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Regulation of vascular endothelial growth factor (VEGF) in prostate cancer

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Key words

Angiogenesis, animal model, androgen, castration-resistant prostate cancer, neuroendocrine, transcription, xenograft

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Abstract

Prostate cancer (PCa) is the most common malignancy affecting men in the western world. While radical prostatectomy and radiation therapy can successfully treat a majority of patients, up to ~30% will experience local recurrence or metastatic disease. Prostate carcinogenesis and progression is typically an androgen dependent process. For this reason, therapies for recurrent PCa target androgen biosynthesis and androgen receptor function. Whilst such androgen deprivation therapies (ADT) are effective initially, the duration of response is typically ≤24 months. While ADT and taxane based chemotherapy have delivered survival benefits, metastatic prostate cancer remains incurable. Therefore it is essential to establish the cellular and molecular mechanisms which enable localized prostate cancers to invade and disseminate. It has long been accepted that metastases requires angiogenesis. In this review we will examine the essential role for angiogenesis in PCa metastases and in particular we will focus on current understanding of the regulation of vascular endothelial growth factor (VEGF) in localized and metastatic PCa. We will highlight recent advances in understanding the role of VEGF in regulating interaction of cancer cells with tumor-associated immune cells during metastatic process of PCa. We will summarize the established mechanisms of transcriptional and post-transcriptional regulation of VEGF in prostate cancer cells and will outline the molecular insights obtained from pre-clinical animal models of prostate cancer. Finally we will summarize the current state of anti-angiogenesis therapies for PCa and how existing therapies impact on VEGF signalling.

Prostate cancer: molecular mechanisms of carcinogenesis and the role of androgens

Prostate cancer (PCa) is the most common malignancy affecting western men (Ferlay, et al. 2013; Siegel, et al. 2015) and is estimated to account for over 220,000 new cases and 27,000 deaths in the United States in 2015. Advances in early diagnosis (Carter, et al. 2013; Heidenreich, et al. 2013), surgical, radio-, chemo- and immuno- therapies (reviewed in Lorente and De Bono 2014; Stewart and Boorjian 2014), are improving patient survival. However, the aging demographics of western countries suggest PCa will remain a leading cause of cancer related mortality in men. Although >90% of PCa are diagnosed as androgen responsive acinar adenocarcinoma (Humphrey 2012), the disease is clinically heterogeneous. Indeed it is currently not possible to accurately distinguish high risk prostate tumors, which require extensive therapeutic intervention, from patients with low risk indolent tumors, many of which would not require any therapy (Cuzick, et al. 2014; Draisma, et al. 2009; Tombal, et al. 2014; Weiner, et al. 2015). Therefore most men with clinically localized PCa undergo radical prostatectomy or radiotherapy with curative intent (Boorjian, et al. 2012; Heidenreich, et al. 2014). Yet, it has been estimated that between 20-30% of cases will experience recurrence (Boorjian et al. 2012). Following local recurrence and metastasis, androgen deprivation therapy, achieved medically or through orchiectomy, is typically effective for <24 months by which time progression to the more detrimental form of castrate resistant PCa (CRPC) is common (Ahmed, et al. 2014). PCa becomes hormone refractory and cancer cells acquire the ability to invade and metastasize to lymph nodes and distant organs (Wegiel, et al. 2005).

The importance of androgen signalling in prostate carcinogenesis has long been recognized (Huggins and Hodges 1941). In the intervening decades it became apparent that androgen signalling plays essential roles in localized and metastatic PCa (Wang, et al. 2009). The androgen receptor (AR) is a member of the ligand dependent transcription factor family of nuclear receptors which also includes the estrogen (ERα/ERβ) and progesterone (PR) receptors, lipophilic ligands (retinoids, vitamin D) and orphan receptors for which ligands have not been identified. In the presence of an agonist, nuclear receptors regulate gene expression by recruiting epigenetic coregulator proteins with histone lysine acetyltransferase (KAT), methyltransferase (KMT) and demethylase (KDM) activity. Consistent with the essential role played by androgens and the AR in hormone dependent (Yu, et al. 2010) and refractory PCa (Wang et al. 2009), nuclear receptor coregulators have also been implicated in prostate carcinogenesis and progression (Debes, et al. 2003; Heemers, et al. 2007; Rahman, et al. 2003). KDMs are key coregulators of AR and ER transcriptional activation and repression (Cheng and Blumenthal 2010; Kooistra and Helin 2012). A subset of KDMs, including

KDM1A/LSD1, are over-expressed in PCa (Kahl, et al. 2006; Kashyap, et al. 2013; Metzger, et al. 2005). Although KDM1A acts predominantly as a transcriptional corepressor, KDM1A can act as a coactivator for AR (Metzger et al. 2005) and ERα (Perillo, et al. 2008) dependent upon promoter context (Cai, et al. 2011). Consistent with this there is evidence that KDM1A can contribute to hormone refractory PCa by sensitizing prostate cells to lower androgen levels (Cai et al. 2011; Cai, et al. 2014). Androgen and estrogen receptors are known to cooperate in gene regulation in PCa and can define transcriptional signatures associated with aggressive disease (Setlur, et al. 2008). As we will discuss in detail later, KDM1A appears to promote PCa recurrence in part by enhancing androgen-regulated VEGF expression (Kashyap et al. 2013). With a clear clinical need for new treatments, nuclear receptor epigenetic coregulators and related proteins are attractive therapeutic targets, due to their feasibility as 'druggable' targets (Asangani, et al. 2014; Dawson and Kouzarides 2012; Rotili, et al. 2014). For this reason recently identified coregulator components of the AR-signaling complex represent potential new targets to circumvent resistance to existing therapies.

Androgen deprivation therapies (ADT) are the standard treatment for locally advanced and metastatic PCa. ADT targets androgen receptor (AR) signaling pathways which are central to gene expression programs driving prostate tumour growth and metastasis. AR signaling persists in hormone refractory PCas which are resistant to ADT (Wang et al. 2009). Although androgen deprivation therapies impede tumor progression, hormone refractory cancers bypass androgen dependency and remain incurable. Recently introduced CRPC therapies include abiraterone, an inhibitor of a key enzyme in androgen biosynthesis, and the potent AR antagonist, enzalutamide. While both abiraterone and enzalutamide have demonstrated survival benefits in the CRPC context, the duration of response to these agents remains disappointing (de Bono, et al. 2011; Scher, et al. 2012). Furthermore, one consequence of prolonged systemic androgen blockade is the increasing emergence of neuroendocrine PCa which is associated with aggressive disease and poor prognosis (Beltran, et al. 2011). Whilst we now have unparalleled insight into the genomic complexity of PCa (Baca, et al. 2013; Barbieri, et al. 2012; Barbieri, et al. 2013; Berger, et al. 2011), there is therefore an urgent need to exploit this knowledge with a view to identifying novel approaches to prevent or delay PCa metastases.

Transcriptional regulation of pro-angiogenesis pathways in prostate cancer

Pro-angiogenic pathways are essential mediators of tumor growth and metastasis, and as a consequence the potential for therapies targeting the tumor vasculature has long been recognized (Folkman 1971; Folkman, et al. 1971). Both normal and pathologic angiogenesis is

regulated predominantly by the vascular endothelial growth factors (VEGF-A, -B, -C and -D) and their cognate cell surface receptors (VEGFR1, VEGFR2, VEGFR3) which can also be activated by neuropilins (Roskoski 2007). VEGF isoforms exhibit distinct receptor affinities and activate the intra-cellular receptor tyrosine kinase signalling cascade. The VEGFs and their receptors also play a role in PCa lymphangiogenesis (Burton, et al. 2008; Wong, et al. 2005). In this review we will focus on the regulation and function of VEGFA (also referred to as simply VEGF) in angiogenesis. VEGF is over-expressed in a variety of haematological malignancies (Krejsgaard, et al. 2006) and the vast majority of solid tumors including PCa (Wegiel et al. 2005)(Figure 1) where it is associated with poorer outcomes (Duque, et al. 1999; Green, et al. 2007). In prostate, in addition to its expression in blood and lymphatic endothelial cells, VEGF is also expressed at low levels in prostatic glandular epithelial cells and in nonvascular cells such as macrophages, fibroblast and mast cells (Hrouda, et al. 2003). Chronic prostatic inflammation and the infiltration of macrophages and other immune cells that express high level of VEGF is believed to be an important event during the malignant transformation. The increased production of cytokines such as interleukin-6 is believed to induce VEGF expression in the infiltrating immune cells (Cohen, et al. 1996). It has been shown that bacterial lipopolysaccharide (LPS) induces the expression of Toll-like receptors (TLRs) in human prostate epithelial PC3 cells after exposure to bacterial infection. This increased expression of TLRs is able to induce VEGF expression which in turn triggers the proliferation and migratory ability of PCa cells (Pei, et al. 2008).

The V*EGF* promoter is regulated by a multiple transcription factor complexes and the function of the hypoxia-inducible factors (HIFs) in the regulation of *VEGF* expression is well understood (Forsythe, et al. 1996; Gray, et al. 2005). However over the last decade it has become apparent that the *VEGF* promoter can be regulated by multiple members of the nuclear receptor family, including the AR (Eisermann, et al. 2013), estrogen (ERα/cMyc) (Buteau-Lozano, et al. 2002; Dadiani, et al. 2009), progesterone (Wu, et al. 2004), vitamin D (Cardus, et al. 2009) and the liver-X receptors (LXR) (Walczak, et al. 2004). Consistent with this, animal studies have indicated a role for androgens and estrogen in prostate vascularization (Daehlin, et al. 1985). In this context it is interesting to note that nuclear receptor-coregulator complexes can regulate splicing events (Auboeuf, et al. 2004; Auboeuf, et al. 2002). Thus a role for aberrant recruitment of nuclear receptor-complexes to the *VEGF* promoter in the induction of pro-angiogenic VEGF splicing during carcinogenesis cannot be excluded (Figure 2).

Interestingly, pro- and anti-angiogenic VEGF splice forms have been identified (Bates et al 2002), which are differentially regulated in cancers, including in PCa (Mavrou, et al. 2014;

Woolard, et al. 2004) and which may be key to the development of future therapies targeting pro-angiogenic VEGF function (Harper and Bates 2008). In the terminal exon of the vegf gene (Exon 8) there are two potential splice sites. A proximal splice site (PSS) encodes 6 amino acids (CDKPRR) before a stop codon is reached, resulting in isoforms such as VEGF-A_{165}a. The use of the PSS results in generation of angiogenic isoforms that increase vascular permeability, stimulate vessel growth and result in vasodilatation. Further into the terminal exon, a distal splice site (DSS), 66 bases downstream of the PSS, results in an alternative open reading frame of the same size (6 amino acids, SLRTKD), resulting in a different C-terminus to the protein. And VEGF-A_{165}b This switches the protein to an anti-angiogenic one that can inhibit vasodilatation (Woolard et al. 2004), and reduce permeability (Oltean, et al. 2012). The splice variants are differentially regulated (e.g. SRPK1 stimulates splicing to VEGF-A_{165}a, and Clk1/4 to VEGF-A_{165}b) (Nowak, et al. 2010; Nowak, et al. 2008) and are differentially reguated post-transcriptionally – e.g. by T-cell intracellular Antigen 1, an RNA binding protein that differentially regulates translation and splicing of VEGF through activation by ras (Hamdollah Zadeh, et al. 2015).

Post-transcriptional Regulation of VEGF in Prostate Cancer

Regulation of VEGF expression can occur at multiple points between transcription and translation, these regulatory effects broadly fall into three different areas; pre-mRNA processing (alternative splicing as discussed above), mRNA transcript stability and control of translation. The latter two categories will be discussed in this section, with a focus on the mechanisms of VEGF post-transcriptional regulation in PCa.

Variations in mRNA transcript stability are commonly seen as a cellular-response to environmental changes such as stress and nutrient availability, acting as a rapid response to maintain protein homeostasis. *VEGF* is tightly regulated at the transcript level and whilst the reported half-life is short, 15-40 minutes *in vitro*, this can be substantially extended during periods of hypoxia and nutrient withdrawal (Dibbens, et al. 1999; Ikeda, et al. 1995; Levy, et al. 1996; Shima, et al. 1995). AU-rich elements (ARE) within the 3'UTR of the VEGF transcript along with other elements within the coding and untranslated regions are potential targets for a range of RNA binding proteins, resulting in both positive and negative effects on transcript stability (Chang, et al. 2013; Claffey, et al. 1998; Coles, et al. 2004; Fellows, et al. 2012; Goldberg-Cohen, et al. 2002; King 2000; Onesto, et al. 2004; Shih and Claffey 1999). Hypoxia-dependent regulation of transcript stability has been well characterised in a number of cancer types and recently reviewed in (Arcondeguy, et al. 2013).

Interestingly, two less well characterised methods of hypoxia-independent regulation of VEGF transcript stability have been observed in studies of PCa. The first occurring when DU145 PCa cells were subjected to glucose deprivation. Under these conditions, VEGF transcript stability was increased as a result of the stimulation of AMP-activated Protein Kinase (AMPK), through an as yet unknown mechanism (Yun, et al. 2005). Further to this, an isoform of the Wilm's Tumour Suppressor Gene (WT1-A) was found to modestly increase VEGF transcript stability in a hormone enhanced mechanism, when WT1 was stably over-expressed in LNCaP PCa cells. Over-expression of other WT1 isoforms lacking the third of four zinc finger domains were unable to mediate VEGF stability, indicating the potential importance of zinc finger domains in this regulatory mechanism (Cash, et al. 2007).

Eukaryotic protein translation predominantly depends on the m^7G cap structure of the mRNA and assembly of the translation initiation complex (cap-dependent translation). However, alternative mechanisms of cap-independent translation have evolved, in order to maintain or activate the translation of essential proteins during periods of cellular-stress when cap-dependent translation is impaired (reviewed (Van Der Kelen, et al. 2009)). Cap-independent mechanisms depend upon the presence of Internal Ribosome Entry Sites (IRES) to enable initiation of translation, whilst originally identified in viruses, multiple eukaryotic mRNAs including VEGF are reported to contain IRES sequences (Jang, et al. 1988; Pelletier and Sonenberg 1988). The VEGF mRNA 5'UTR features two IRESs; IRES-A and IRES-B 293 and 947 nucleotides upstream of the canonical AUG start site respectively, the position of IRES-B is also just over 40 nucleotides upstream of an alternative CUG start codon (Akiri, et al. 1998; Huez, et al. 1998; Miller, et al. 1998). A single-nucleotide polymorphism (SNP) of the VEGF gene (-634 C>G substitution) has been linked with increased risk of PCa (Sfar, et al. 2006). This SNP was found to impair IRES-B function, reducing translation initiated from the alternative CUG start codon (Lambrechts, et al. 2003). Furthermore, a 17 nucleotide sequence within VEGF IRES-A has been shown to promote the formation of an intramolecular G-quadruplex structure (Morris, et al. 2010). G-quadruplex formation potentially regulates multiple aspects of RNA regulation, in the case of VEGF, mutations of this 17 nucleotide sequence prevents G-quadruplex formation and results in inhibition of IRES-A function (Morris et al. 2010). The contribution of G-quadruplex regulation to VEGF expression in PCa remains to be determined, but given the role of IRESs in mediating VEGF translation under stress conditions these intramolecular structures warrant further investigation.

Translation efficiency of VEGF can be further modified by microRNAs (miRNAs), a class of small non-coding RNA. MicroRNAs regulate translation by binding to specific sequences

within the target mRNA, usually these binding sites reside within the 3'UTR but can also occur in the 5'UTR and coding regions (Tay, et al. 2008). Target binding is mediated by the miRNA-associate RNA Induced Silencing Complex (mi-RISC) and results in either the repression of translation or mRNA degradation, with the net result of both processes being reduced protein expression (reviewed in (Huntzinger and Izaurralde 2011)). Analysis of prostate tissue and cell lines have identified multiple miRNAs, the expression of which are consistently altered in prostate tumors, leading to further analysis of downstream gene targets and their potential contribution to carcinogenesis. Szczyrba *et al*. reported a significant reduction of miR-29b expression in PCa and subsequently demonstrated miR-29b as a direct regulator of VEGF in PCa cell lines LNCaP and DU145 (Szczyrba, et al. 2010; Szczyrba, et al. 2013).

In addition to miR-29b, the VEGF transcript is predicted to contain binding sites for multiple miRNA types (as highlighted in Figure 2C), such as miR-145 and miR-205, the expression of which are reduced in PCa and have been shown to regulate VEGF in other cancer types (Boll, et al. 2013; Fan, et al. 2012; Szczyrba et al. 2010; Yue, et al. 2012). However, it remains to be determined how effectively these miRNAs repress VEGF translation in PCa. Indeed it is also possible that such repression may only occur in specific cellular contexts. In relation to this latter point, an investigation of the anti-angiogenic effects of melatonin on hypoxic PCa PC3 cells, determined a melatonin-dependent increase in the expression of miR-374b. Subsequent studies confirmed miR-374b mediated the anti-angiogenic effects of melatonin by inhibiting VEGF expression (Sohn, et al. 2015).

VEGF, bone metastasis and niches

The dissemination of cancer cells from the primary tumor site to distant organs is a key step during cancer progression. Once cancer cells invade into the bone, liver and lung, no curable treatment exists. PCa cells preferentially invade into the bone. It is estimated that 70% of patients with metastatic PCa develop bone metastasis (Semenas, et al. 2012; Shah, et al. 2004). These studies suggest that altered VEGF expression in endothelial cells leads to impaired blood vessel invasion. As blood vessels serve as a way of transporting circulating cancer cells, the increased blood vessels beds will increase the transporting of cancer cells into the blood-vessels enriched organs including liver and lung.

The spread of PCa cells metastasis to bone is a complex process involving local infiltration of tumour cells into adjacent tissue, migration from the primary tumour site into vessels (intravasation), survival and dissemination through the vascular system, extravasation, and finally invasion and subsequent proliferation in bone. There is increasing evidence showing

that VEGF signaling plays an important role in promoting bone metastasis of PCa. It has been shown that VEGF signalling initiate metastatic niches to allow cancer cells to home to the bone marrow during bone metastasis (Kaplan, et al. 2005). VEGF may stimulate the proliferation and migration of the infiltrated immune cells that secondarily infiltrate tumor tissue to promote PCa cells to enter into the blood vessels and to disseminate into the distant organs. The expression of VEGF is also detected in osteoblasts (Maes, et al. 2010).

Previous reported studies have shown that VEGF has autocrine and paracrine effects on the growth and survival activity of osteoblasts (Dai, et al. 2004; Midy and Plouet 1994; Street, et al. 2002). Further, bone morphogenesis proteins (BMPs) contribute to PCa–mediated osteoblastic activity *in vitro* partly through VEGF (Dai et al. 2004). It has also been shown that VEGF contributes to PCa induced bone remodelling at bone metastatic sites in mouse models (Kitagawa, et al. 2005). These studies suggest that altered expression of VEGF in both PCa cells and cells of invaded bone tissue may result in increased activity of bone cells, leading to an imbalance of bone formation and resorption. VEGF is also functionally linked to adhesion molecules such as fibronectin and extracellular matrix. These proteins may assist tumour cells to attract and adhere to the bone microenvironment through VEGF receptors VEGFR1 and VEGFR2 (Chen, et al. 2004; Sterling, et al. 2011).

VEGF, in addition to its angiogenic role, suppresses the immune system (Figure 3). It has been shown that VEGF directly or indirectly exerts multiple immunosuppressive activities. It has been reported that VEGF secreted by mouse tumor cells prevented dendritic cells from maturing, thus hampering tumor antigen presentation (Gabrilovich, et al. 1996). VEGF expression is present in cytotoxic T cells and it has been shown that increased expression of VEGF and VEGFR2 suppressed the activity of T cell receptor CD47 and cytotoxic T cell function (Kaur, et al. 2014). Altered VEGF signaling may also suppress the function of dendritic cells and indirectly inhibit T-cell infiltration of tumor tissue. Consistent with this, VEGF blockade has resulted in increased T-cell homing to tumors and has enhanced the efficacy of immunotherapy in mouse models (Mellman, et al. 2011).

Mouse models of PCa and relevant aspects of angiogenesis/VEGF signalling

The need for a better understanding of the molecular and pathological events involved in PCa progression has driven the development of animal models. Animal models of PCa can be distinguished into two broad groups, the first being xenograft of human PCa into immune-compromised mice and the second genetically modified mice (GEM) that will develop prostatic cancer during their lifetime (Gingrich, et al. 1999; Gray, et al. 2004). Although informative,

mouse models have several limitations. These include the inability to encompass the full complexity of the human disease and the inherent resistance to the development of invasive PCa. Nevertheless, several mouse models have been developed for the study of PCa and these have been comprehensively reviewed elsewhere (Berman-Booty and Knudsen 2015; Grabowska, et al. 2014; Wu, et al. 2013). Here we will focus on those that more closely recapitulate the progression of the human disease (Table 1).

Several xenograft animal models have been developed to recapitulate progression of human PCa. The PC3 and LNCaP, derived from an osteolytic and a lymph node metastasis respectively, are two of the most frequently used cell lines used to study PCa (Kaighn et al., 1979, Horoszewicz, 1980). Several sublines were derived from these original cell lines with enhanced tumorigenicity *in vivo*, including LNCaP-Pro3-5, LNCaP-LN3-4, PC3M, PC-3M-LN4 (Wu et al. 2013). LNCaP-LN3 and LNCaP-Pro5 xenografts are thought to resemble prostatic adenocarcinomas as xenografts express AR and PSA and are shown to be androgen sensitive (Pettaway et al., 1996, Yonou et al., 2001). Intravenous or orthotopic injections of LNCaP in mice are able to metastasize to subcutaneously implanted human adult bone but not murine bone (Yonou, et al. 2001). Interestingly, one androgen independent subline, LNCaP C4-2, is able to metastasize to the bone and cause osteoblastic lesions (Thalmann, et al. 1994). PC3M xenografts are androgen-insensitive and stain negative for PSA and AR, with the subline PC-3M-LN4 forming bone, lymphatic and lung metastases after orthotopic or intravenous injection into mice (Pettaway et al., 1996, Yonou et al., 2001). Overall, this data suggests LNCaP xenografts may model an earlier stage PCa progression than PC3.

The WISH-PC2 xenograft model was derived from a poorly differentiated adenocarcinoma that was treated with androgen deprivation and histologically consistent with a neuroendocrine (NE) PCa upon implantation (Pinthus et al., 2000). WISH-PC2 orthotopic xenografts are able to metastasize to the lymph nodes, lung and liver, and when injected locally can form tumors within bone and liver tissues (Pinthus et al., 2000). Other NE PCa relevant models include the LTL352 and LTL370 derived from metastatic NE PCa resected from urethral and penile areas, respectively. Like WISH-PC2, these xenografts stain negative for PSA and AR, and can grow in androgen deprived mice with rapid doubling time. A major limitation of xenograft models is that most tissues are obtained from advanced and aggressive PCas and therefore tend to model later stages of the disease. Furthermore, one intrinsic limitation of xenografts is that these systems depend upon effective murine vascularization of human cancer cell masses and may therefore not fully recapitulate all aspects of tumors in patients.

Nevertheless, the xenograft models, especially LNCaP xenografts, have been instrumental for understanding PCa and for many preclinical studies.

Transgenic mouse models can approximate the different stages of PCa progression, from low grade to high grade prostate intraepithelial neoplasia (PIN), adenocarcionoma and metastatic cancer. Early models utilised expression of viral oncogenes (such as small and large SV40 tumour antigens under the control of the prostate-specific probasin (PB) promoter) in the prostate epithelium. The viral oncogene models differ from human PCa as they present a rapid progression of the disease and predominant NE differentiation. However, they have been recognised as relevant models for PCa, and very useful for the investigation of CRPC that progresses to NE carcinoma (Berman-Booty and Knudsen 2015). In the TRAMP (transgenic adenocarcinoma mouse prostate) model a rapid progression of PCa with lymph node and lung metastasis was observed, with bone metastasis only reported for the FVB mouse background (Gingrich, et al. 1996). The TRAMP mice also respond to castration and can progress to hormone refractory disease associated with NE differentiation and increased metastasis rate (Gingrich, et al. 1997; Kaplan-Lefko, et al. 2003). Similarly some of LADY mouse model lines (e.g 12T-7s-f/PB-hepsin, and 12T10), drive invasive carcinoma and NE carcinoma with metastasis to the liver, lung and bone (Klezovitch, et al. 2004; Masumori, et al. 2001). The second generation mouse models were based on human PCa genetic alterations, including loss of the tumour suppressor genes *PTEN, NKX3.1, p53, Rb* and amplification of the *MYC* oncogene. Interestingly, none of the single gene deletion models shows a significant PCa phenotype but their synergistic inactivation results in the cancer onset. For instance, simultaneous inactivation of p53 and Rb results in the formation of highly metastatic tumors that are resistant to castration and showing NE differentiation (Zhou, et al. 2006). The best of these new models incorporate multiple genetic lesions with Cre-gene targeting. The most utilised models are based on the conditional targeted deletion of PTEN and they seem to recapitulate the disease progression seen in humans, including the development of CRPC with activation of PI3K/Akt signalling (Grabowska et al. 2014; Wang, et al. 2003).

Despite being the main angiogenic factor involved in PCa progression and metastasis, few studies have examined the role of VEGF in PCa animal models. Xenografts of PCa and benign prostate primary tissue exhibit maturation of vascularisation at 30 days with the presence of small vessel of human origin containing red blood cells within (Gray et al. 2004; Montecinos, et al. 2012; Presnell, et al. 2001). These xenograft tumors exhibit a surge of angiogenesis at day 6 post-implantation into mice, preceded by an up-regulation of VEGF in the stromal counterpart of the tumour at day 2 (Montecinos et al. 2012). A further increase in VEGF

protein is also shown to modulated through the addition of human testosterone pellets implanted into castrated mice when compared to the controls (Montecinos et al. 2012). This data suggests a role for VEGF in angiogenesis establishment and PCa progression through androgen regulation. During androgen deprivation (AD), a marked reduction in microvascular density (MVD) is seen after 2 days followed by vascular reestablishment from days 7 and 14 (Godoy, et al. 2011). The expression of VEGF and VEGFR2 increased in epithelial cells 2 days post AD suggesting a compensatory role for these molecules in survival of PCa and progression (Godoy et al. 2011). This data suggests androgen-dependent and independent mechanisms for VEGF induction. As described above, most xenograft models use primary PCa tissue, however PCa cell lines have been exploited in a subset of studies. For example, PC3 has been used to investigate the use of drugs to inhibit VEGF signalling (Anai, et al. 2011; Pang, et al. 2011a; Pang, et al. 2011b). Similarly, the LNCaP-LN3 orthotopic xenograft has been used to evaluate the response of bone metastasis to the anti-VEGF receptor antibody DC101 (Sweeney, et al. 2002).

The TRAMP model has been used to study angiogenic responses. Pathologically, the TRAMP mice of the FVB genetic background show highly vascularised tumors with early onset of angiogenic switch, together with loss of E-cadherin expression indicative of epithelia-to-mesenchymal transition (EMT) (Chiaverotti, et al. 2008; Gingrich et al. 1999; Kaplan-Lefko et al. 2003). Based on histological and immunohistochemical analysis, TRAMP mice tumors also show high VEGF and FGF-2 expression, with increased microvessel density. Importantly, these mice recapitulate the stimulation of angiogenesis observed in the aged mouse prostate, which is sensitive to treatment with antiangiogenic drugs (TNP-470 alone or in combination with SU5416) and finasteride (Montico, et al. 2014). The role of VEGF in advanced PCa has also been studied in *Pten* conditional knockout mice. PCa cells in these mice express the VEGF receptor NRP2 and activate signalling leading to expression of the Polycomb transcriptional repressor Bmi-1, which is implicated in the onset of PCa induced by Pten deletion (Goel, et al. 2012). This highlights an important role of VEGF/NRP2 signalling in PCa and the need to develop new therapies specifically targeting of this pathway (Geretti, et al. 2010).

Anti-VEGF therapies in clinical management of prostate cancer

High tumor VEGF levels have been associated with poor treatment outcome in PCa and higher VEGF serum levels has been described in patients with metastatic disease than in those with localized disease (Duque et al. 1999; Green et al. 2007). The use of anti-VEGF therapies in preclinical and clinical studies has been associated with increased side effects including

hypertension, gastrointestinal bleeding, intestinal perforation and pulmonary embolism (Mangoni, et al. 2012; Ogita, et al. 2012). Although bevacizumab has shown some promise with improved progression free survival, no significant improvement in overall survival has been achieved even in combination therapies (reviewed in Armstrong, et al. 2013; Small and Oh 2012). A newer anti-angiogenesis agent derived from the extra-cellular domains of the VEGFR (aflibercept) in combination with docetaxel and prednisone also offered no improvement in overall survival (Tannock, et al. 2013). Yet given the comparative success of trials of newer agents targeting VEGF signalling in other cancer types (Grothey, et al. 2013; Qi, et al. 2011), further studies are required of these agents in the PCa setting. Indeed Cediranib, a VEGFR receptor tyrosine kinase inhibitor was tested in a phase II trial on docetaxel pre-treated CRPC patients as monotherapy and was found to be well tolerated with some anti-tumour activity (Dahut, et al. 2013). There are ongoing phase II trial using Cediranib in combination with docetaxel plus prednisone or with abiraterone (ClinicalTrials.gov identifier NCT00527124 and NCT01574937 respectively) in hormone refractory PCa. A phase I trial combining abiraterone with cabozantinib is also ongoing (NCT01574937) likewise a phase II trial combining bevacizumab, lenalidomide, docetaxel, and prednisone (ART-P) for treatment of metastatic castrate-resistant PCa (NCT00942578). Given the immuno-suppressive and pro-angiogenic actions of VEGF new combinations therapies targeting VEGF signalling and promoting immune function are likely to emerge (reviewed in Cheng and Fong 2014). However further studies are required to not only identify the optimal therapeutic combinations, but also the sequencing of therapies with respect to cytotoxic chemotherapy use. This is of particular significance given that reduced tumor angiogenesis achieved by anti-VEGF therapies may impair optimal delivery of chemotherapeutics within tumor masses (Carmeliet and Jain 2011).

Effect of radiation therapy on angiogenesis

Radiation therapy is an important treatment modality for the management of malignancies. Preclinical studies have demonstrated that in addition to inducing cell death, radiation also damages tumor vasculature and prevents tumor angiogenesis (El Kaffas, et al. 2013). However local treatment failures occur in many patients after initial response to radiation therapy. Such recurrent diseases are noted to be more aggressive, resistant to therapy and have poor prognosis (Punnen, et al. 2013). Recurrence has been partly attributed to subsequent improvements in the tumour vasculature induced by radiation treatment. It has been reported that following radiation therapy, pro-angiogenic factors including VEGF are induced in remaining malignant and stromal cells in the tumour. Mobilization of pro-angiogenic CD11b positive

myelomonocytic cells from the bone marrow to the tumour stroma has also been noted to improve the revascularization of the tumor bed (Martin 2013 and references therein). Thus anti-VEGFs such as bevacizumab may both sensitize the tumor to radiotherapy and block post-therapy re-vascularization (Zhuang, et al. 2014). However the combination of radiation therapy with anti VEGF therapies in PCa has not been extensively studied clinically. A phase II study reported by Vuky and colleagues (2012) examined long-term androgen suppression with bevacizumab and intensity-modulated radiation therapy (IMRT) in high-risk PCa with acute and late toxicity as end points. It was reported that the addition of bevacizumab did not appear to worsen the effect of radiotherapy in PCa. A phase I trial which has recently completed recruitment is also studying the toxicity associated with the combination of sunitinib with hormone ablation and radiotherapy in patients with PCa (ClinicalTrials.gov. identifier NCT00631529). More trials with overall survival as endpoint are needed to assess the effect of combining anti VEGFs with radiation therapy in prostate CRPC.

Conclusion

Tumors must exploit pro-angiogenesis pathways to metastasize. For this reason targeting VEGF signalling remains an attractive approach to prevent, delay or reverse tumor metastasis. The clinical utility of anti-angiogenesis therapy for metastatic PCa has been disappointing to date. Such therapies have almost exclusively targeted circulating VEGF or the tyrosine kinase activity of VEGF receptors. However recent advances in understanding of the regulation of *VEGF* in prostate cells (Kashyap et al. 2013) raises the potential to pharmacologically target epigenetic complexes involved in the hormonal regulation of *VEGF* expression. Indeed with the approval of the HDAC inhibitors, vorinostat(SAHA) and romidepsin, for the treatment of cutaneous T-cell lymphoma and with trials of epigenetic targeted therapies for PCa ongoing (Campbell and Tummino 2014), the simultaneous targeting of pro-androgenic, pro-estrogenic and pro-angiogenic pathways with small molecular inhibitors of nuclear receptor coregulators is an increasingly attractive approach.

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Figure Legends

Figure 1. A. Immunohistochemical analysis of the expression of cyclin A1 (a,b,c), vascular endothelial growth factor (VEGF) (d,e,f) and prostate specific antigen (PSA) (c,f,i) in benign prostate hyperplasia (a,d,g) and moderately (b,e,h) and poorly differentiated (c,f,i) PCa specimens. Adapted and reproduced with permission from (Wegiel et al. 2005). B. Evaluation of vascular endothelial growth factor (VEGF) in PCa specimens. Tissue microarrays of sections from benign tissue and adjacent tumor tissue designated as Gleason grade 3 (81%) or Gleason grade 4–5 (18%) were immunostained with antibodies against VEGF. Differences in the expression of VEGF (tumor n = 864, benign n = 787), between groups were assessed using the paired Wilcoxon signed rank test ($P < .001$). The mean values of intensities of staining (horizontal lines) with error bars representing 95% confidence intervals for the mean are shown. The outliers are labelled by open circles. The boxes represent the distribution of the expression of each protein in the groups. The dot plot shows the expression of genes encoding VEGF in tumour specimens from patients with BPH (n = 6), primary PCa (n = 7), and metastatic PCa (Met, n = 6), analysed by cDNA microarray. Differences between metastatic cancers (Met) and nonmetastatic disease (benign PCa and primary tumours in localized cancer) were assessed using the Mann-Whitney test. *P* values from two-sided tests are indicated. Adapted and reproduced with permission from (Wegiel, et al. 2008).

Figure 2. (A). The *VEGF* promoter is regulated by a diverse array of transcription factors hypoxia-inducible factors (HIFs), specificity protein-1 (Sp1) and most notably in the context of this review, multiple nuclear receptors including the androgen (Eisermann et al. 2013), estrogen (Buteau-Lozano et al. 2002; Dadiani et al. 2009) indicated in red and yellow respectively. IN addition the *VEGF* promoter is regulated by progesterone (Wu et al. 2004), vitamin D (Cardus et al. 2009) and the liver-X nuclear receptors (LXR) (Walczak et al. 2004). Nuclear receptors recruit multiple, enzymatically diverse epigenetic coregulators including p160/p300 lysine acetyltransferase, demethylases which cooperate with the mediator complex to stabilize recruitment of the basal transcriptional machinery and RNA polymerase II. (B) Evidence from genomewide chromatin immuno-precipitation studies indicate recruitment of AR in LNCaP, 22Rv1, VCaP PCa cells (GSM698597)(Sharma, et al. 2013) and ERα in VCaP (GSM1076110) (Chakravarty, et al. 2014) to the *VEGF* promoter. (C) Positions of microRNA target sites and Internal Ribosome Entry Sites (IRES) in relation to the coding sequence of the *VEGF.*

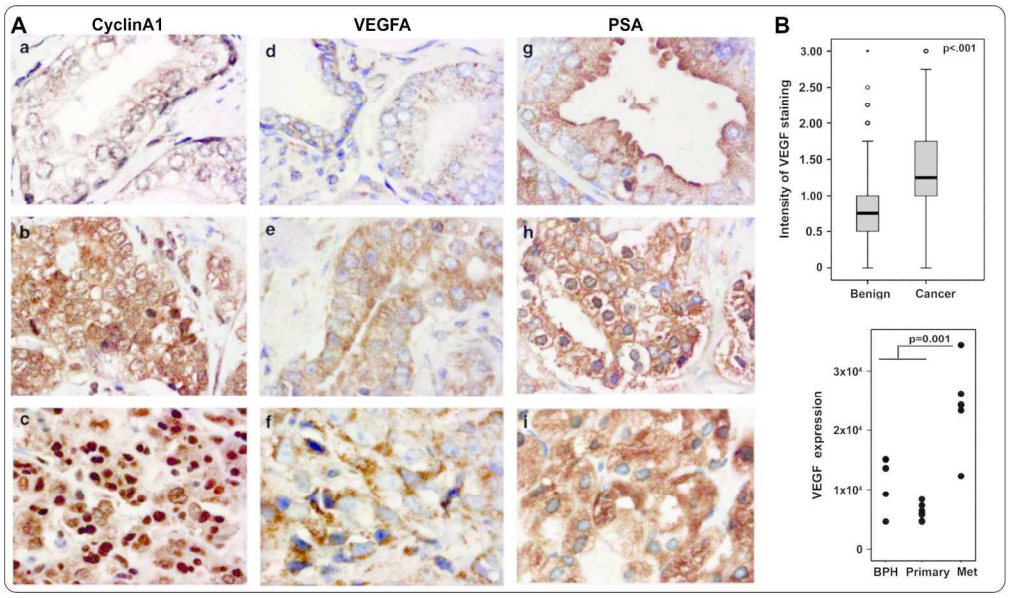
Figure 3. VEGF influences multiple convergent mechanisms contributing to metastases. VEGF promotes angiogenesis in response to intra-tumoral hypoxia and deregulated hypoxia inducible factor function (A), promotes local invasion and distant metastases by facilitating PCa cell colonisation of niches within the bone marrow (B) and suppresses function of cytotoxic T, anti-tumor macrophages and dendritic cells thereby enabling disseminating tumor cells to evade immune surveillance (C).

Figure 4. Therapies targeting receptor tyrosine (RTK) activity of VEGF receptors. Results have been disappointing for nintedanib (Molife, et al. 2014). However dovitinib, (Porta, et al. 2015; Wan, et al. 2014). cabozantinib (Smith, et al. 2014), pazopanib (Sridhar, et al. 2014), axitinib (Eswaraka, et al. 2014) have shown some promising activity in patient subsets in PCa clinical trials or pre-clinical models. The structures of FDA approved RTK inhibitors, sorafenib and sunitinib, are shown for comparison. Trials of tivozanib are underway (NCT01885949).

Table 1. Selected mouse models for the study of prostate cancer (PCa) progression.

Model	PCa type	Metastasis	CRPC model	NE PCa model	VEGF studies
Mouse xenografts					
LNCaP (Sublines: LNCaP-Pro3-5, LNCaP-LN3-4, LNCaP-IL6, LNCap-abl, LNCaP C4-2)	AD, MC	V, L	NR	No	(Sweeney et al., 2002)
PC3 (Subline: PC3M, PC3-AR, PC-3M-LN4, PC-3M-luc-C6, PC-3M-Pro4)	AD, MC	V, B, L	Yes	No	(Pang et al., 2011a and 2011b, Anai et al., 2011)
WISH-PC2	MC, NE	V, L	Yes	Yes	NR
LTL352, LTL370	MC, NE	Yes, NR	Yes	Yes	NR
Genetically engineered mice					
TRAMP	AD, NE	V, B, L	Yes	Yes	(Montico et al. 2014)
LADY (12T-7s-f/PB-hepsin)	MC, NE	V, B	NR	Yes	NR
LADY (12T-10)	MC, NE	V, B, L	NR	Yes	NR
$P53^{PE-/-}$ $Rb^{PE-/-}$	MC, NE	V,L	Yes	Yes	NR
$Pten^{flox/flox}$	MC	V, L	Yes	No	(Geretti et al. 2010)
$Pten^{flox/flox}$ NKX3.1-Cre^{ERT2}	AD	L	Yes	NR	NR
$Pten^{flox/flox}$ NKX3.1-$Cre^{ERT2}$$Braf^{LSLflox/+}$	AD, MC	V, L	NR	NR	NR
$Pten^{flox/flox}$ NKX3.1-$Cre^{ERT2}$$Kras^{LSLflox/+}$	AD, MC	V, L	NR	NR	NR
$Pten^{flox/flox}$, $Smad4^{flox/flox}$	MC	V, L	NR	NR	NR
Z-Myc, $Pten^{flox/+}$, $p53^{flox/flox}$	AD, MC	L, B	NR	No	NR

AD: adenocarcinoma; MC: metastatic carcinoma; AI: androgen independent; NE: neuroendocrine, CRPC: castrate-resistant prostate cancer (PCa); SQ: squamous differentiation; V: visceral; B: bone; L: lymp nodes; NR not reported



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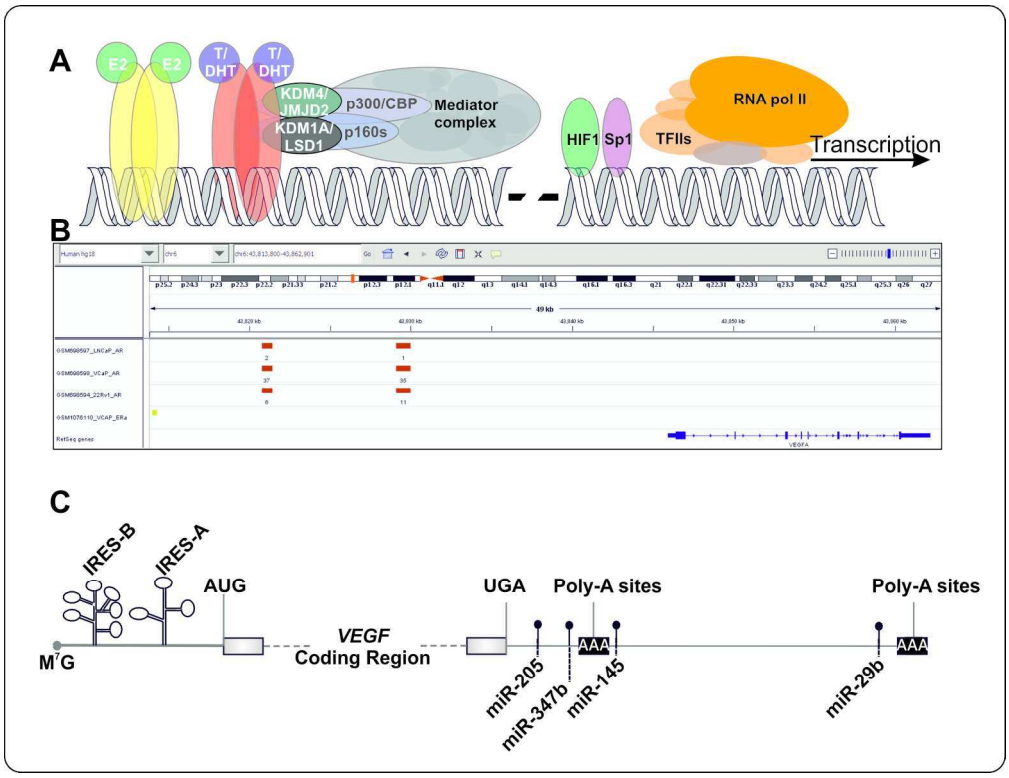

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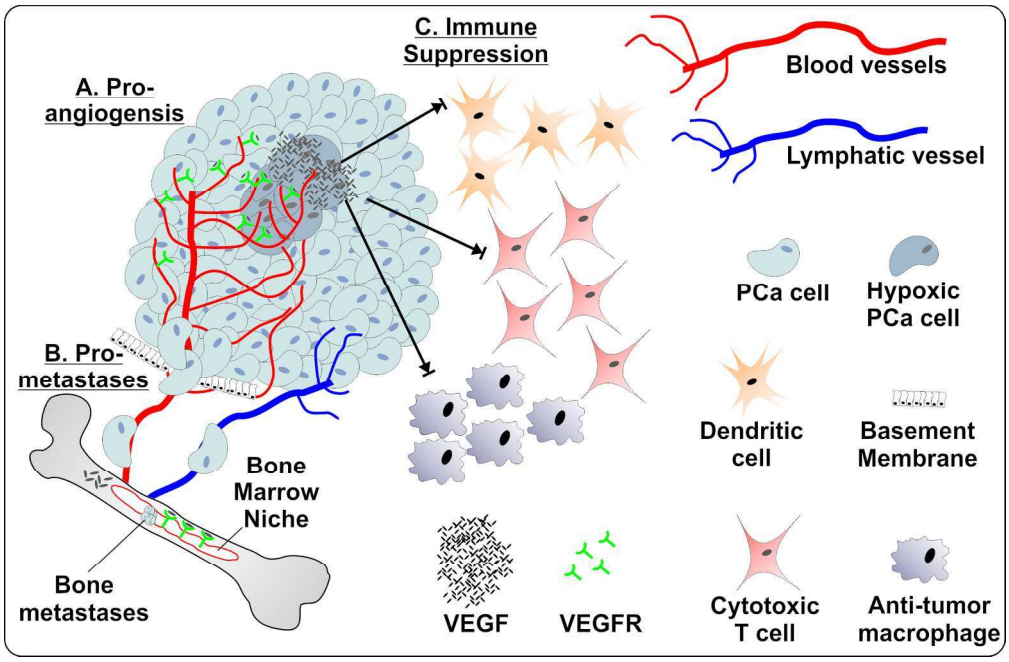

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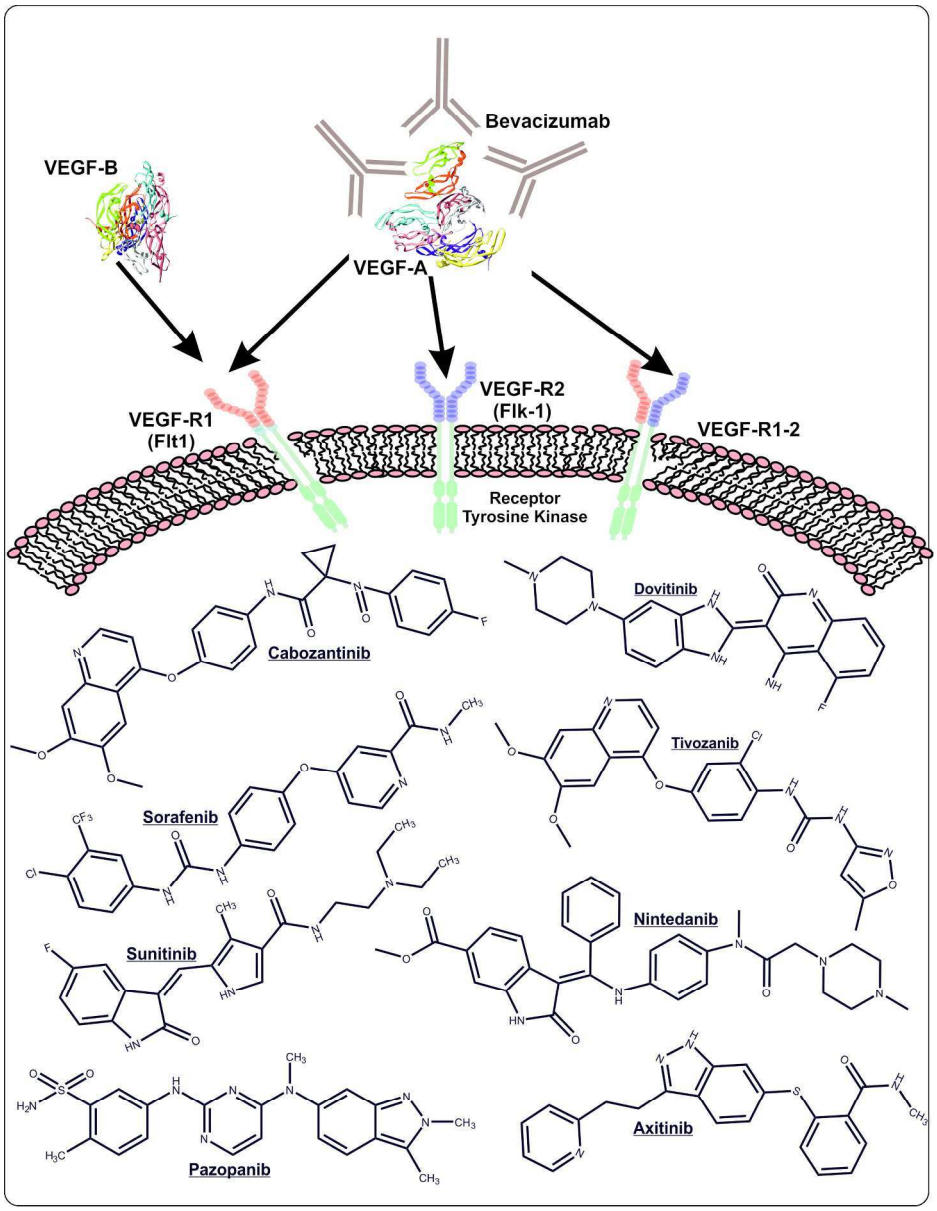
176x115mm (300 x 300 DPI)



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200x259mm (300 x 300 DPI)