are few circumstantial clues, which suggest the coordinated crosstalk of Wnt signaling with other pro-angiogenic pathways involved in compensatory angiogenesis. For example, in Zebrafish model studies, there is a clear evidence of Rspo1/Wnt mediated signaling, which promotes angiogenesis via VEGF-C/VEGF-R3. R-spondins are a group of ligands (Rspo1-4) that are known to coordinate upregulation of Wnt signaling. VEGF-C expression has been shown to be dependent on Rspo1 and Wnt. The findings showed that VEGF-C and VEGF-R3 are required to promote developmental vascularization downstream of Rspo1-Wnt. It has been anticipated that the Rspo-Wnt-VegfC-Vegfr3 pathway might be playing an inscrutable alternative pro-angiogenic role during tumor angiogenesis (Gore et al., 2011). Of note, these findings bear importance in the mainstream of alternate angiogenesis, as aberrant Wnt and VEGF-C signaling is frequently observed in different tumors and tumor-derived cell lines (Polakis, 2007). Nevertheless, VEGF-C has been reported to play a significant role in driving angiogenesis under VEGF-A blockade (Ye et al., 2013)

Tumor associated macrophages (TAMs) have been described as an important determinant imparting anti-VEGF tumor refractoriness, as they are known to modulate tumor growth by regulating angiogenesis (Newman and Hughes, 2012). Additional studies on TAMs have shown that “invasive” TAMs in breast cancer are found to possess components of Wnt signaling pathway, while the progression of colon cancer was found to be correlated with upregulation of Wnt5a in TAMs (Ojalvo et al., 2010). Macrophage-derived Wnt5a has been reported to induce expression of Tie-2 in human umbilical vein endothelial cells (HUVEC). Receptor Tie-2-ANGs signaling is known for its role in driving redundant angiogenic signaling and promoting survival and maturation of ECs (Huang et al., 2009). Furthermore, Wnt5a-mediated autocrine signaling in macrophages induced expression of different pro-inflammatory cytokines such as IL-6, IL-8 and

IL-1β (Pereira, et al., 2008). Moreover, Wnt5a-mediated signaling has been also shown to induce expression of pro-inflammatory cytokines, IL-6 and IL-8 in ECs (Corada et al., 2010). Thus, the macrophage-derived Wnt5a-mediated signaling seems to be associated with compensatory angiogenesis as the role of IL-6 and IL-8 is well documented as pro-angiogenic factors in anti-VEGF escape mechanism (Dmitrieva et al., 2016; Huang et al., 2010). The better understood Wnt signaling pathway is coordinated by the transcriptional activity of β-catenin: a bifunctional protein coordinating gene transcription and cell-cell adhesion (Dejana, 2010). Deviations in Wnt signaling pathways are correlated with a vascularization pattern. For instance, endothelial-specific β-catenin gain-of-function mutant mouse was observed to have aberrant vasculature and more interestingly, the remodeled vasculature was associated with overexpression of Dll4/Notch signaling (Corada et al., 2010). The significance of Dll4/Notch signaling in compensatory angiogenesis is well-documented (Li et al., 2011). Thus, the current circumstantial literature supports the plausible contribution of Wnt signaling in driving compensatory angiogenesis, perhaps, by activating other pro-angiogenic pathways implicated in conferring tumor resistance towards anti-VEGF agents.

2.4 Role of Stromal Cells in Restoring Vasculature under anti-VEGF Treatment

There is mounting evidence towards the role of tumor-infiltrating cells in tumorigenesis and angiogenesis. There is ample evidence about the infiltration of bone marrow derived cells (BMDCs) into the stroma of growing tumors. The current literature about the inscrutable involvement of BMDCs in promotion of tumor angiogenesis and growth is increasing in a logarithmic fashion (Huijbers, et al., 2016). The observations made in animal model studies and clinical trials on human subjects have proved that BMDCs are associated with tumor growth and differentiate into a variety of stromal cells that conspire to regulate tumor angiogenesis independent of VEGF and thereby contribute more significantly towards tumor refractoriness against currently used anti-angiogenic agents (Bergers and Hanahan, 2008). In a current state-of-the-art study, it has been well established that a variety of stromal cells such as TAFs, different inflammatory cells including BMD myeloid cells expressing different markers, myeloid-derived suppressor cells, dendritic cells, Tie 2 expressing monocytes, macrophages, ECs, pericytes, platelets etc. in concert with tumor cells via different cytokines/chemokines inscrutably and sabotagely promote tumor growth and angiogenesis under VEGF blockade (Crawford and Ferrara, 2009; Huijbers, et al., 2016). The role of BMDC myeloid and stromal cells in promoting tumor

progression and angiogenesis under anti-VEGF environment is summarized in **Figure 3.** The following sections are an overview of the molecular underpinnings associated with stromal/tumor cells in restoring vasculature under a selective pressure of VEGF blockade.

2.4.1 Pericytes: A Pro-angiogenic Wrapper

Pericytes (peri: around; cyte: cell) are contractile perivascular cells that wrap around the newly formed blood capillaries. Although pericytes (PCs) were discovered a century ago, their functional attributes are critically assessed in relation to their contributions in tumor angiogenesis and tumor refractoriness (Bergers and Song, 2005; Huijbers et al., 2016). Despite the growing evidence about the pro-angiogenic functions of PCs, the enigma of the origin of PCs is still not fully resolved. Recently, in a glioblastoma xenograft model study by Cheng et al., (2013), it was found that glioblastoma stem cells differentiate into vascular PCs and coordinate vasculature function and tumorigenesis (Cheng et al., 2013). The origin of PCs is of immense importance from an angiogenesis point of view; however, the detailed discourse in this regard is beyond the scope of the current review. Altogether, it will remain an excitement to determine whether the transformation of cancer stem cells into tumor vascular PCs is a universal mechanism in all vascular-rich malignancies. Therefore, the quest of resolving the identity of PCs either from mesenchymal and other stem cells is an evolving area of research as both cells are predominantly observed in vascular niches (Armulik, et al., 2011). PCs and PC-like cells act as mediators of several pathophysiological processes of cancers including tumor angiogenesis and metastasis. Particularly in the case of initial stage angiogenesis, the release and migration of ECs towards the localized angiogenic cues initiates only after ECs are relieved from the embracement of PCs. This clearly point out that pericyte-mediated signaling coordinates the initial growth phase of angiogenesis. From a functional point of view, PCs are involved in stabilization of blood vessels and quiescence of ECs. This process is referred as vascular maturation (Teichert, et al., 2017).

The molecular mechanisms of PC-mediated angiogenic sprouting are still not clear, however the variable expression patterns of pericyte markers have unraveled the presence of pericytes in the newly assembled vascular plexus, indicating that perhaps, pericyte-driven signaling might be a basic prerequisite for the initial growth phase of neovascularization. A recent study has reported that pericytes regulate VEGF-induced EC sprouting through VEGFR1 (Eilken et al., 2017). Sizable evidence has accumulated recently linking the role of PCs in pro-angiogenic

signaling in vascular morphogenesis and shown to have regulatory control over EC proliferation or quiescence (Ding et al., 2012). A coordinated crosstalk between PCs and ECs is very important for the promotion of PC-mediated pro-angiogenic functions and stabilization of new blood vessels. PCs in concert with ECs and VSMCs play an important role in active remodeling of vasculature during angiogenesis (Dulmovits and Herman, 2012). It is imperative to take an overview of factors involved in recruiting PCs into tumor vessels, as the degree of PC coverage is correlated with tumor resistance towards anti-angiogenic agents. The PC-EC crosstalk involving paracrine signaling by VEGF, PDGF-B, and Ang/Tie2 plays a key role in recruiting PCs into tumor vessels (Raza et al., 2010; Teichert, et al., 2017). PCs secrete VEGF in a hypoxic environment, which drives proliferation of ECs through paracrine signaling. Nevertheless, mature PCs having overexpression of VEGF-A are recruited into newly formed vessels. VEGF in association with other pro-angiogenic factors vasodilates the vessels and increases the permeability of the EC barrier. During PDGF-mediated angiogenesis, VEGF acts as an inhibitor of PDGF signaling in mural cells and reduces PC coverage of newly formed vessel sprouts, which in turn results in vessel destabilization (Eilken, et al., 2017). A scientific report describes that apart from paracrine action of VEGF-A, PCs also promote EC survival and confer protection through upregulation of autocrine VEGF-A signaling and expression of Bcl-w protein in tumor ECs. PC-mediated expression of Bcl-w protein protect ECs from apoptosis (Franco et al., 2011). This pro-angiogenic role of PCs is significant owing to the involvement of anti-angiogenic agents in pruning of neovasculature and inducing apoptosis in ECs. The autocrine VEGF-A signaling pathway of ECs in concert with PCs, offers an understanding of how PCs are involved in protecting ECs under anti-VEGF stress and recurrence of angiogenesis after the withdrawal of anti-angiogenic drugs.

The crosstalk between PCs and ECs mediated through paracrine PDGF signaling is another key factor in recruiting PCs into tumor vessels. PCs express PDGF receptor-β (PDGFR-β) while activated tumor ECs express ligand PDGF-B. The consequence of receptor-ligand binding leads to recruitment of PCs into tumor vessels. For example, PDGF-B and PDGFR-β knockout mice die at embryonic stage mostly due to irregular, tortuous and leaky blood vessels having overall aberrant orchestration of neovasculature (Stapor et al., 2014; Lindahl et al., 1997). Moreover, there is clear evidence of overexpression of PDGF-B in tumor cells on increasing the PC coverage. For example, Song et al., have shown that tumor-derived PDGF-B increases PC coverage in tumor vessels by activation of stromal-derived factor 1α. Altogether, PDGF-B-mediated paracrine

signaling loop appears to be a critical factor in the recruitment of PCs to newly formed vessels (Song et al., 2009). PCs also express ANG1, which signals via binding to endothelial Tie2 receptor. Sizable amount of literature describes the role of ANG1/Tie2 signaling in recruiting PCs into tumor vessels, blood vessel stabilization and in counteracting VEGF-induced permeability (Raza et al., 2010; Teichert et al., 2017). Moreover, knockout of the ANG1 gene during embryonic day 10.5 and 12.5 resulted in aberrant vascular network comprising an increased number of vessels with dilated diameters (Koh, 2013). However, in contrast to the above literature, conditional global ANG1 gene inactivation studies conducted by Jeansson et al., (2011) demonstrated that early ANG1 deficiency is a cause of defective vascular morphogenesis, which are caused by secondary flow disturbance and cardiac defects, but it does not affect recruitment of PCs. It was also observed that in postnatal angiogenic developments, ANG1 deficiency was associated with accelerated angiogenesis, which indicates that ANG1 was not indispensable for quiescent vessels rather regulating the vascular cues after injury (Jeansson et al., 2011; Teichert et al., 2017). Patient derived-PCs from infantile hemangioma, (a type of a rapidly growing vascular tumor in newborns), showed promising pro-angiogenic potential; however, the isolated PCs were not able to stabilize the vessels and expressed reduced levels of ANG1 (Boscolo et al., 2013). Conversely, overexpression of ANG1 was associated with increased coverage of PCs over vessels under conditions of VEGFR-2 blockade. Perhaps a few more tailored investigations might resolve the imbroglio of PC-derived ANG1 in recruiting PCs into tumor vessels, as the extent of PC coverage is closely associated with the degree of tumor resistance towards anti-angiogenic agents (Teichert et al., 2017).

Two aspects are very clear in assigning the role of PCs in compensatory angiogenesis and related tumor resistance. PCs were reported to play an inscrutable pro-angiogenic role by activating PDGFR-mediated signaling in anti-VEGF environment. The outcome of several findings have shown that ECs are able to induce PC recruitment to obtain protection against anti-VEGF agents via a PDGF-mediated pathway. The overall influence of PDGF-mediated signaling leads to an increase in PC coverage (Song et al., 2009; Birbrair et al., 2014). In the current state-of-the-art, it has been well established that anti-angiogenic drugs act more effectively on ECs lacking PCs, with no significant effect on PCs present in mature vessels. Moreover, in tumors, unlike normal vasculature, PC coverage is decreased and often tumor-associated PCs express abnormal markers and morphology. Nevertheless, tumor vessels also attenuate their dependency

on VEGF-mediated survival signal by increasing PC coverage to provide integrity (Scott et al., 2015). Interestingly, low to moderate doses and transient treatment duration of anti-angiogenic agents especially, bevacizumab and other VEGFR2 inhibitors, have significant contribution to the normalization of aberrant, torturous and leaky architecture of tumor vasculature, thereby improving the therapeutic performance of anti-angiogenic agents (Chatterjee et al., 2014). From a disease progression point of view, a low degree of PC coverage on ECs or ECs lacking PCs, are strongly correlated with increased metastasis in prostate, colorectal, pancreatic and breast cancers (Cooke et al., 2012). Indeed, a proper formulation of the dose and treatment duration of anti-angiogenic therapy, might improve the therapeutic index of this impressive regimen.

The second line of evidence in relation to the involvement of PCs in alternate angiogenesis is centered on the role of PC progenitor cells derived from bone marrow. The lineage of bone marrow derived (BMD) hematopoietic cells have been considered a hub of pro-angiogenic progenitor cells. The BMD hematopoietic lineage of cells especially expressing a variety of PC markers such as PDGFRβ, Desmin, NG2, αSMA, and ScaI are reported to coordinate tumor angiogenesis (Song et al., 2005; Gao and Mittal, 2009; Ding, et al., 2012), However, the detailed molecular complexities of these BMD cells and their pro-angiogenic activities under VEGF blockade, are yet to be deciphered. Of note, different types of stromal BMD cells play an important role in tumor refractoriness against anti-angiogenic agents (Clarke et al., 2013). PCs from infantile hemangioma tumor (human Hem-PCs) origin showed significant pro-angiogenic activities. When the matrigel-based mixture of Hem-PCs and cord-blood derived endothelial colony-forming cells were injected into immunocompromized nude mice, there was a remarkable production of a higher number of blood vessels and human $CD31^{+}$ vessels. Interestingly, pro-angiogenic activity of Hem-PCs was many fold higher than the retinal PCs (Boscolo et al., 2013). Future experimental settings probing the pro-angiogenic functions of PCs in tumor vessels under VEGF blockade might offer a clear understanding regarding the role PCs in compensatory angiogenesis.

2.4.2 Bone Marrow: A Hub of Angiogenic Progenitor Cells

The frequency of clinical reports linking the role of BMDCs in regulation of tumor angiogenesis, progression, metastasis, inflammation and tumor resistance towards current chemotherapy, has increased significantly in recent years. BMDCs are mobilized by the tumors as pro-angiogenic precursor cells, which later developed into tumor vasculature. The current literature has revealed that an increase in the infiltrated titer of BMDCs in the stromal bed of tumors is strongly associated

with poor prognosis (Lee et al., 2015). Perhaps, such prognostic clinical clues have inspired researchers to probe the molecular underpinnings conspiring pro-angiogenic and tumor-supportive activities of BMDCs. The outcome of series of tailored preclinical and clinical endeavors have resulted in maneuvering BMD tumor microenvironment as an attractive therapeutic target on the eve of confronted hurdles experienced by the anti-angiogenic therapy in clinical practice. Moreover, the research excitement has resulted in launching few therapeutic candidates targeting BMD components of tumor microenvironment in clinical trials (Gao and Mittal, 2009).

A cursory look at the literature accumulated in relation to implications of BMDCs in driving pro-angiogenic functions indicates that the infiltrated BDMCs in the tumor stroma are involved in regulation, responsiveness and resistance to anti-angiogenic therapy in a very sabotaging manner (Lee et al., 2015). Amongst the most notable cell lineages of BMDCs promoting tumor progression by angiogenesis, includes the lineage of $GR1^+CD11b^+$ (Yang et al., 2004), $CD11b^+CD13^+$myeloid cells (Dondossola et al., 2013), $CXCR4^+VEGFR1^+$ hemangiocytes (Jin et al., 2006), TAMs expressing $CD11b^+F4/80^+$ (Chanmee et al., 2014), Tie2-expressing monocytes (Coffelt et al., 2010) and $PDGFR^+$ pericyte progenitors (Song et al., 2005), the populations of $CD45^+CD11b^+$ myeloid cells (Grunewald et al., 2006), tumor infiltrated mast cells and neutrophils (Soucek et al., 2007; Nozawa et al., 2006), vascular endothelial-cadherin$^+$ $CD45^+$ vascular leukocytes, and a variety of BMDCs orchestrate both angiogenesis and immune evasion to support tumor growth and angiogenesis in the midst of anti-VEGF environment (Li et al., 2017). Besides the abovementioned cell lineages, a variety of different pro-angiogenic BMDCs and myeloid cells playing pro-angiogenic roles were extensively reviewed elsewhere (Quail and Joyce, 2013). In contrast to the role of different lineages of BMDCs in regulating the angiogenic switch, the molecular mechanisms governing the pro-angiogenic functions in general and their specific involvement in compensatory angiogenesis/tumor resistance remain a vibrant research area in the mainstream of tumor angiogenesis.

Various papers are available, linking the specific role of some of the lineages of tumor infiltrated BMDCs in regulation of angiogenesis under the blockade of VEGF and the related tumor resistance towards the currently used anti-angiogenic agents (Ferrara, 2010). The angiogenic drive of the $CD11b^+Gr1^+$ lineage was evidenced, when a mixture of $CD11b^+Gr1^+$ and tumor cells was injected into mice; there was a significant increase in tumor growth and angiogenesis. More clearly, transfer of resistant tumor-derived $CD11b^+Gr1^+$ cells to sensitive tumors sustained tumor

growth and angiogenesis in the presence of anti-VEGF antibodies. The authors found that granulocyte-colony stimulating factor (G-CSF), IL-6 and stromal-derived factor 1α (SDF-1α) secreted by tumor stroma and tumor cells invites the mobilization of $CD11b^{+}Gr1^{+}$ myeloid cells in the bone marrow. After mobilization, $CD11b^{+}Gr1^{+}$ cells are infiltrated and recruited to the tumor and trigger neovascularization, thereby sustaining angiogenesis and imparting resistance to anti-VEGF therapy (Shojaei et al., 2007). The scientific craze of the same research group, further clarified that the $CD11b^{+}Gr1^{+}$-driven angiogenesis in a coordinated manner involving granulocyte-macrophage colony stimulating factor (GM-CSF), SDF-1α, placenta growth factor, G-CSF and the G-CSF-induced Bv8 (Bombina variagata peptide 8) protein. Of note, these chemokines are specifically overexpressed in anti-VEGF refractory tumors. In brief, $CD11b^{+}Gr1^{+}$ cells mediate angiogenesis via a Bv8-dependent pathway, which is activated by G-CSF that escapes VEGF, thereby rendering tumors more resistant towards anti-VEGF drugs (Shojaei and Ferrara, 2008; Shojaei et al., 2009).

A subset of $CD11b^{+}CD13^{+}$ myeloid cells of BM origin specifically localize in tumor microenvironment and showed significant pro-angiogenic activities by direct involvement in regulating tumor blood vessel formation by producing different proangiogenic factors. However, their interactive gossip in the tumor microenvironment in relation to anti-VEGF resistance is yet to be deciphered (Dondossola et al., 2013). BMD circulating endothelial progenitors (CEPs) have been suggested as a culprit for rebounds of tumor growth and vascularization after relapse of vascular disrupting agents (VDA). VDAs are the microtubule-destabilizing agents that cause the rapid destruction of tumor blood vessels, which ultimately lead to tumor necrosis and shrinkage. Investigation carried out in this respect suggested that treatment with VDA causes an increase in the number of CEPs in the peripheral blood of mice and recruit them into tumor vasculature and thereby promote angiogenesis. However, after the initial phase of VDA treatment, aggressive tumor regrowth and revascularization was observed (Shaked et al., 2008). In contrast to these findings, the experiments carried out in mice and clinical trials in humans, demonstrated that the CEP-induced drug resistance towards VDA could be reversed by using a combination of VDA and anti-VEGFR2 antibody or sunitinib (Taylor et al., 2015). The anti-angiogenic agent mediated reversal of VDA-induced tumor resistance is of special interest, as the anti-angiogenic drugs like anti-VEGFR2 antibody or sunitinib did not affect the recruitment of BMDC infiltration in tumors which have not experienced the exposure of VDA (Taylor et al., 2015). Indeed, it seems to be an

adaptive feedback of the tumor to enable evasive resistance to anti-angiogenic agents. Alternatively, it has been suggested that CEPs and immature myeloid cells may promote resistance to anti-angiogenic therapy by incorporating these cells into tumor vessels (Bailey et al., 2006). Besides few reports in favor of pro-angiogenic concerns of CEPs, series of studies have questioned the identity and contribution of CEPs to neovessel formation (Gao and Mittal, 2009). In an *in vitro* experimental setting, TNF*α* stimulated eosinophils to secrete several pro-angiogenic factors such as IL-6, bFGF, IL-8, PDGF, and MMP-9 (Nissim Ben Efraim and Levi-Schaffer, 2014). Of note, these factors are known to play a key role in compensatory angiogenesis and tumor drug resistance, however, mimicry of similar effects in tumors under the blockade of VEGF, might ascertain the role of eosinophils in anti-VEGF escape mechanism and tumor drug refractoriness.

Another population of BMD myeloid cells playing a plausible role in sustaining tumor vasculature and conferring resistance are TAMs. Series of clinical and experimental reports correlated the high levels of infiltrating TAMs in tumor microenvironment (TME) with poor prognosis and resistance to chemotherapy (Chanmee et al., 2014; Zhu et al., 2017). Besides stimulating tumor angiogenesis, TAMs also induce tumor revascularization in response to cytotoxic chemotherapy. In advanced stage of tumor progression, TAMs are transformed into M2-like phenotype that has pro-tumorigenic/pro-angiogenic properties, and secrete different pro-angiogenic cytokines (Qian and Pollard, 2010). Mounting experimental and clinical evidence suggest that the extent of TAMs infiltration in TME determines the operational kinetics of the angiogenic switch (Hao et al., 2012). A variety of factors such as colony stimulating factor-1 (CSF-1) also known as macrophage-CSF (M-CSF), VEGF-A, monocyte chemoattractant protein-1 (MCP-1/CCL2) and endothelin are instrumental in ascertaining destination of TAMs from BM to TME for promoting tumor angiogenesis. M-CSF also plays an important regulatory role in survival, proliferation, differentiation and chemotaxis of macrophages in mice harboring tumors (Aharinejad et al., 2004). Paralyzing the functions of CSF-1 resulted in suppression of the infiltration of TAMs in TME and significantly impaired the process of tumor progression and angiogenesis (Lin et al., 2001). Moreover, TAMs are known to play an important role in remodeling of the ECM by producing ECM degrading matrix metalloproteinases (MMPs) such as MMP-2, MMP-7, MMP-9 and MMP-12. MMP-9 produced by TAMs and other infiltrating myeloid cells plays a crucial role in tumor angiogenesis, progression and metastasis (Zhu et al., 2017).

Series of animal model studies demonstrated that TAMs mediate regulation of angiogenesis (Chanmee et al., 2014; Hao et al., 2012; Zhu et al., 2017). Besides the significant production of VEGF-A by TAMs, they also have the ability to produce notable pro-angiogenic factors such as bFGF, placenta growth factor (PlGF), PDGF, TGF-β, IL-1β and IL-8. Notably, prominent pro-angiogenic factors produced by TAMs are known to play an important role in compensatory angiogenesis. PLGF acts a chemo-attractant for TAMs, however when functions of PLGF are blocked using monoclonal antibodies (αPlGF), the recruitment of TAMs to murine tumors were suppressed and ultimately resulted in growth inhibition of VEGFRI-resistant tumors. Upregulated levels of PLGF were observed upon anti-VEGFR treatment, PLGF-mediated proangiogenic signaling and TAMs implicated in drug resistance might be the possible mechanism for VEGF rescue mechanism and tumor resistance (Kim et al., 2012).

In a clinical phase II study of sunitinib treatment in HCC patients, rapid disease progression and high mortality of HCC patients was associated with elevated levels of SDF-1α (Zhu et al., 2009; Karagiannis et al., 2012). The involvement of TAMs in tumor progression and angiogenesis cannot be ruled out, because SDF-1α has proven as a chemokine for pro-angiogenic progenitor cells involved in anti-VEGF rescue mechanisms (Shojaei et al., 2007; Shojaei et al., 2009). Zhang et al., (2010) have demonstrated a better insight on the role of TAMs in alternate angiogenesis and tumor resistance in sorafenib-treated human HCC xenografts in a nude mouse model. Treatment with sorafenib induced the recruitment of BMD vascular modulatory pro-angiogenic cells, like $F4/80^{-}$ and $CD11b^{+}$ that, might drive tumor angiogenesis and invasion. Nevertheless, elevated levels of macrophage-inviting chemokines such as of CSF-1, SDF-1α and VEGF were found to be associated with sorafenib treatment (reviewed by Guo et al., 2013; Chanmee et al., 2014). In another experimental setting involving an HCC tumor model, TAMs have been suspected for their role in driving angiogenesis under sorafenib treatment. The authors supported their argument of TAMs involvement in tumor angiogenesis and progression by performing elimination of TAMs using Zoledronic acid or clodrolip, which strongly enhanced the anti-angiogenic and anti-tumor potential of sorafenib in mice treated with sorafenib alone. Moreover, TAMs depletion is also associated with enhancing the synergistic anti-angiogenic efficacy of VEGF/VEGFR2 antibodies in amelioration of subcutaneous human cancer xenografts. Thus, the current state-of-the-art outlines the central position of TAMs in invasive TME and perhaps can be maneuvered as a target for arresting tumor growth and angiogenesis (Noy and Pollard, 2014). Altogether, TAMs have

been considered a primary cause of resistance to anti-VEGF agents and they are more likely to contribute significantly to alternative or compensatory pro-angiogenic conspiracy that leads to tumor arrogance under anti-VEGF selective pressure (Shojaei and Ferrara, 2008; Noy and Pollard, 2014).

In an excursion of finding potential BMDCs as drivers of compensatory angiogenesis, the subpopulation of BMD myeloid cells expressing angiopoietin receptor Tie2 indeed a qualified candidate to be considered as potential cell lineage that might contribute to alternate angiogenesis and tumor resistance (Coffelt et al., 2010). These cells, also known as Tie2 expressing macrophages/monocytes (TEMs), have been identified in both murine and human tumors and preferentially accumulate in the close vicinity of tumor blood vessels. TAMs, TEMs display the upregulation of the pro-angiogenic VEGF-A and MMP-9 as compared to Tie2-negative monocytes (Coffelt et al., 2010). De Palma and colleagues have clearly shown that TEMs can be selectively recruited to orthotopic tumors, where they promote angiogenesis through a paracrine loop and perhaps act as a key mediator for most of the pro-angiogenic transactions of the BMD myeloid cells in TME. Interestingly, TEMs demonstrated profound angiogenic activity than TAMs in mouse tumor model. Function gain and loss experiments showed that TEM knockout completely resulted in prevention of human glioma angiogenesis in the mouse brain along with substantial regression in tumor growth (De Palma et al., 2017). Several reports state that anti-angiogenic agents are known to induce hypoxia, which preferentially mobilize and recruit a variety of BMD myeloid cells including TEMs (Rivera et al., 2014). The enhanced recruitment of TEMs in TME under anti-VEGF environment might play an inscrutable role in promoting angiogenesis and thereby conferring tumor resistance as the lineage of TEMs are implicated in neovascularization in concert with ANG expressing ECs through a paracrine signaling loop (De Palma and Naldini, 2009). Nevertheless, the ANG2/Tie2 signaling cues play a crucial role in sustaining angiogenesis and conferring adaptive tumor resistance during VEGF blockade (Rigamonti et al., 2014). In a recent investigation involving a chimeric mouse model, BMD myeloid cells such as Gr1+ CD11b+, F4/80+, CD202b+, VEGFR2+, have been found to be actively involved in orchestrating anti-angiogenic resistance in glioblastoma through coordinated molecular networks (Achyut et al., 2015).

2.4.3 Tumor-Associated Fibroblasts and Resistance to Anti-VEGF Therapy

Recent mainstream of tumor biology research has identified TME or stroma as a hub of physiologically vibrant hotspot where the decisions of supporting the tumor progression at different levels are committed and executed. Conceptualization of tumors as 'a wound that does not heal' not only offered a new comparative discourse on wound healing and tumorigenesis, but also expanded our knowledge of the role of TME and CAF in tumor progression. Although tumor resemble a dysfunctional organ, but it recapitulates the features of development and within TME, tumor cells signals corrupt instructions to the surrounding stromal cells and hijack their support for growth and invasion. Within the TME, a variety of cells are educated, trained and equipped in favor of tumor progression and metastasis (Daniela and Joyce, 2013). Fibroblasts in general are heterogeneous, non-vascular, non-inflammatory and non-epithelial cells, occurring in various phenotypes, and are embedded within the ECM of the connective tissue. However, the fibroblasts within the tumor stroma also called cancer/carcinoma associated fibroblasts (CAFs), TAFs and reactive stromal fibroblasts are activated and acquire a modified phenotype (Kalluri, 2016). TAFs are associated with tumor cells at various stages of tumor growth and metastasis. Their growth factor production ability, chemokines and role in remodeling of ECM enables them to recruit pro-angiogenic ECs and pericytes. Besides sizable investigations that focus on the involvement of fibroblasts in tumor growth, angiogenesis, metastasis and tumor resistance, the concrete molecular mechanisms unravelling the baseline underpinnings are still evolving and poorly understood. The circumstantial reviews published elsewhere, clearly described a variety of pro-angiogenic attributes like angiogenesis, invasion, progression, and metastasis (Kharaishvili et al., 2014; Kalluri, 2016; Tao et al., 2017).

CAFs were observed at the frontline of the tumor invasive port in a variety of cancers like breast, lung, pancreas, prostrate and colon (Kharaishvili et al., 2014). The clue of involvement of fibroblasts in angiogenesis comes from the fact that they secrete different pro-angiogenic factors including VEGF, FGF, CXCL12, PDGF-C, IL-8/CXCL8 and MMPs in ECM. The pro-angiogenic factors released in the ECM, recruit the other pro-angiogenic stromal cells including the ECs and their progenitor and stimulate tumor angiogenesis and vasculogenesis (reviewed by De Veirman et al., 2014). Notably, fibroblasts also secrete SDF-1α which mobilizes BMDCs including CEPs and not only recruit them into tumor vessels but also retain BMDCs in a close vicinity to the angiogenic port (Watnick, 2012). CAFs associated with pancreatic tumors, secrete pro-angiogenic factor CXCL12 and stimulate carcinoma cells to secrete CXCL8. The synergistic action of

CXCL12 and CXCL8 leads to increase in EC proliferation, migration and thereby activation of the angiogenic pathway (Matsuo et al., 2009). Tumor transplantation studies, demonstrated that CAFs promote angiogenesis, cancer cell proliferation, invasion, and metastasis. When cancer cells admixed with CAFs were transplanted into mice, the resultant tumors were more malignant than its normal counterpart. The findings indicate the tutor role of the CAFs in conferring added malignancy to the cancer cells (Luo et al., 2015; Tao et al., 2017).

There is a consensus over immune suppressive implications of the TME that discourages the suppression of tumor progression. In general, the inscrutable activities of stromal compartments are linked with reduced efficacy of anti-VEGF therapy. Precisely, TAF-driven sabotaging of the pro-angiogenic roles might be a contributing factor in conferring resistance against anti-VEGF agents (reviewed by Kalluri, 2016; Öhlund et al., 2014). As a counter action of combating TAF-mediated anti-VEGF tumor resistance, several novel tailored therapeutic regimens targeting TAF-regulating genes and the signaling pathways are evolving and a few agents are in clinical and preclinical trials (Togo et al., 2013). Experiments designed with an intension of tumor specific role of TAFs, perhaps the contribution by Crawford, Y. et al. (2009), indeed, a focused attempt of its kind, which unraveled largely the role of TAF-driven angiogenesis during VEGF blockade. Tumor-derived TAFs either sensitive or resistant to anti-VEGF treatment, were assessed for their abilities to stimulate tumor angiogenesis and growth. TAFs derived from anti-VEGF resistant tumors promoted angiogenesis and tumor growth even after treatment with bevacizumab. While understanding the role of TAFs under an anti-VEGF environment, it was experimentally confirmed that TAF- secreted PDGF-C was instrumental in driving angiogenesis under the anti-VEGF environment (Crawford et al., 2009). Consistent with the similar line of investigation, it was proved that the stroma-generated PDGF-C overexpression was strongly attributed to resistance to anti-VEGF in a glioblastoma mouse model (di Tomaso et al., 2009). The involvement of PDGF-C in tumor growth and angiogenesis was also outlined by previous studies. The overexpression of PDGF-C and PDGF-A in some tumor cells is associated with recruitment of VEGF-producing fibroblasts. Indeed, the stromal fibroblasts play a crucial role in RTK treatment resistance by generating angiogenic factors such as HGF, which *per se* play an important role in compensatory angiogenesis (Wang et al., 2009). In pancreatic cancer model studies, HIF-1 upregulates the c-MET expression in cancer cells and induced production of HGF by fibroblast cells (Ide et al., 2006). These findings focus on the significance of the HGF/c-MET pathway as a

driver of VEGF-independent angiogenesis (Shojaei et al., 2010; Kopetz et al., 2010; Miyahara et al., 2011). Unravelling the concrete molecular signatures of TAF associated pro-angiogenic functions might help in tailoring TAF inhibitors.

2.4.4 Cancer Stem Cells: Memory of Pro-angiogenic Signatures

The previous presumption that tumors are a homogenous clone of cancer cells proved to be an illusion. Now it has been well-established that tumors consist of heterogeneous subclones of cells having intra-tumoral interactions at different levels of tumor progression. The chaos of genomic instability in tumors may regulate the type and amount of pro-angiogenic factors expressed in a tumor. Tumor heterogeneity has seeded several enquiries and imposed challenges in relation to its causes and consequences, concern with improving the drug efficacy and challenge of ameliorating the drug resistance (Easwaran et al., 2014). Perhaps the speculative suggestion that tumors might harbor hierarchies of organized stem cells was a paradigm shift in the mainstream of tumor biology, which opened a new avenue in tumor pathophysiology/progression and improved rationales for design and development of novel anti-tumor agents. Stem cell-like phenotypes of CSCs have been reported in series of cancer types like breast, brain, head and neck, prostate, melanoma, colorectal and hepatocellular. Although not necessarily exclusive, several competing models have been proposed to demonstrate tumor heterogeneity and progression of CSCs (Prasetyanti and Medema, 2017).

Mounting experimental evidence supports the notion that apart from the self-renewal and proliferative abilities of CSCs, they may also actively participate in tumor angiogenesis. A discourse in relation to pro-angiogenic activities of CSCs in concert with anti-angiogenic agents has been critically reviewed elsewhere (Ribatti, 2012; Melero-Martin and Dudley, 2011). The pro-angiogenic functions of CSCs are mediated through a coordinated crosstalk between CSCs and ECs. Moreover, CSCs that express markers of ECs and form tumor blood vessels were reported (Ribatti, 2012). For example, Wang et al., have showed that glioblastoma stem cells, through an intermediate $CD133^{+}/CD144^{+}$ progenitor cell, could give rise to ECs or tumor cells, where ECs participate in neovascularization (Wang et al., 2010). There are ample reasons towards patrolling CSCs as players in promoting redundant angiogenesis and thereby conferring drug resistance. For example, in recent cell culture studies, glioblastoma stem-like cells secrete the pro-angiogenic VEGF-A factor in extracellular vesicles and interestingly, these extracellular vesicle-derived

VEGF-A contributes to the *in vitro* elevation of permeability and angiogenic potential in human brain ECs (Treps et al., 2017), which perhaps might be boosting strong angiogenic cues after the relapse of VEGF blockade. Of note, anti-angiogenic treatment is associated with generation of a hypoxic microenvironment (Rivera et al., 2014), thereby creating a more conducive environment for CSC-mediated pro-angiogenic functions. Like normal stem cells, CSCs can also switch to different phenotypes. For instance, in xenograft tumor model studies, a specific population of ovarian cancer cells has demonstrated stem-cell like characters and formed blood vessels, which were specifically lined by human $CD34^+$ cells. Furthermore, these cells mimicked the behavior of normal ECs and formed extensive branching and mature vessel-like structures within 7 days (Alvero et al., 2009). Folkins et al., (2009) have demonstrated that tumors possessing a larger population of CSCs, recruited an increased number of endothelial progenitor cells (EPCs), suggesting that CSCs are implicated in local angiogenesis by stimulating VEGF- and SDF-1-mediated mobilization of EPCs (Folkins et al., 2009). BMP (bone morphogenic protein)-mediated pathway has been considered as one of the crucial signaling pathways regulating the pro-angiogenic functions of CSCs. Reports published by Shao et al., showed that BMP-9 can suppress VEGF expression and angiogenesis through the BMP-9/ALK1 pathway; on the other hand, TGFβ1/ALK5 signaling has a counter effect. BMP-4 has been suggested to play a balancing role in between these two pathways and maintain vascular integrity (Shao et al., 2009). Interestingly, the investigation by Piccirillo suggested that the BMP-4/BMPR/SMAD signaling pathway significantly inhibits the tumorigenic activity of glioblastoma cancer stem cells (Piccirillo et al., 2006). The circumstantial findings link the role BMP in CSC-mediated tumorigenesis and angiogenesis.

Recent findings provide evidence that intra-tumoral hypoxia induced by the anti-angiogenic treatment, also enhances the formation of CSCs. Investigations in relation to glioblastoma have shown that the transcription factors HIF-1α and HIF-2α play a crucial role in stimulation of CSCs (Seidel et al., 2010). Conley et al., (2011) have demonstrated the similar trend of hypoxia-mediated increase in breast CSCs in xenograft model studies. The authors further explained that the increase in the number of CSCs in sunitinib- and bevacizumab-treated breast cancer is associated with upregulation of HIF-1α and thereby activation of the Wnt-signaling pathway via Akt/β-catenin signaling (Conley et al., 2012). The suggested mechanism bears importance in the discourse of resistance to anti-VEGF agents, as these agents not only prune the

neovessels but also induce apoptosis in ECs (Franco et al., 2011). TAMs, through a complex coordinated network of growth factors involving cytokines, chemokines and ECM molecules, have been shown to regulate the self-renewal and drug resistance of CSCs. Yi and colleagues (2011) observed that glioma-initiating cells expressed higher levels of VEGF-A, CCL2, CCL5 and neurotensin than regular glioma cells. The result outlines the involvement of CSCs in TAM recruitment by secreting macrophage-attracting chemokines (Yi et al., 2011). Indeed, CSCs might have a linkage in an imbroglio of compensatory angiogenesis and tumor resistance, as TAMs have been shown to play a crucial role in such activities. Agents that may control or eliminate the population of CSCs are required, as CSCs possess remarkable tumor-recurring capabilities along with high metastatic potential (Ribatti, 2012). The findings of a recent investigation advocate the inclusion of doxycycline with anti-angiogenic agents for arresting the hypoxia-induced survival and propagation of CSCs (De Francesco et al., 2017). Notch signaling, which is known to operate under anti-VEGF stress, has also been shown to coordinate glioblastoma angiogenesis and self-renewal of CSCs. Treatment with the Notch inhibitor DAPT (γ-secretase inhibitor) significantly reduced the self-renewal potential of CSCs, the number of $CD133^+$ expressing tumor cells, expression of vascular markers such as CD31, CD105, and von Willebrand factor (Hovinga et al., 2010). Owing to the current findings linking the role of CSCs in pro-angiogenic functions, the inscrutable direct or indirect involvement of CSCs in alternate angiogenesis cannot be ignored, however, more focused studies unravelling the pro-angiogenic role of CSCs under VEGF blockade need to be performed so as to understand the gossip of CSCs in tumor angiogenesis, recurrence and progression.

2.5 Role of Hypoxia in Activating Compensatory Angiogenic Mediators

Hypoxia and angiogenesis have been identified as hallmarks of cancer. HIF-1α is a critical transcription factor under oxygen deprivation and is a potential candidate for the induction of compensatory pathways activated by cancer cells under hypoxic conditions. HIF-1α mediates the transcription of genes involved in angiogenesis, oxygen consumption, glycolytic metabolism, migration and invasion of cancer cells. Under hypoxic environment, HIFs is a major regulator of pro-angiogenic factors and factors involved in vascular remodeling such as VEGF, FGF2, PlGF, VEGFR-1, CXCL12/CXCR4, PDGF, ANG-1, ANG-2, SCF etc. (Semenza, 2012). A discourse on anti-angiogenic therapy-mediated functional consequences in the tumor microenvironment is an

ongoing, poorly understood controversial issue, wherein the concrete understandings in this regard are still evolving. Two schools of thoughts have been proposed to minimize the chaos in this scientific discourse. The first school opines that the treatment with anti-angiogenic agents leads to decrease in intra-tumor hypoxia and interstitial pressure, which may further improve the tumor perforation, circulation and better delivery of chemotherapy; the so called process of 'normalization of vasculature'. Supporters of this hypothesis argue that it is essential to alleviate hypoxia for improvement of cancer chemotherapy (Jain, 2005). However, the second school hypothesizes that anti-angiogenic drugs induce intra-tumor hypoxia which leads to selection of more aggressive metastatic clones of cancer cells that might confer resistance to chemotherapeutic drugs including anti-angiogenic agents (Conley et al., 2012). Evidence from preclinical model studies and clinical observations favor the latter hypothesis that anti-angiogenic therapy might be a cause of increasing intra-tumor hypoxia and a more invasive malignant phenotype (Conley et al., 2012; Rey et al., 2017). Consistent with these preclinical data, the clinical findings observed in patients with NSCLC and primary liver cancer also demonstrated increased intra-tumor hypoxia following treatment with bevacizumab (Yopp et al., 2011). The cause and effects of intra-tumor hypoxia and its significance as a tumor-specific target have been a subject of interest in the area of tumor biology. Targeting hypoxia is emerging as a research priority for alleviating the poor clinical performance of anti-angiogenic drugs (Rapisarda and Melillo, 2012).

A vast body of literature is constantly accumulating in the recent years linking the role of hypoxia in activation and upregulation of a variety pro-angiogenic factors and bypass angiogenic pathways along with infiltration and activation of a variety of specialized cells in the TME (Mahase et al., 2017; Semenza, 2013). FGFs have been identified as the first angiogenic factors in preclinical model studies that act as drivers of compensatory angiogenesis and confer tumor resistance to anti-angiogenic agents. Relapsing tumors, which had an exposure to anti-angiogenic agents, expressed high levels of FGF2 and SDF-1. Further, it was observed that HIF-1α was involved in controlling these elevated levels of FGF2 and SDF-1 (Calvani et al., 2006). A similar trend of results was confirmed in clinical settings where high levels of FGF2 were observed in serum of patients treated with anti-VEGF therapy (Motzer et al., 2014). The most significant impact of hypoxia is recruitment of BMDCs in TME (Mahase et al., 2017; Semenza, 2013). Perhaps the first evidence of hypoxia-induced recruitment of BMDCs was obtained in an experimental model of ischemia, wherein BMDCs and EPCs expressing CXCR4 were recruited to

the ischemic area by a HIF-1α-mediated upregulation of VEGF and SDF-1 (Ceradini et al., 2004). It was also suggested that pro-angiogenic factors like VEGF, SDF-1, and SCF secreted by hypoxic cells, signal their effects by binding to their cognate receptors located on the surface of bone marrow cells, which later are released into the circulation and recruited to TME where they stimulate angiogenesis (Mahase et al., 2017). Because of a massive induction of hypoxia, a remarkable mobilization of pro-angiogenic circulating BMDCs has been shown to occur rapidly after treatment with VDA in tumor-bearing mice. In this experimental setting, CECs have been shown to contribute significantly to the rapid recurrence of tumor growth (Shaked et al., 2008). Arresting the functions of HIF in immunocompromized mice carrying human prostate cancer xenografts, was associated with reduced expression levels of VEGF, SCF and SDF-1 mRNAs. More interestingly, disruption of HIF also arrested the infiltration of a variety of BMD pro-angiogenic cells like $VEGFR2^{+}CD117^{+,}$ $VEGFR2^{+}CD34^{+}$, and $CXCR4^{+}$ $Sca1^{+}$ which ultimately inhibited tumor growth and angiogenesis (Lee et al., 2009). Prolyl hydroxylase 2 (Phd2) is a negative regulator of HIF-1α and a molecular oxygen sensor. Down-regulation of Phd2 in human colon cancer resulted in infiltration of pro-angiogenic $CD11b^{+}$ clad of tumor-associated myeloid cells and promotion of angiogenesis (Chan et al., 2009).

TAMs have been identified as one of the culprits in driving compensatory angiogenesis as they are known to secrete several pro-angiogenic growth factors including VEGF, bFGF, EGF, PDGF and TGF-α (Newman and Christopher, 2012). Hypoxia acts as an active "consigliere" in inducing accumulation of TAMs in TME and activating their role in compensatory angiogenesis (Chanmee et al., 2014). TAMs preferentially localize in hypoxia-rich areas in a variety of human cancers like colon, breast, bladder, endometrium, oral cavity and ovary (Semenza, 2012). Interestingly, hypoxia not only recruits TAMs in TME but also remodels them towards a pro-tumorigenic phenotype by modifying the gene expression pattern. It has been suggested that retention of TAMs in hypoxic areas is achieved by reducing their mobility through hypoxia-mediated upregulation of mitogen-activated protein kinase phosphatase enzymes, which nullifies the TAMs response to chemoattractants outside the hypoxic areas by inactivating ERK1/2 and p38 MAP kinase signaling pathways (Grimshaw and Balkwill, 2001). MMPs help in sequestering VEGF from the ECM to expedite tumor vascularization. TAMs have been shown to secrete hypoxia-activated proteolytic enzymes like MMP-1 and MMP-7. Moreover, hypoxia recruited $CD45^{+}$ monocytic cells into tumors and accelerated tumor angiogenesis by secreting MMP-9 (Du

et al., 2008). Hypoxia was shown to upregulate Tie-2 expression in monocytes, thereby contributing to angiogenesis (Murdoch et al., 2007).

Hypoxia, especially HIF-1α, induces the expression of several pro-angiogenic factors including PDGF-B. Stromal CAFs activated by PDGF-B, produce PDGF-C, erythropoietin and FGF, which collectively coordinate VEGF-independent angiogenesis (Cao, 2013). The HGF/c-Met signaling pathway plays a crucial role in driving compensatory angiogenesis. Hypoxia induces the HGF/c-Met signaling pathway especially in pancreatic cancer model studies by upregulating expression of c-MET in cancer cells and HGF production by fibroblast cells (Ide et al., 2006). CSCs play a crucial role in tumor angiogenesis, recurrence and drug resistance. In general, CSCs mostly accumulate in hypoxic-rich regions where HIFs are upregulated (Vlashi et al., 2009). Both HIF-1α and HIF-2α have been implicated in stimulation of CSCs in glioblastoma. The hypoxic niche not only maintains glioblastoma stem cells but also stimulates reprogramming towards a CSC phenotype (Seidel et al., 2010). In an animal model setting, the treatment with anti-angiogenic drugs leads to an increase in hypoxia and in the number of breast CSCs (Conley et al., 2012). In glioblastoma animal model studies, Hu et al., (2012) demonstrated that hypoxia triggers autophagy, tumor cell survival and confers adaptive resistance (Hu et al., 2012). The roles of hypoxia in activation of a series of pro-angiogenic factors, signaling pathways and drug resistance are presented in **Figure 4.** The present state-of-the-art clearly outlines the crucial role of hypoxia-mediated regulation of different pathways/factors that directly or indirectly promote compensatory angiogenesis and limit the efficacy of currently used anti-angiogenic agents. Under such circumstances, targeting hypoxia and development of novel anti-hypoxic agents might circumvent the emerging resistance towards angiogenesis inhibitors. As a representative example of targeting hypoxia, metformin-treated tumors resulted in significant reduction in HIF-1α expression and improved tumor blood perfusion with suppression of hypoxia-induced excessive tumor angiogenesis (Wang et al., 2017).

2.6 Alternative Mechanisms of Remodeling Angiogenesis-Independent Tumor Vasculature

Being a hallmark of tumor growth, angiogenesis was considered indispensable for tumor growth and metastasis, however, this understanding turned out as an illusion owing to the presence of angiogenesis-independent vascular remodeling mechanisms like vessel cooption, vascular mimicry and intussusceptions. Processes like postnatal vasculogenesis, looping angiogenesis and

glomeruloid angiogenesis also serve as a vasculature under different circumstances (Holash et al., 1999). These angiogenesis-independent vascular remodeling processes might serve as pacemakers to tumors, which experience the exposure of anti-angiogenic stress and may sustain tumor growth under such critical conditions. Series of investigations have reported the association of these angiogenesis-independent vascular mechanisms as one of the important factors in limiting the efficacy of anti-angiogenic therapy and compensating for the requirement of classical angiogenesis during anti-VEGF stress. Well-studied processes like vessel cooption, intussusceptive microvascular growth, vascular mimicry and their angiogenesis- independent mechanisms are described below.

2.6.1 Vessel Cooption

Perhaps, Holash and colleagues were the pioneers to demonstrate that the initial growth of some tumors is angiogenesis-independent and could be mediated by vessel cooption with existing normal vessels of the host tissue (Holash et al., 1999). In general, a vessel cooption mechanism is more frequently encountered in densely vascularized organ cancers such as lung, brain, and liver; in most of the cases, the primary tumor cells including metastases coopt with the adjacent existing quiescent cells and migrate along the vasculature of the host organ tissue. Accumulating experimental evidence clearly indicates that many human solid tumor types may opt for vessel co-option, which might profoundly demonstrate resistance towards anti-angiogenic agents (Kuczynski et al., 2016). Series of recent research findings identified vessel cooption as one of the important mechanisms in sustaining tumor growth under anti-angiogenic stress. For example, Sorafenib-resistant tumors sustained tumor growth by extensive incorporation of liver parenchyma and the co-option of liver-associated vessels. Interestingly, up to 75% of total vessels were provided by a vessel co-option mechanism in resistant tumors as compared to 23% in untreated tumors (Kuczynski et al., 2016). In a mouse model carrying cerebral brain metastases of the human angiogenic melanoma cell line Mel57-VEGF-A, even after treatment with ZD6474, a VEGFR2 inhibitor, the tumor sustained tumor progression via vessel co-option (Leenders et al., 2004).

In an orthotopic intracerebral glioma model study, Kunkel and colleagues showed that the treatment with DC101 (antibody against VEGFR-2) significantly reduced tumor growth and microvascular density, however, this treatment resulted in the formation of central cores of coopted vessels. It was also observed that tumor cells travelled a long distance along the coopted vessels

of the host to reach the surface and spread over the meninges (Kunkel et al., 2001). In preclinical mechanistic animal model settings, it was observed that vessel co-option mediates resistance to bevacizumab therapy in liver metastases. The motility-related actin protein 2/3 complex (Arp2/3) was involved in mediating vessel co-option in liver metastases. The authors also observed that combined inhibition of angiogenesis and vessel co-option was more effective than the inhibition of angiogenesis alone (Frentzas et al., 2016). In a genetically engineered mouse model of pancreatic neuroendocrine tumors which were resistant to VEGFR-2 treatment, were found to possess blood vessels with more coverage of PCs expressing smooth muscle actin (α-SMA). The authors opined that the blood vessels carrying α-SMA^+ PCs residing within resistant tumors were derived from coopted blood vessels (Franco et al., 2011). Recent preclinical lung metastasis model studies revealed that vessel co-option in human lung metastases is frequently observed with anti-angiogenic treatment and also found to be implicated in mediating drug resistance against anti-angiogenic drugs (Bridgeman et al., 2017). Vessel cooption was evident in brain metastases of cerebral melanoma treated with the ZD6474 anti-angiogenic agent (Leenders et al., 2004). Interestingly, the coopted vessels from different tumors demonstrated differential response towards anti-angiogenic agents.

For instance, in primary and metastatic lung cancer and liver metastasis from distant primary origins, over 10- 30% of the tumors are observed to be dependent on vessel cooption for retrieving blood supply. Describing the mechanisms of vessel cooption is beyond the scope of the present review; however, current literature links the process of vessel cooption as one of the mechanisms of acquired resistance to anti-angiogenic therapy. Indeed, in the early stage of cooption, perhaps the tumor growth is angiogenesis-independent, also tumor vessels acquire phenotype of normal vessels, hence might be less sensitive to anti-angiogenic agents (Donnem et al., 2013).

2.6.2 Intussusceptive Microvascular Growth

Splitting angiogenesis is another name of intussusceptive microvascular growth (IMG) which involves the development of two vessels by fission of a parent vessel without sprouting. Experimental evidence accumulated clearly demonstrating that IGM is involved in remodeling vasculature under anti-VEGF treatment in a variety of cancers and is a kind of adaptive response to stress conditions and hypoxia (Ribatti and Djonov, 2012). Previous reports clearly indicate the

induction of IMG after a relapse of anti-angiogenic treatment. For example, treatment with PTK787/ZK222854 (a VEGF tyrosine kinase inhibitor) of mammary carcinoma allograft resulted in drastic reduction in tumor growth and vascularization. However, the post relapse observations revealed the development of extensive IMG with characteristic sinusoidal-like vessels consisting of multiple transluminal tissue pillars. The authors opine that the significant reduction in intra-tumoral microvascular density might be associated with intussusceptive pruning that leads to a minimal decrease in the total microvascular exchange area. Nevertheless, IMG contributes to improving the tumor oxygen supply and the overall perfusion of the tumor mass (Hlushchuk et al., 2008). A clear evidence of rapid revascularization in the form IGM was observed in RIP-Tag 2 and Lewis lung carcinoma models harvested after treatment with VEGFR inhibitors (Mancuso et al. 2006). Similar changes were observed in murine RCC vasculature (Drevs et al., 2002).

Tumors might possibly opt towards IMG during anti-angiogenic treatment as it is faster, metabolically feasible and thermodynamically stable as compared to classical sprouting angiogenesis. Moreover, it has optimal vascular permeability and neither it involves high rate of EC proliferation nor it requires degradation of basement membrane for invasion (Hlushchuk et al., 2008; Ribatti and Djonov, 2012). Thus, the state-of-the-art literature clearly links the IMG as one of the adaptive responses towards anti-angiogenic agents and a potential compensatory angiogenic mechanism; however, more tailored experimental settings might provide deeper insights to IMG under anti-VEGF microenvironment. In a recent research investigation designed to unravel the molecular mechanisms underlying intussusceptive angiogenesis, the results revealed that the two molecules implicated in vascular growth and differentiation, namely endoglin (ENG) and chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) were involved in intussusceptive angiogenesis. It was observed that ENG inhibition and upregulation of COUP-TFII was strongly correlated with progression of intussusceptive angiogenesis (Hlushchuk et al., 2017).

2.6.3 Vascular Mimicry

The pioneer investigation by Maniotis et al., in 1999, discovered a VEGF-independent tumor supporting vascular process called as vasculogenic mimicry (VM). In an experimental setting, the authors demonstrated that invasive human melanoma cells were similar in function and phenotype to ECs and interestingly, these cells generated tunnel-like vascular phenotype that was foolproof

in carrying red blood cells without involvement of ECs (Maniotis et al., 1999). After opening of this research avenue, a plethora of investigations have enhanced our understanding regarding mechanistic insights and formation of VM in different human malignant tumors like breast, melanoma, bladder, kidney, gliomas, glioblastomas, prostrate, ovarian, lung, sarcomas of different tissues, RCC and astrocytoma (Kirschmann et al., 2012). The complex orchestration of VM generation is an integration of three different aspects, the primary requisite is the plasticity of aggressive tumor cells and remodeling of the ECM which finally leads to establishment of connections of the VM channels with the existing vasculature of host microvessel network (Fan and Sun 2010). Tumor cells having potential of VM, possess a high degree of plasticity and demonstrate multiple phenotypes similar to those of embryonic stem cells (Qiao et al., 2015). Several signaling molecules/cells like stem cells (Nodal, Notch4), vascular factors (VE-cadherin, EphA2, and VEGFR1) and hypoxia-related HIF are critical regulators of VM (Paulis et al., 2010). Each of these pathways warrants contextual investigations to maneuver them as potential vascular targets, indicators of drug resistance, plasticity and aggressive metastatic phenotype.

Clinically, it was observed that the occurrence of VM in patients is mostly associated with poor prognosis, high risk of metastasis, relapse of cancer and worse survival for patients of different cancers like breast, ovarian, melanoma, primary gall bladder, RCC, malignant esophageal stromal carcinoma, hepatocellular carcinoma, mesothelial sarcomas and alveolar rhabdomyosarcomas (Fan and Sun, 2010). There is evolving evidence of direct involvement of VM under anti-angiogenic environment. The resistance of VM to anti-angiogenic agents like TPN-470 and endostatin was observed in B16F10 murine melanoma model and melanoma tumor model (van der Schaft et al., 2004). It is interesting to note that CSCs possessing inherent recurrent abilities play a crucial role in hypoxia-induced VM (Liu et al., 2013). Therefore, the hypothesis that hypoxia induced by anti-angiogenic agents accelerates the formation of VM channels for sustaining tumor growth is indeed rationale, as administration of anti-angiogenic agents leads to induction hypoxia (Semenza, 2012; Ray et al., 2017). Moreover, in a recent investigation an attempt has been made to test the previous hypothesis in a nude mouse model and in clinical setting as well. The results of the study concluded that treatment with sunitinib leads to accumulation of hypoxia in this triple-negative breast cancer (TNBC) mouse model. Moreover, sunitinib-induced hypoxia further activates the VM regulating consigliere the gene Twist1, which in turn accelerated VM by increasing the CSC population of $CD133^{+}$ cells. A similar trend was observed in human

patients with TNBC. The authors also claim VM as a culprit for the recurrence of TNBCs after the relapse of sunitinib treatment and appeal the need of targeting Twist 1 for increasing the therapeutic index of anti-angiogenic regimens (Zhang et al., 2014). In xenograft model co-cultured with glioblastoma cell lines, the treatment with sunitinib significantly impaired vascular functions. However, blockade of VEGF activity using bevacizumab failed to recapitulate the impact of sunitinib. The outcome of the experiment suggested that Flk-1-mediated VM is independent of VEGF. Moreover, the analysis of xenotransplanted tumors revealed the presence of robust mural cell linked vascular channels in anti-VEGF environment (Francescone et al., 2012). In recent xenograft animal model studies, sunitinib treatment leads to tumor invasion via VM in TNBC (Sun et al., 2017).

As a part of designing novel anti-VM agents for increasing the efficacy of antiangiogenic drugs, results of preclinical animal model studies demonstrated that Everolimus can adversely affect VM and tumor cell differentiation in sunitinib-resistant RCC tumors in mice (Serova et al., 2016). With a similar motive, the studies conducted using glioblastoma xenograft animal model clearly observed that Vatalanib induced an enhanced VM predominantly in the hypoxic core region of the tumor. Interestingly, the potent 20-HETE (20-Hydroxyeicosatetraenoic acid) biosynthesis inhibitor HET0016, significantly attenuated the growth of GBM tumors by targeting VM structures at the core and at the periphery of the GBM tumors (Angara et al., 2017). Ascribing VM as a potential factor in conferring tumor resistance can be speculative, as the current anti-angiogenic regimen has not been proved effective in ameliorating VM-mediated vascular network (Pauliset al., 2010). The study of VM in context with development of anti-VM agents is in its infancy; however, the future settings should capitalize more on inhibiting the plasticity of tumor cells, signaling pathways of VM and remodeling of ECM in favor of VM; perhaps VM may serve as an attractive therapeutic target for the management of compensatory vascular mediators.

3.0 Dynamics of Multidrug Drug Resistance in Cancer: A Molecular Chess?

Despite the great strides of efforts that have been made towards the discovery and improvements of novel anti-cancer drugs, a satisfactory chemotherapy outcome is consistently limited by the emerging multidrug resistance (MDR) both in classical chemotherapy and targeted therapy against variety of human cancers **(**Shapira et al., 2011; Gonen and Assaraf, 2012; Livney and Assaraf, 2013; Taylor et al., 2015; Zhitomirsky and Assaraf, 2016; Li et al., 2016; Gottesman et al;., 2016;

Bar-Zeev et al., 2017). Although patients may respond well at the initial phase of treatment, recurrence after "drug holidays" is inevitable for many patients harboring distinct types of cancers. The last 40 years of research in cancer biology has unraveled a variety of inscrutable secretes of resistance to anti-cancer drugs. The most notable mechanisms within neoplastic cells include: impaired drug uptake, resistance via expression of multidrug efflux pumps, alteration of drug targets, up-regulation or expression of drug detoxification mechanisms, down-regulation of genes imparting reduced susceptibility to apoptosis, enhanced ability to repair DNA damage and alternative modes of cancer cell proliferation. Nevertheless, increasingly sophisticated state-of-the-art instrumentation is being made available for measuring and tackling drug resistance mechanisms at the clinical levels (Cree and Charlton, 2017).

The current preclinical and clinical data also attribute the stroma/tumor microenvironment and local immunity as major contributing factor towards the development of cancer drug resistance (Gacche, 2015). One of the most important aspects of cancer drug resistance is the evolving tumor heterogeneity wherein the positive selection of drug-resistant clones can act as drivers of cancer drug resistance (Caswell and Swanton, 2017). In the past two decades, research observations from preclinical and clinical studies speculate about the possibility of the dependency of tumors on the therapeutic drugs to which they have acquired resistance. This concern of drug resistance certainly represents vulnerability with potential applications in management of cancer treatment. A recent investigation in this relation clearly revealed that the ERK2-dependent pathway relays the cancer drug addiction phenomenon in lung and melanoma cancer. The finding offers a 'proof of concept' that underpins the drug addiction in cancer cells (Kong et al., 2017). However, the concrete molecular mechanisms of drug addiction trait are yet to be established. In the midst of emerging complexities of MDR in cancer, the clinical oncologist should be at least one step ahead of developing tailored anti-cancer agents targeting drivers of cancer drug resistance, which perhaps may act as 'checkmate' for cancer progression.

3.1 Tumor Drug Resistance: An Emerging Challenge in Anti-angiogenic Therapy

Before the launching of anti-angiogenic agents in the clinic, they were appreciated based on the fact that the anti-angiogenic regimen may not encounter resistance because of its specific targeting of ECs, which *per se* do not demonstrate genetic instability (Boehm et al., 1997). Genetically quiescent recognition of ECs and the resistance forecast of anti-angiogenic therapy was logical on

one hand, but proved to be an illusion in the TME due to epigenetic alterations conspired by the coordinated crosstalks of ECs, cancer cells and stromal cells. Moreover, karyotype analysis of tumor ECs showed characteristics of aneuploidy. The research findings showed that ECs from tumors derived from different metastatic capacity levels also demonstrate different angiogenic capabilities. In brief, this EC heterogeneity, although may provoke a mere consequence of local tumor growth environment or a crosstalk between tumor cell and ECs, it adversely affects the therapeutic index of anti-angiogenic drugs (Hida et al., 2013). In addition, ECs and tumor blood vessels exhibit a significant heterogeneity with respect to its sensitivity towards anti-angiogenic agents, wherein some vessels are sensitive, while others are recalcitrant. Nevertheless, the more serious part is that the status of EC heterogeneity is in concert with the malignancy status of the tumor. There are clear instances in preclinical settings where VEGF-targeted therapy prunes the newly formed tumor vessels, but the more mature and established vasculature is comparatively less sensitive. Moreover, the ECs in the peripheral tumor vasculature are heterogeneous, possess stem cell or EPC-like properties and play an indispensable role in tumor angiogenesis. The lack of susceptibility of these ECs towards anti-angiogenic agents might contribute towards anti-angiogenic drug resistance (Bergers and Hanahan, 2008; Hida et al., 2015). Perhaps, the insult to genetic stability of ECs might be a cause for manifestation of altered sensitivity to anti-angiogenic therapy. Anti-angiogenic modality has achieved an appreciable success over the past decade, where to date the FDA has approved eleven anti-angiogenic agents targeting VEGF or its receptors for the treatment of cancer (**Table1**). However, the overall clinical benefits have been more muted than expected. Moreover, the progression-free survival was not always in tune with overall survival of the patients. In the midst of such rebounds, the paradigm of "resistant to drug resistance" turns out as an illusion and now the therapy is being given similar status to other therapies, which have displayed inherent/acquired resistance towards the therapeutic modalities in patients, leading to disease recurrence.

An impressive repertoire of tailored investigations in preclinical and clinical settings have unraveled several molecular factors, tumor/stromal cells, signaling pathways and their coordinated crosstalk in driving compensatory angiogenesis and thereby conferring resistance to anti-angiogenic therapy (discussed in various sections of the review). The issue of resistance to anti-angiogenic therapy has gone up to the doorsteps of the FDA, which withdrew its approval in 2010 for bevacizumab for the treatment of metastatic breast cancer (HER2-negative) because of the lack

of apparent survival benefits to patients. Nevertheless, even the treatment of these patients with sunitinib could not extend the patients disease free survival for a considerable period (Welti et al., 2013). A vast body of preclinical and clinical findings addressed the rebound invasiveness of tumor cells and emergence of a highly aggressive tumor phenotype in a variety of cancers after the relapse of the anti-VEGF/R treatment. More precisely, intrinsic resistance against bevacizumab, sorafenib, and sunitinib treatment in various human cancers is well documented in the clinical and preclinical settings (Ebos and Kerbel, 2011; Ebos and Pili, 2012; Welti et al., 2013; Jeffrey and Hurwitz, 2013; Vasudev and Reynolds, 2014; Wragg et al., 2017; Tomida et al., 2017; Simon et al., 2017). Besides the preclinical data of anti-angiogenic drug resistance, the data from clinical trials is also disappointing towards the clinical benefits. Moreover, there are also a few reports describing the emergence of adaptive resistance to combination chemotherapy comprising of anti-angiogenic drugs (Kopetz et al., 2010; Tomida et al., 2017). Indeed, it is practically impossible to address the vast body of clinical data of resistance to anti-angiogenic agents that is encountered during series of clinical trials. Series of reviews published elsewhere might offer a clear understanding in this regard (Kopetz et al., 2010; Welti et al., 2013; Vasudev and Reynolds, 2014; Jeffrey and Hurwitz, 2013; Tomida et al., 2017). A cursory look at the current clinical trials in 'Anti-angiogenic Drugs' or 'Angiogenesis Inhibitors' posted at ClinicalTrial.gov (accessed in October 2017) shows that out of total 4479 registered data trial entries, 2110 trials were completed (47%), 461 trials were terminated (10%), 133 trials were withdrawn (almost 3%), 23 (0.51%) trials were suspended, 574 trials are active (not recruiting, 12 %) and only 4 trials have been approved for marketing.

Intra-tumor heterogeneity is one of the emerging challenges which is limiting the efficacy of current cancer treatment regimens including anti-angiogenic drugs. The clinical data clearly focus on the adverse effects of emerging tumor heterogeneity limiting the efficacy of cancer chemotherapy drugs (Bedard et al., 2013). The evolving nature of tumor heterogeneity is at par with a dynamic evolving ecosystem. The intra-tumor mutations are a rule rather than an exception and the mutated cells, which do not adapt to the TME are marginalized, whereas the cells adapting with the ongoing intra-tumor metabolic trend are emerging as more aggressive and "arrogant" clones than their parental counterparts **(Figure 5).** Owing to the cellular dynamics of tumor heterogeneity and its concerns towards drug efficacy, this area of research is in the frontline of tumor biology labs across the world (Easwaran et al., 2014). A heterogeneous response to anti-angiogenic therapy is observed sometimes in patients with multiple metastases. For example, in

the same patient some lesions are sensitive to treatments while others are refractory and progressive (Vasudev et al., 2013). This is critical for patient management and prognosis. Histopathological observations on human liver and lung cancer revealed remarkable inter- and intra-tumor heterogeneity in adapting either to angiogenesis or to co-opted vessel to retrieve the vascular connections (Van den Eynden et al., 2012). The observations clearly indicate that there exist more than one mechanism of tumor vasculature and perhaps may be the cause of differential sensitivity towards anti-angiogenic agents. In addition, genetic profiling revealed the existence of intra- and inter-tumor genetic heterogeneity (Meacham and Morrison, 2013). Heterogeneous tumors harbor several subclones of different characteristics and obey Darwinian Theory of 'survival of fittest', therefore it is likely that some of the subclones might have acquired resistance to chemotherapy including to anti-angiogenic agents. Sizable literature has accumulated in the recent years correlating the intra-tumor heterogeneity with resistance to treatment and poor prognosis (Caswell and Swanton, 2017). In the words of Marcel Proust, "The real act of discovery consists not in finding new lands, but in seeing with new eyes." Indeed, it applies to the current cancer chemotherapy. On the eve of emerging imbroglio of tumor heterogeneity, it is imperative to change the approach of developing novel anti-tumor agents. Perhaps, new ways of viewing cancer might open new avenues for the design of more effective therapies with improved therapeutic index.

Pharmacokinetic resistance refers to the inability to deliver the right dose of a drug molecule to the right destination, for a proper duration. There is every possibility that failure of many anti-angiogenic agents might be associated with pharmacokinetic resistance. Based on the preclinical and clinical data, Jain and others are consistently advocating that judicious selection of the right dose and dosing duration of anti-angiogenic agents might play a crucial role in normalization of complex and abnormal tumor vasculature by alleviating tumor hypoxia, thereby improving the tumor perfusion or oxygenation and effective uptake of chemotherapeutic agents (Jain, 2005; Chatterjee et al., 2014; Jain, 2014). Administration of higher doses of anti-angiogenic agents might increase hypoxia that may fuel metastasis which clearly harbor CSCs. Conversely, lower doses might improve tumor perfusion and oxygen uptake. In breast cancer model studies, low doses of an anti-VEGFR2 antibody like 10 or 20 mg/kg increased perfusion as compared to a high dose of 40 mg/kg (Huang et al., 2012). Nevertheless, short time exposure to anti-angiogenic drugs also improved the delivery of cytotoxic agents and therapeutic impact in lung cancer

(Chatterjee et al., 2014). Although dose-response and time schedule might be associated with increased tumor perfusion and overall survival (Jain, 2014), the subsequent challenge is to find answers to why the same dose/time schedule of anti-angiogenic agents is indiscriminate at patient's level?

Lastly, a cursory look at the current state-of-the-art literature accumulated in the mainstream of angiogenesis inhibitors, reveals that apart from emerging EC heterogeneity there are also few more emerging challenges. For example, emergence of anti-angiogenic VEGF and its suspicious role in anti-VEGF resistance, interactive gossip of extracellular vesicles secreted by tumor cells and stromal cells in conferring drug resistance, sequestration and accumulation of anti-angiogenic drugs in the lysosomes and endocytic vesicles and emergence of related acquired resistance (Zhitomirsky and Assaraf, 2015, 2016, 2017), glycosylation-driven resistance (as galectin-1 activates VEGFR2 signaling in the absence of VEGF), and genetic polymorphisms in the VEGF pathway. However, the molecular underpinnings of these emerging mechanisms are evolving and yet to be established (reviewed by van Beijnum et al., 2015).

4.0 Opportunities in Future Setting of Anti-angiogenic Modalities

Before one plans the design and development of novel strategies in anti-angiogenic regimen, it will be prudent to have a cursory look at the ground realities in the mainstream of targeting angiogenesis. First, blood vessels are not the exclusive targets of the anti-angiogenic agents, rather, they also influence the off target physiological functions. For example, besides key role in recruiting new blood vessels, VEGF can block or regulate the maturation of dendritic cells, can serve as a survival factor, promote epithelial-mesenchymal transition (EMT), and promote the stem cell phenotype in cancer cells (Goel and Mercurio, 2013). Moreover, the VEGF family member PlGF acts as a survival factor for medulloblastoma cells and promotes their spread via neuropilin 1 signaling (Snuderl et al., 2013). Nevertheless, besides targeting VEGFR, sunitinib also interacts with c-KIT, which is usually mutated in gastrointestinal stromal tumor cells. Anti-angiogenic agents directly or indirectly interact with several stromal and cancer cells (Jain, 2014). Second, one may need to think beyond our current understanding that all cancers vascularize in a similar manner. Interestingly, cancers such as RCC and neuroendocrine tumors which mostly depend on VEGF-mediated angiogenesis are sensitive towards anti-VEGF treatments, however, breast, melanoma and pancreatic cancers which are moderately sensitive to anti-VEGF therapy,

may utilize distinct mechanisms of vascularization. Another important enquiry is whether the phenotype of vasculature of primary tumors and its cognate metastasis is similar or different. If we assume that organ-specific TME is influential in tumor growth, there is likely to be a difference in metastatic vasculature as it is not a homogenous network, but possesses significant heterogeneity of phenotype and function in different organs (Langenkamp and Molema, 2009). Moreover, tumor cells are often re-educated for vascularization in a new microenvironment (Quail and Joyce, 2013; Vasudev and Reynolds, 2014). Although selective pressure of tumor evolution is primarily linked as a plausible explanation, the concrete understanding of such diverse angiogenic mechanisms that might operate at different levels of cancer progression are still not clear. Efforts in this direction may help in developing personalized anti-angiogenic therapy.

It is not always important to "punish the criminals, but it is more important to punish the agency promoting crime". Hypoxia is such an instrumental crime partner, promoting a variety of redundant angiogenic factors, signaling pathways, BMD myeloid cells, stromal cells, CSCs etc. for coordinated and integrated sabotaging functions in tumor progression under anti-angiogenic environment. Therefore, targeting hypoxia-induced factors, signaling pathways and specialized cells, should be the baseline principle for the design and development of novel strategies towards targeting tumor angiogenesis. Nevertheless, owing to the emerging resistance to the currently available anti-angiogenic agents, future settings should capitalize more on developing novel anti-angiogenic agents targeting compensatory angiogenic mechanisms (Ebos and Kerbel, 2013), which may be used in combination with currently available anti-angiogenic agents. In the current state-of-the-art, combination therapeutic approach seems to be a possible strategy for the management of emerging tumor resistance. Several clinical and preclinical reports have demonstrated positive impact of combination therapy in improving the clinical benefits of anti-angiogenic drugs. As suggested by Jain and others (Jain, 2005; Chatterjee et al., 2014; Hida et al., 2015), the clinical benefits of cytotoxic chemotherapy have been appreciated when it is administered in combination with bevacizumab. More precisely, the clinical benefits of such combinations were more significant to patients with metastatic colorectal cancer, metastatic breast cancer, non-squamous and NSCLC, RCC, head and neck cancer and ovarian carcinoma (Li, et al., 2011; Ebos and Kerbel, 2013). Developing inhibitors against alternative pro-angiogenic factors and testing them in combination therapy has been suggested as a remedy for combating emerging resistance to anti-angiogenic agents (Ebos and Kerbel, 2013; Jain, 2014).

Although several combinatorial clinical trials have been terminated on several grounds (ClinicalTrials.gov.in), series of preclinical and clinical model studies demonstrate that the combination of inhibitors of compensatory angiogenic factors with bevacizumab or cytotoxic chemotherapy improve the anti-angiogenic effects and tumor resistance. Combined inhibition of bFGF and VEGF using the dual inhibitor brivanib prolonged tumor metastasis and angiogenic arrest when used as a first-line therapy, or as a second-line treatment after the relapse of previous episode of anti-angiogenic treatment. A therapeutic strategy of combining Dll4/Notch and Ephrin-B2/EphB4 inhibitors has been found to be highly effective in blocking tumor angiogenesis (Djokovic et al., 2010). In xenograft model studies, anti-ANG2 antibodies effectively blocked tumor growth and angiogenesis and significantly improved the anti-angiogenic efficacy of anti-VEGF agents (Daly et al., 2013). Moreover, a combination of ANG inhibitors and cytotoxic chemotherapy improved the progression-free survival of ovarian cancer patients (Karlan et al., 2012). In a mouse tumor model study, crizotinib, a dual inhibitor of c-Met and ALK pathways in combination with sunitinib, effectively inhibited the frequency of sunitinib-induced metastasis (Shojaei et al., 2012). Similarly, in an experiment involving resistant tumor model, combination of sunitinib and a selective inhibitor of c-Met demonstrated significant inhibition of tumor growth as compared to the individual treatment of these agents (Shojaei et al., 2010). PDGF-C has been identified as an important mediator of TAF-induced VEGF-independent angiogenesis (Crawford et al., 2009). In an *in vivo* murine lymphoma tumor model setting, a combination of anti-PDGF-C with bevacizumab remarkably reduced the growth of admixture tumors as compared to monotherapy with bevacizumab. As compared to individual treatment of bevacizumab, almost 74% of tumor growth inhibition was observed in combinatorial treatment (Crawford et al., 2009). A combined treatment of bevacizumab with anti-IL-17A improved the efficacy of anti-VEGF antibodies and reduced the tumor growth by ~50% as compared to reference treatment in mice bearing EL4 tumors (Chung et al., 2013). Human IgG1 Fc fragment fused Wnt inhibitory factor 1 (WIF1-Fc) and secreted frizzled-related protein 1 (sFRP1-FC), not only inhibited Wnt-mediated tumor growth and vascularization in HCC but also promoted the apoptosis rate in tumor cells and inhibited the migration of human microvascular ECs and mouse EPC (Hu et al., 2009). Combined treatment of bevacizumab with PF-03446962 (a human IgG2 monoclonal anti-ALK1 antibody) significantly improved the efficacy of VEGF/VEGFR targeting agents (Hu-Lowe et al., 2011). The agents that target BMD stromal and tumor cells have been tested in combination with anti-

angiogenic agents. For example, combination of an anti-Gr1 antibody (against $CD11b^{+}Gr1^{+}$ cells) with bevacizumab, successfully delayed the onset of tumor refractoriness (Shojaei et al., 2007). In a nude mouse model study designed to explore the tumor homing mechanism of $CD11b^{+}Gr1^{+}$ lineage, the combined treatment of anti-VEGF and anti-G-CSF (or anti-Bv8) dramatically reduced tumor growth as compared with the treatment of anti-VEGF-A alone. However, such combination failed to completely block tumor growth (Shojaei et al., 2009). With Judicious and rational formulation of combination therapies indeed indispensable, as combination of anti-angiogenic agents with drugs that target oncogenic pathways has resulted in discouraging results in clinical trials. Although the preclinical results of combined efficacy of VEGF- and EGFR-targeted drugs were impressive in colorectal and NSCLC models, all the phase III clinical trials of such combination failed (Tol et al., 2009). Similar frustrations were encountered in phase III trials comprising a combination of VEGF- and HER2-targting agents in $HER2^{+}$ breast cancer patients (Gianni et al., 2013). The possible mechanism of such failure suggests that the selected dose of bevacizumab might have reduced the size of pores in the tumor vessel walls and may have compromised the delivery of antibodies (Chauhan et al., 2013). Besides the promising efficacy of combination strategies in preclinical studies, there are some practical difficulties in testing such combinations in the clinic. For example, anti-angiogenic agents have remarkable toxicity (Gacche and Meshram, 2014; Jain, 2014) and at present, the toxicity data of combination therapy is lacking. Moreover, finding a druggable target and formulating a combination strategy with an acceptable toxicity profile is troublesome (Garcia et al., 2012).

Besides the intrinsic and adaptive tumor resistance to anti-angiogenic agents, there are other subsidiary, rather important issues that limit the administration of anti-angiogenic drugs. For example, in the midst growing complexities of cancer progression and unpredictable outcome of the current therapeutic modalities in general, and anti-angiogenic therapy in particular, the physician's prescriptions are still not guided by the valid biomarkers of anti-angiogenic therapy. Developing anti-angiogenic therapy-related biomarkers not only will help physicians in management of the disease, but will facilitate correlating the levels of such valid tissue/circulating biomarker with treatment outcome, hence may unravel potential pathways or provide cues conferring refractoriness to anti-VEGF therapies. For example, upregulation of VEGFR1 before treatment was associated with a poor performance of bevacizumab in HCC, rectal carcinoma and metastatic colorectal carcinoma patients (Jain, 2014). Increased levels of sVEGFR1 are associated

with mild side effects in liver cancer and rectal cancer patients. Perhaps, the most extensively explored biomarker in current anti-angiogenic therapy is VEGF. Besides the significant correlation of VEGF in promotion of angiogenesis, its usage as a biomarker has not been validated in the context of the cancer type and disease progression (Jain et al., 2009).

Another important aspect that needs to be prioritized in the mainstream of angiogenesis research is the need for radical improvements in animal models that are used in the preclinical and clinical settings. In fact, FDA has primarily approved anti-angiogenic agents for the amelioration of metastatic diseases; however, paradoxically, the majority of the preclinical settings use primary tumor models for investigating the anti-angiogenic effects. Apart from the lack of sensitivity of bevacizumab to mouse VEGF, several murine model studies incorporate mouse VEGF in their protocols, which perhaps mask the contribution of host VEGF in the experimental inference. Many preclinical studies have tested both bevacizumab and aflibercept as monotherapies, apart from the fact that they have shown impressive overall patient survival only when used in combinations with chemo- or immune therapies (Jain, 2014). Preclinical studies should prioritize the investigations in metastatic or adjuvant settings, as impressive metastatic murine models are now being developed (Francia et al., 2011). The paradox of experimental outcomes in preclinical and clinical settings might affect the formulation of doses, combinations and overall management of disease. A few questions still need to be addressed using cutting edge experimental approaches; for example, based on the current understanding in the mainstream tumor angiogenesis, is it possible to identify early predictors of response to anti-angiogenic drugs and translate them into clinical practice? As hypoxia has been identified as one of the culprits in causation of resistance to anti-angiogenic drugs, will it be prudent to develop combination therapy targeting hypoxia? Although, impressive preclinical data is in favor of such possibilities, it is yet to become a clinical reality.

Conclusions

The clinical benefits of anti-angiogenic therapy have been appreciated both for malignant and non-malignant human diseases. Initially, this therapy was appreciated on certain limited understandings; however, the previously forecasted straightforward assumptions in relation to its unbeatable efficacy and ‘resistant to drug resistance’ candidature turned out to be an illusion and now this therapeutic regimen is described as “the great discovery and greater complexity” owing to frequently emerging drug resistance phenomena. The impressive repertoire of tailored

investigations carried out in the past two decades unraveled the fact that tumors employ multiple mechanisms of vascularization that compensate for the treatment of the currently used anti-angiogenic agents. The discouraging performance of anti-angiogenic agents in the clinics fueled the revisitation of research in tumor angiogenesis. Several combinatorial approaches that target the compensatory angiogenic factors or pathways have been tested in series of preclinical and clinical settings and many novel approaches are evolving for circumventing tumor resistance to anti-angiogenic agents. In the midst of toxicity and other negative concerns of the anti-angiogenic regimens, judicious and rationale efforts are needed while designing novel combinatorial treatment approaches, else the threat of 'several cooks spoil the meal' cannot be ruled out. The combinatorial approach of cancer therapy seems to be indispensable owing to a "Target-rich and lead-poor" imbalance, reflecting an insufficiency of chemists pursuing drug discovery of current anti-cancer drug development. Taking into consideration the emerging, intrinsic and adaptive tumor resistance, the inscrutable acts of compensatory angiogenic factors/pathways and their conspiracy in crosstalks, the dynamics and daunting tumor heterogeneity and the limitations of the currently used anti-angiogenic therapy, one needs to make a paradigm-shift from 'one drug-one target' to a 'one drug-multi-targets' approach for the development of novel anti-cancer leads. Our current understandings are mostly centered on VEGF *per se* which seems to be a key player in angiogenesis, while our knowledge of other molecular underpinnings conferring drug resistance is still evolving and is in its infancy. The current situation warrants deeper understandings of the molecular signatures that govern compensatory angiogenesis, which perhaps may lead to the development of novel anti-angiogenic agents that may target all types of tumor vessels and improve the therapeutic index of the anti-angiogenic modality. When we change the approach we may observe novel avenues, and we may hence achieve the target. New approaches of looking at the complex and dynamic nature of cancer may lead to the design of more effective and cost-compromised anti-cancer therapies, which might reduce life-threatening malignancies and economic burden of cancer patients and make them realize that cancer is not a capital punishment.

Conflict of Interest

The authors declare no conflict of interest

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Fig. 1. VEGF dependent redundant angiogenic signalling under anti-VEGF environment.

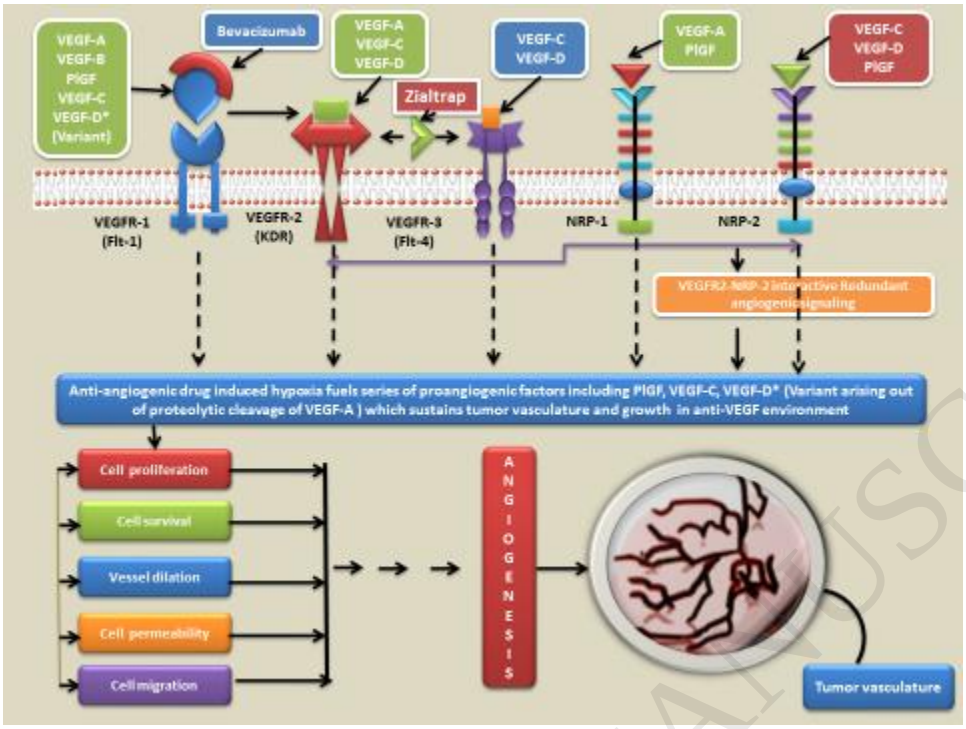

Fig. 2. VEGF independent compensatory proangiogenic signalling pathways in tumor progression and angiogenesis.

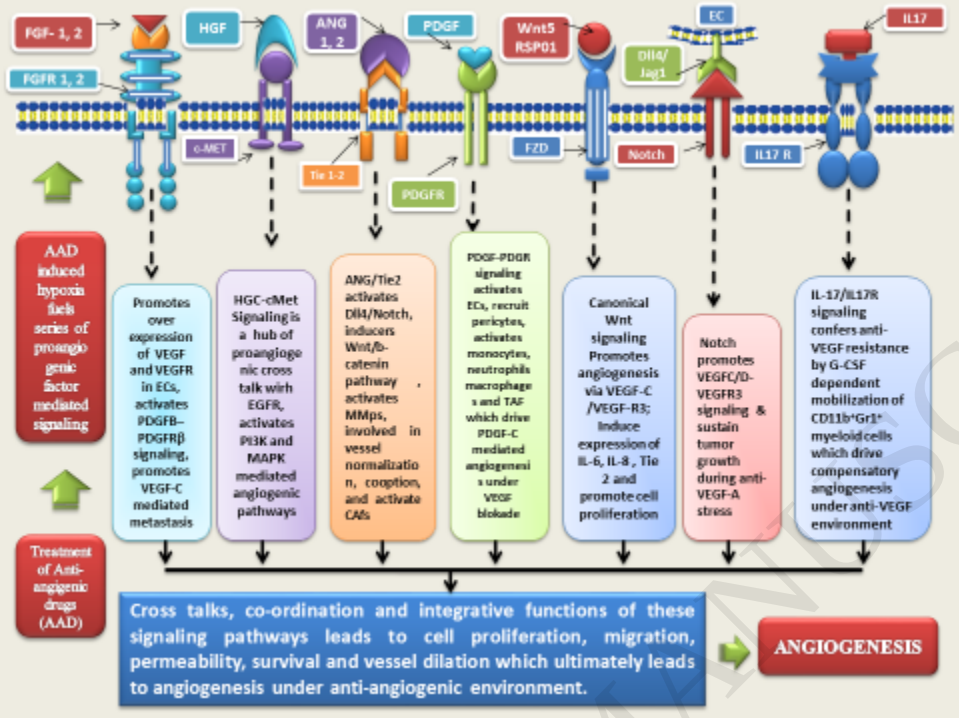

Fig. 3. Role of bone marrow derived myeloid and stromal cells in promoting tumor progression and angiogenesis under anti-VEGF treatment.

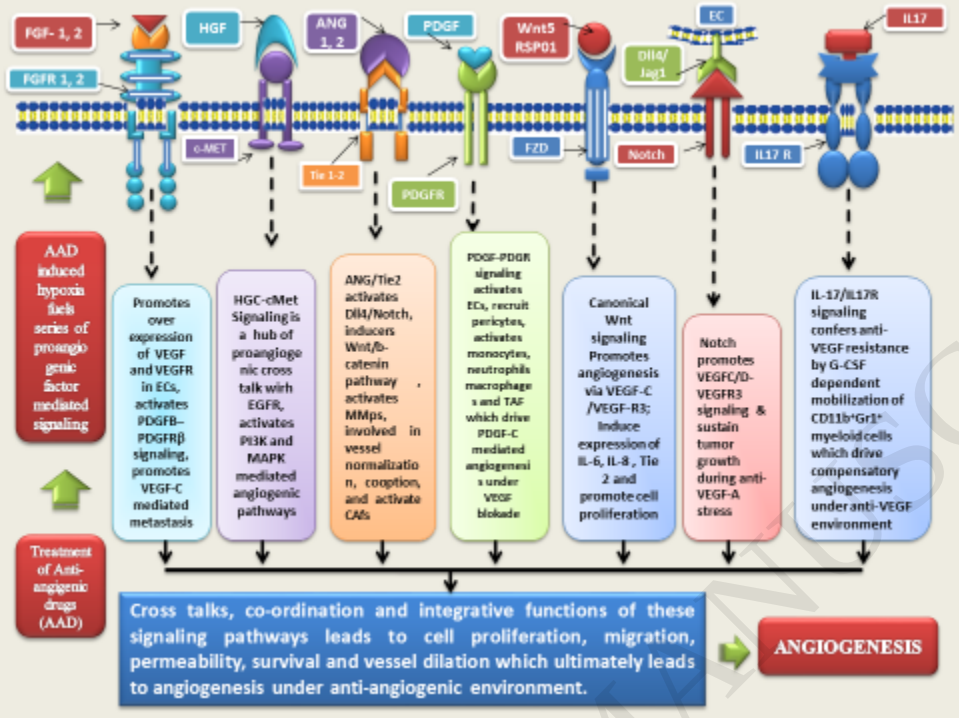

Fig. 4. Role of hypoxia in conferring tumor resistance to anti-angiogenic drugs.

Fig. 5. Evolving tumor heterogeneity and selection of drug resistant clones.

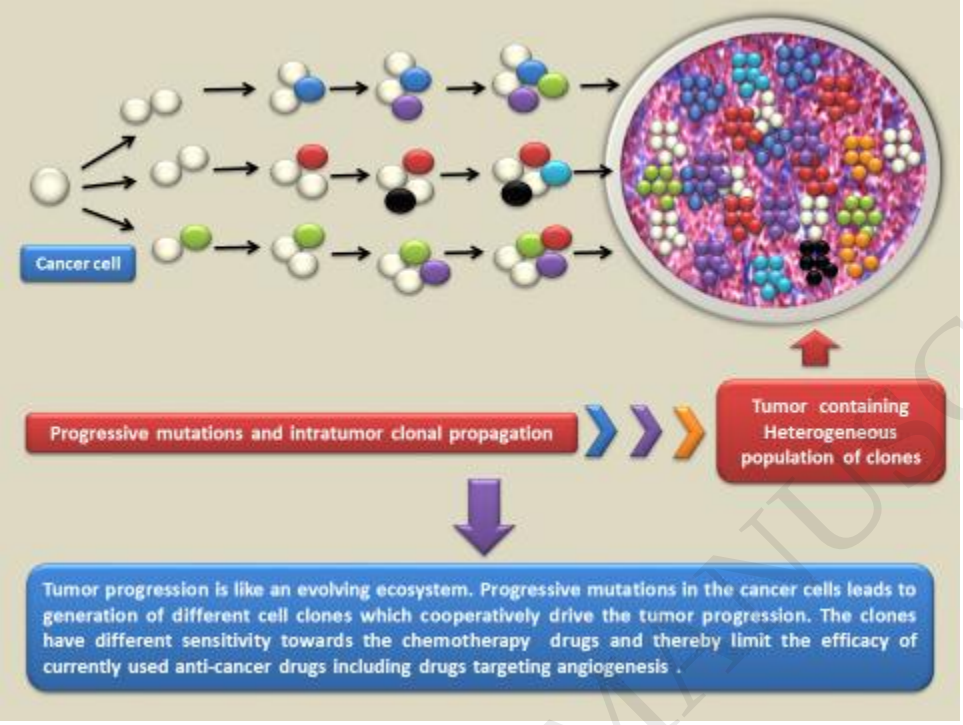

Table 1: Profile of FDA approved anti-angiogenic drugs.

Name of the Approved drug (Trade Name)	Therapeutic Targets	Year of Appr-ovals	Types of Cancer Treated	Guidelines for Treatment
Monoclonal Antibodies/chimeric fusion proteins				
Bevacizumab (Avastin)	VEGF-A	2004 2006 2009 2014 2009	Metastatic colorectal cancer (MCC) Non-small-cell lung cancer (NSCLC) Renal cell carcinoma (RCC) platinum-resistant recurrent ovarian cancer (OC) Approval withdrawn for treating breast cancer (BRCA)	First and second line treatment for MCC, first line for NSCLC, with interferon for RCC, with chemotherapy for OC.
Aflibercept (Zaltrap): a chimeric VEGF/PlGF neutralizing receptor	VEGFA, VEGFB, PLGF	2012	colorectal cancer (CRCA), pancreatic cancer (PACA), NSCLC	Second-line metastatic treatment for CRCA, with chemotherapy for PACA & NSCLC
Ramucirumab (Cyrazma)	VEGFR2	2014 2014 2015	Gastric or gastro-oesophageal junction adenocarcinoma (GOAC) NSCLC MCC	Refractory with or without chemotherapy for GOAC; Refractory with chemotherapy for NSCLS and MCC
Small molecule tyrosine kinase inhibitors with anti-VEGFR activity				

Axitinib (Inlyta)	VEGFR 1-3	2012	RCC	Second-line single drug therapy.
Cabozantinib (Cometriq)	All VEGFRs	2012	Progressive metastatic medullary thyroid cancer	Second line therapy with chemotherapy
Pazopanib (Votrient)	All VEGFRs	2009 2012	Renal cell carcinoma Soft tissue sarcoma, Recommended treatment for RCC, NSCLC	Second-line treatment with chemotherapy
Regorafanib (Stivarga)	All VEGFRs	2013	Resistant metastatic colorectal cancer	Single drg treatment for resistant advanced gastrointestinal stromal tumors, second-line treatment for MCC
Sorafenib (Nexavar)	All VEGFRs	2005 2007 2013	Renal cell carcinoma HCC Differentiated thyroid cancer Recommended treatment for melanoma and NSCLC	Second-line treatment for metastatic or recurrent thyroid carcinoma and advanced renal cell carcinoma
Sunitinib (Sutent)	All VEGFRs	2006 2011	RCC Pancreatic neuroendocrine tumors; also recommended for RCC, gastrointestinal stromal tumor (GIST), BRCA, HCC, CRCA	Single drug, first line treatment for RCC, Single drug for treatment of progressive well differentiated pancreatic neuroendocrine tumours

Lenvatinib (**Lenvima**)	All VEGFRs	2015	Thyroid cancer	Treatment of locally recurrent or metastatic, progressive, radioactive iodine-resistant differentiated thyroid cancer
Vendatanib (Caprelsa)	All VEGFRs	2011	NSCLC, medullary thyroid carcinoma (MTC)	Unresectable, locally advanced, or metastatic MTC