Regulation of vascular endothelial growth factor in prostate cancer

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Abstract

Prostate cancer (PCa) is the most common malignancy affecting men in the western world. Although radical prostatectomy and radiation therapy can successfully treat PCa in the 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. Such androgen deprivation therapies (ADT) are effective initially, but the duration of response is typically ≤24 months. Although ADT and taxane-based chemotherapy have delivered survival benefits, metastatic PCa remains incurable. Therefore, it is essential to establish the cellular and molecular mechanisms that enable localized PCas to invade and disseminate. It has long been accepted that metastases require angiogenesis. In the present review, we examine the essential role for angiogenesis in PCa metastases, and we focus in particular on the current understanding of the regulation of vascular endothelial growth factor (VEGF) in localized and metastatic PCa. We highlight recent advances in understanding the role of VEGF in regulating the interaction of cancer cells with tumor-associated immune cells during the metastatic process of PCa. We summarize the established mechanisms of transcriptional and post-transcriptional regulation of VEGF in PCa cells and outline the molecular insights obtained from preclinical animal models of PCa. Finally, we summarize the current state of anti-angiogenesis therapies for PCa and consider how existing therapies impact VEGF signaling.

Key Words
- angiogenesis
- animal model
- androgen
- castration-resistant prostate cancer
- neuroendocrine
- transcription
- xenograft

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Prostate cancer: the 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 it is estimated to account for more than 220 000 new cases and 27 000 deaths in the USA in 2015. Advances in early diagnosis (Carter *et al*. 2013, Heidenreich *et al*. 2013), surgical, radio-, chemo-, and immunotherapies (reviewed in Lorente & De Bono (2014) and Stewart & Boorjian (2014)) are improving patient survival. However, the aging demographics of western countries suggest that PCa will remain a leading cause of cancer-related mortality in men.

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Although >90% of PCa cases 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 low-risk indolent tumors, many of which do not require any therapy (Draisma *et al*. 2009, Cuzick *et al*. 2014, 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 and 30% of cases will experience recurrence (Boorjian *et al*. 2012). Following local recurrence and metastasis, androgen deprivation therapy (ADT), which is 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). At that point, 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 signaling in prostate carcinogenesis has long been recognized (Huggins & Hodges 1941). In the intervening decades, it became apparent that androgen signaling plays an essential role 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, Rahman *et al*. 2003, Heemers *et al*. 2007). KDMs are key coregulators of AR and ER transcriptional activation and repression (Cheng & Blumenthal 2010, Kooistra & Helin 2012). A subset of KDMs, including KDM1A/LSD1, are overexpressed in PCa (Metzger *et al*. 2005, Kahl *et al*. 2006, Kashyap *et al*. 2013). Although KDM1A acts predominantly as a transcriptional corepressor, it can act as a coactivator for AR (Metzger *et al*. 2005) and ERα (Perillo *et al*. 2008), depending on 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, 2014). ARs and ERs are known to cooperate in gene regulation in PCa and can define transcriptional signatures associated with aggressive disease (Setlur *et al*. 2008). As we discuss in detail later in the present review, 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 because of their feasibility as 'druggable' targets (Dawson & Kouzarides 2012, Asangani *et al*. 2014, Rotili *et al*. 2014). For this reason, recently identified coregulator components of the AR-signaling complex represent potential new targets for circumventing resistance to existing therapies.

ADTs are the standard treatment for locally advanced and metastatic PCa. ADT targets AR signaling pathways, which are central to the gene expression programs that drive prostate tumor growth and metastasis. AR signaling persists in hormone refractory PCas that are resistant to ADT (Wang *et al*. 2009). Although ADTs 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. Although 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 (NE) PCa, which is associated with aggressive disease and poor prognosis (Beltran *et al*. 2011). Although we now have unparalleled insight into the genomic complexity of PCa (Berger *et al*. 2011, Barbieri *et al*. 2012, 2013, Baca *et al*. 2013), 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 PCa

Pro-angiogenic pathways are essential mediators of tumor growth and metastasis, and as a consequence, the potential for therapies that target 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

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growth factors (VEGFA, VEGFB, VEGFC, and VEGFD) and their cognate cell surface receptors (VEGFR1, VEGFR2, and VEGFR3), which can also be activated by neuropilins (Roskoski 2007). VEGF isoforms exhibit distinct receptor affinities and activate the intracellular receptor tyrosine kinase signaling cascade. The VEGFs and their receptors also play a role in PCa lymphangiogenesis (Wong *et al.* 2005, Burton *et al.* 2008). In the present review, we focus on the regulation and function of VEGFA (also referred to as simply VEGF) in angiogenesis. VEGF is overexpressed in a variety of hematological malignancies (Krejsgaard *et al.* 2006) and the vast majority of solid tumors, including PCa (Wegiel *et al.* 2005) (Fig. 1), where it is associated with poorer outcomes (Duque *et al.* 1999, Green *et al.* 2007). In PCa, 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 cells, and mast cells (Hrouda *et al.* 2003). Chronic prostatic inflammation and the infiltration of macrophages and other immune cells that express high levels of VEGF are believed to be important events 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 induces the

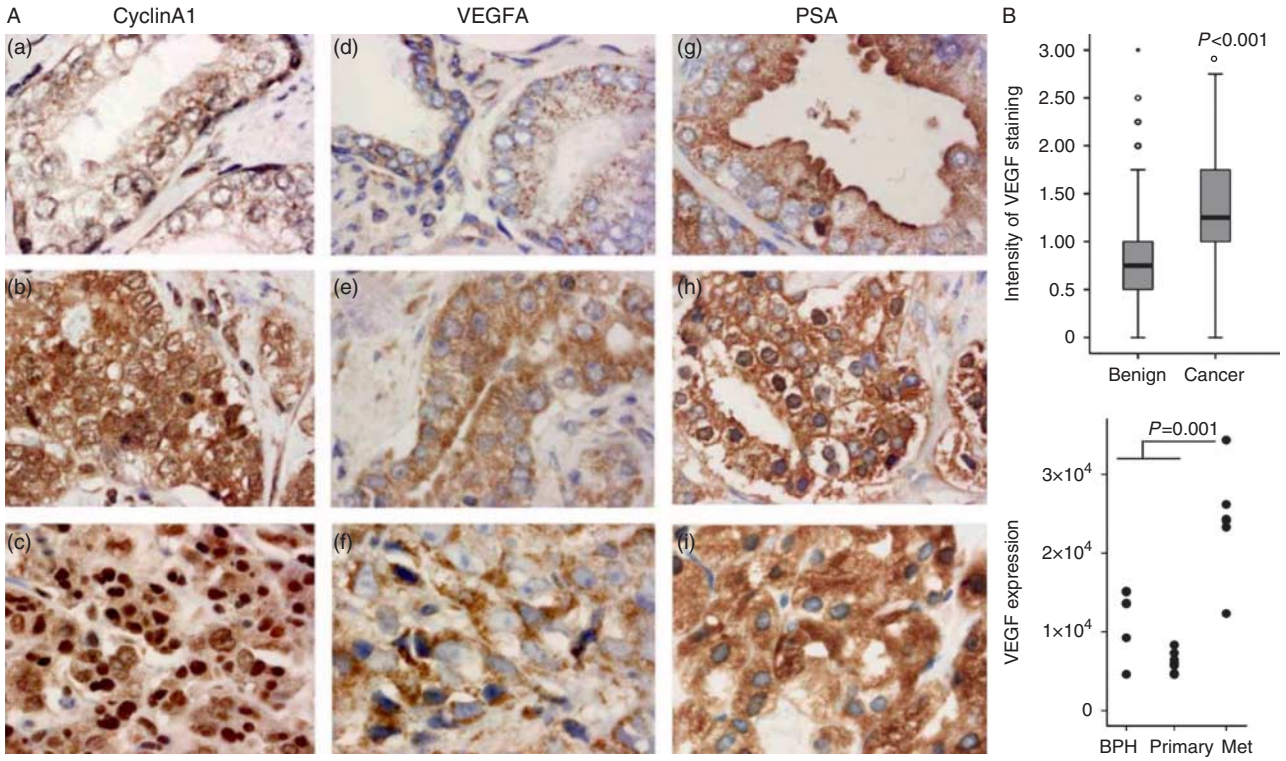

Figure 1

(A) Immunohistochemical analysis of the expression of cyclin A1 (a, b and c), vascular endothelial growth factor (VEGF) (d, e and f) and prostate-specific antigen (PSA) (c, f and i) in benign prostate hyperplasia (a, d and g) and moderately (b, e and h) and poorly differentiated (c, f and i) prostate cancer (PCa) specimens. Adapted and reproduced, with permission from Macmillan Publishers Ltd: *Oncogene*, from Wegiel B, Bjartell A, Ekberg J, Gadaleanu V, Brunhoff C & Persson JL 2005 A role for cyclin A1 in mediating the autocrine expression of vascular endothelial growth factor in prostate cancer. *Oncogene* **24** 6385–6393, copyright 2005. (B) Evaluation of VEGF in PCa specimens. Tissue microarrays of sections from benign tissue and adjacent tumor tissue designated as Gleason grade 3 (81%) or Gleason grades 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 ($P<0.001$). The mean values of intensities of staining (horizontal lines) with error bars representing 95% CIs for the mean are shown. The outliers are labeled by open circles. The boxes represent the distribution of the expression of each protein in the group. The dot plot shows the expression of genes encoding VEGF in tumor specimens from patients with BPH ($n=6$), primary PCa ($n=7$), and metastatic PCa (Met, $n=6$), as analyzed by cDNA microarray. Differences between metastatic cancers (Met) and nonmetastatic disease (benign PCa and primary tumors in localized cancer) were assessed by the Mann–Whitney *U* test. *P* values from two-sided tests are indicated. Adapted and reproduced, with permission from Oxford University Press, from Wegiel B, Bjartell A, Tuomela J, Dizeyi N, Tinzl M, Helczynski L, Nilsson E, Otterbein L, Härkönen P & Persson JL. 2008 Multiple cellular mechanisms related to cyclin A1 in prostate cancer invasion and metastasis. *Journal of the National Cancer Institute* **100** 1022–1036, copyright 2008.

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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 *VEGF* promoter is regulated by multiple transcription factor complexes, and the function of the hypoxia-inducible factors in the regulation of *VEGF* expression is well understood (Forsythe *et al*. 1996, Gray *et al*. 2005). However, over the past 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 (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*. 2002, 2004). Thus, a role for the aberrant recruitment of nuclear receptor complexes to the *VEGF* promoter in the induction of pro-angiogenic VEGF splicing during carcinogenesis cannot be excluded (Fig. 2).

Interestingly, recent studies have identified pro- and anti-angiogenic VEGF splice forms (Bates *et al*. 2002), which are differentially regulated in cancers, including in PCa (Woolard *et al*. 2004, Mavrou *et al*. 2014), and which

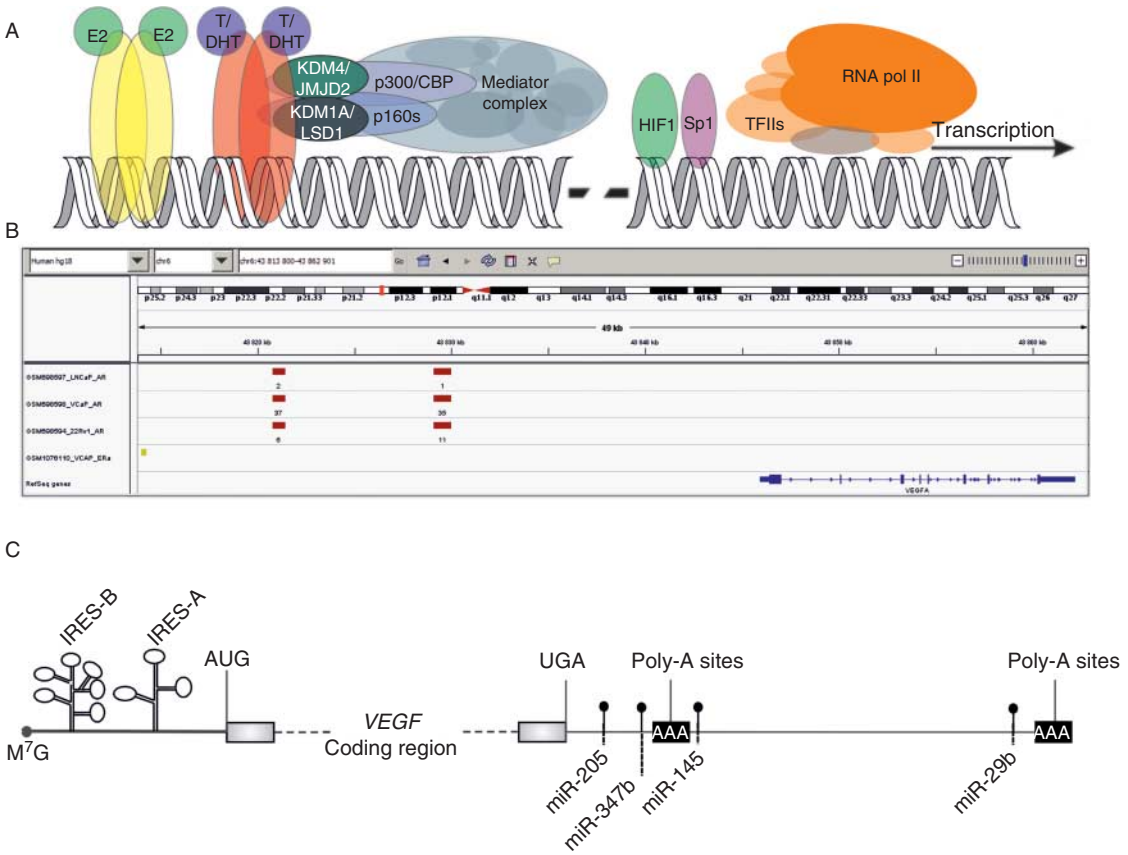

Figure 2

(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 the present review, multiple nuclear receptors, including androgen (Eisermann *et al*. 2013) and estrogen (Buteau-Lozano *et al*. 2002, Dadiani *et al*. 2009), which are 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 that cooperate with the mediator complex to stabilize recruitment of the basal transcriptional machinery, and RNA polymerase II. (B) Evidence from genome-wide chromatin immunoprecipitation studies indicate the 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*.

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may be key to the development of future therapies that target pro-angiogenic VEGF function (Harper & Bates 2008). In the terminal exon of the *VEGF* gene (exon 8), there are two potential splice sites. A proximal splice site (PSS) encodes six amino acids (CDKPRR) before a stop codon is reached, which results in isoforms such as $VEGFA_{165}a$. The use of the PSS results in the generation of angiogenic isoforms that increase vascular permeability, stimulate vessel growth, and result in vasodilatation. Further into the terminal exon, a distal splice site, 66 bases downstream of the PSS, results in an alternative open reading frame of the same size (six amino acids, SLRTKD), which in turn results in a different C-terminus to the protein. Furthermore, $VEGFA_{165}b$ 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 $VEGFA_{165}a$, and Clk1/4 stimulates splicing to $VEGFA_{165}b$) (Nowak *et al*. 2008, 2010) and are differentially regulated post-transcriptionally – for example, 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 signaling in PCa

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 in the previous section), mRNA transcript stability, and control of translation. The latter two categories are discussed in the present section, with a focus on the mechanisms of VEGF post-transcriptional regulation in PCa.

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

Interestingly, two less well-characterized methods of hypoxia-independent regulation of VEGF transcript stability have been observed in studies of PCa. The first occurred 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 through a mechanism that is still unknown (Yun *et al*. 2005). In addition to this, an isoform of the Wilms' tumor suppressor gene (*WT1-A*) was found to modestly increase VEGF transcript stability in a hormone-enhanced mechanism when *WT1* was stably overexpressed in LNCaP PCa cells. Overexpression of other *WT1* isoforms the lacked the third of fourth zinc finger domains was unable to mediate VEGF stability, which indicates 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 by Van Der Kelen *et al*. (2009)). Cap-independent mechanisms depend on the presence of internal ribosome entry sites (IRES) to enable the initiation of translation. Although they were originally identified in viruses, multiple eukaryotic mRNAs, including *VEGF*s, have also been reported to contain IRES sequences (Jang *et al*. 1988, Pelletier & 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 slightly more than 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 an increased risk of PCa (Sfar *et al*. 2006). This SNP was found to impair IRES-B function by 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

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multiple aspects of RNA regulation, in the case of VEGF, mutations of this 17-nucleotide sequence prevent G-quadruplex formation and result in the 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.

The translation efficiency of VEGF can be further modified by microRNAs (miRNAs), a class of small, non-coding RNA. miRNAs regulate translation by binding to specific sequences within the target mRNA. Usually these binding sites reside within the 3′UTR, but they 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 and results in either the repression of translation or mRNA degradation, with the net result of both processes being reduced protein expression (reviewed by Huntzinger & Izaurralde (2011)). Analyses of prostate tissue and cell lines have identified multiple miRNAs, the expressions of which are consistently being altered in prostate tumors, which has led to further analysis of downstream gene targets and their potential contribution to carcinogenesis. Szczyrba *et al*. (2010, 2013) 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.

In addition to miR-29b, the VEGF transcript is predicted to contain binding sites for multiple miRNA types (as highlighted in Fig. 2C), such as miR-145 and miR-205, the expressions of which are reduced in PCa and have been shown to regulate VEGF in other cancer types (Szczyrba *et al*. 2010, Fan *et al*. 2012, Yue *et al*. 2012, Boll *et al*. 2013). 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 point, an investigation of the anti-angiogenic effects of melatonin on hypoxic PCa PC3 cells identified a melatonin-dependent increase in the expression of miR-374b. Subsequent studies confirmed that miR-374b mediated the anti-angiogenic effects of melatonin by inhibiting VEGF expression (Sohn *et al*. 2015).

VEGF signaling, 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 lungs, no curable treatment exists. PCa cells preferentially invade into the bone. It is estimated that 70% of patients with metastatic PCa develop bone metastasis (Shah *et al*. 2004, Semenas *et al*. 2012). These studies suggest that altered VEGF expression in endothelial cells leads to impaired blood vessel invasion. Because blood vessels serve as a way of transporting circulating cancer cells, the increased blood vessel beds will increase the transporting of cancer cells into the blood vessel-enriched organs, including the liver and lungs.

The spread of PCa cell metastasis to bone is a complex process that involves the local infiltration of tumor cells into adjacent tissue, migration from the primary tumor site into vessels (intravasation), survival and dissemination through the vascular system, extravasation, and finally, invasion and subsequent proliferation into the 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 signaling initiates 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 distant organs. The expression of VEGF has also been 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 (Midy & Plouet 1994, Street *et al*. 2002, Dai *et al*. 2004). Furthermore, bone morphogenesis proteins 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 remodeling at bone metastatic sites in mouse models (Kitagawa *et al*. 2005). These studies suggest that the altered expression of VEGF in both PCa cells and cells of invaded bone tissue may result in the increased activity of bone cells, which leads 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 tumor cells to attract and adhere to the bone microenvironment through the VEGF receptors VEGFR1 and VEGFR2 (Chen *et al*. 2004, Sterling *et al*. 2011).

VEGF, in addition to its angiogenic role, suppresses the immune system (Fig. 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, thereby hampering tumor antigen presentation

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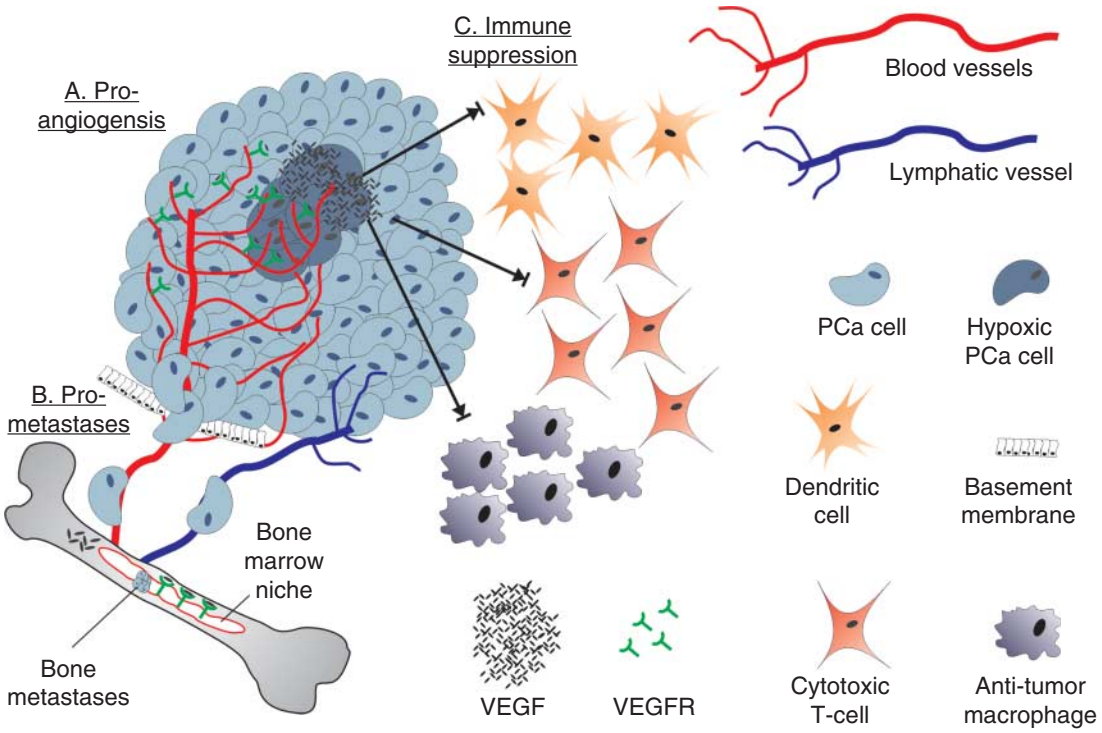

Figure 3

VEGF influences multiple convergent mechanisms that contribute to metastases. VEGF promotes angiogenesis in response to intratumoral hypoxia and deregulated hypoxia inducible factor function (A), promotes local invasion and distant metastases by facilitating PCa cell colonization of niches within the bone marrow (B), and suppresses the function of cytotoxic T, anti-tumor macrophages and dendritic cells, which thereby enables disseminating tumor cells to evade immune surveillance (C).

(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 the 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; Fig. 4).

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

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 being genetically modified mice that will develop prostatic cancer during their lifetime (Gingrich *et al*. 1999, Gray *et al*. 2004). Although they are 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 (Wu *et al*. 2013, Grabowska *et al*. 2014, Berman-Booty & Knudsen 2015). In the present review, we focus on those that more closely recapitulate the progression of the human disease (Table 1).

Several xenograft animal models have been developed to recapitulate the progression of human PCa. The PC3 and LNCaP, which are derived from an osteolytic and a lymph node metastasis respectively, are two of the cell lines that are most frequently 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, because xenografts express AR and prostate-specific antigen (PSA) and are shown to

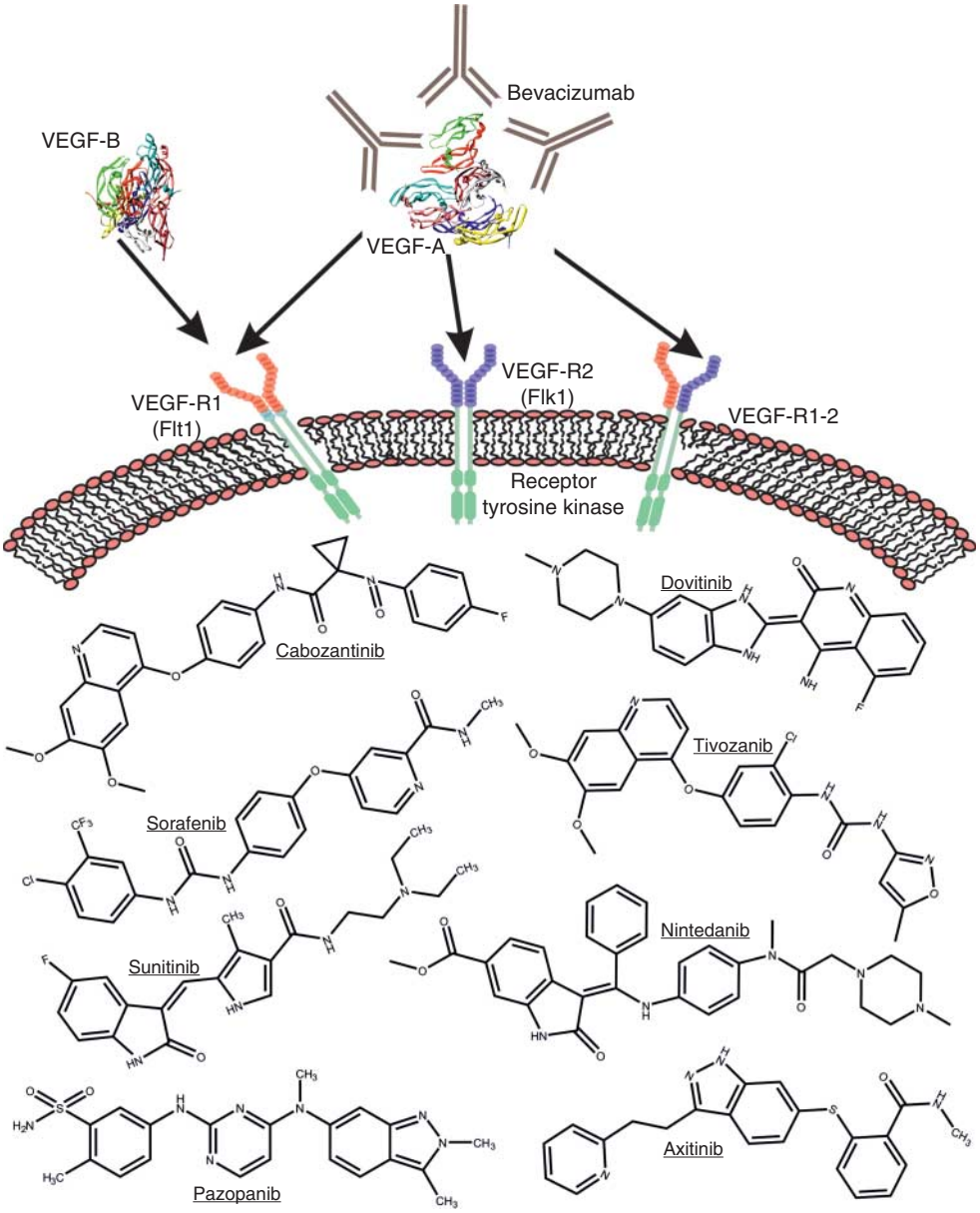

Figure 4

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

be androgen-sensitive (Pettaway *et al*. 1996, Yonou *et al*. 2001). I.v. or orthotopic injections of LNCaP in mice are able to metastasize to subcutaneously implanted human adult bone but not to 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, and the subline PC-3M-LN4 forms bone, lymphatic, and lung metastases after orthotopic or i.v. injection into mice (Pettaway *et al*. 1996, Yonou *et al*. 2001). Overall, these data suggest that LNCaP xenografts may model an earlier-stage PCa progression than PC3 xenografts do.

The WISH-PC2 xenograft model was derived from a poorly differentiated adenocarcinoma that was treated

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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	Anai *et al*. (2011) and Pang *et al*. (2011*a*,*b*)
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, lymph nodes; NR, not reported.

with androgen deprivation (AD) and was histologically consistent with a NE PCa upon implantation (Pinthus *et al*. 2000). WISH-PC2 orthotopic xenografts are able to metastasize to the lymph nodes, lungs, and liver, and when they are injected locally, they can form tumors within bone and liver tissues (Pinthus *et al*. 2000). Other NE PCa-relevant models include the LTL352 and LTL370, which are derived from metastatic NE PCa resected from urethral and penile areas respectively. Like WISH-PC2, these xenografts stain negative for PSA and AR, and they can grow in androgen-deprived mice with rapid doubling time. A major limitation of xenograft models is that most of the tissues are obtained from advanced and aggressive PCas, and they therefore tend to model later stages of the disease. Furthermore, one intrinsic limitation of xenografts is that these systems depend on the effective murine vascularization of human cancer cell masses, and they 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, adenocarcinoma, and metastatic cancer. Early models utilized the expression of viral oncogenes (such as small and large SV40 tumor antigens under the control of the prostate-specific probasin (PB) promoter) in the prostate epithelium. The viral oncogene models differ from human PCa, insofar as they present a rapid progression of the disease and predominant NE differentiation. However, they have been recognized as relevant models for PCa, and they are very useful for the investigation of CRPC that progresses to NE carcinoma (Berman-Booty & Knudsen 2015). In the transgenic adenocarcinoma mouse prostate (TRAMP) model, a rapid progression of PCa with lymph node and lung metastasis has also observed, and bone metastasis has only been reported for the FVB mouse background (Gingrich *et al*. 1996). The TRAMP mice have also responded to castration and progressed to hormone refractory disease associated with NE differentiation and increased metastasis rate (Gingrich *et al*. 1997, Kaplan-Lefko *et al*. 2003). Similarly, some LADY mouse model lines (e.g., 12T-7s-f/PB-hepsin and 12T10) have been shown to drive invasive carcinoma and NE carcinoma with metastasis to the liver, lungs, and bone (Masumori *et al*. 2001, Klezovitch *et al*. 2004). The second-generation mouse models were based on human PCa genetic alterations and included a loss of the tumor-suppressor genes *Pten, Nkx3.1*, *p53*, and *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 cancer onset. For instance, simultaneous inactivation of *p53* and *Rb* results in the formation of highly metastatic tumors that are resistant to castration and show NE differentiation (Zhou *et al*. 2006). The best of

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these new models incorporate multiple genetic lesions with *Cre*-gene targeting. The most-utilized 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 the activation of PI3K/Akt signaling (Wang *et al*. 2003, Grabowska *et al*. 2014).

Even though VEGF is 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 vascularization at 30 days, when small vessels of human origin containing red blood cells become present (Presnell *et al*. 2001, Gray *et al*. 2004, Montecinos *et al*. 2012). At day 6 post-implantation into mice, these xenograft tumors exhibit a surge of angiogenesis, which is preceded by an up-regulation of VEGF in the stromal counterpart of the tumor at day 2 (Montecinos *et al*. 2012). A further increase in VEGF protein has also been shown to be modulated through the addition of human testosterone pellets implanted into castrated mice as compared to the controls (Montecinos *et al*. 2012). These data suggest a role for VEGF in angiogenesis establishment and PCa progression through androgen regulation. During AD, a marked reduction in microvascular density was seen after 2 days, and it was followed by vascular reestablishment from days 7 to 14 (Godoy *et al*. 2011). The expression of VEGF and VEGFR2 increased in epithelial cells 2 days post-AD, which suggests a compensatory role for these molecules in the survival and progression of PCa (Godoy *et al*. 2011). These data suggest androgen-dependent and androgen-independent mechanisms for VEGF induction. As described earlier in the present review, 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 signaling (Anai *et al*. 2011, Pang *et al*. 2011*a*,*b*). 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 with an FVB genetic background show highly vascularized tumors with early onset of angiogenic switch, together with a loss of E-cadherin expression, which is indicative of epithelial–mesenchymal transition (Gingrich *et al*. 1999, Kaplan-Lefko *et al*. 2003, Chiaverotti *et al*. 2008). Based on histological and immunohistochemical analysis, TRAMP mice tumors also showed high VEGF and FGF-2 expression, with increased microvessel density. Importantly, these mice recapitulate the stimulation of angiogenesis that has been 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 signaling that leads to the 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 signaling in PCa and the need to develop new therapies that specifically target this pathway (Geretti *et al*. 2010).

Anti-VEGF signaling therapies in the clinical management of PCa

High tumor VEGF levels have been associated with poor treatment outcomes in PCa, and higher VEGF serum levels have 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 by Small & Oh (2012) and Armstrong *et al*. (2013)). A newer anti-angiogenesis agent derived from the extracellular 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 that target VEGF signaling in other cancer types (Qi *et al*. 2011, Grothey *et al*. 2013), further studies are required of these agents in the PCa setting (Fig. 4). Indeed, cediranib, a VEGFR receptor tyrosine kinase inhibitor, was tested in a phase II trial on docetaxel pretreated CRPC patients as monotherapy and was found to be well tolerated, with some anti-tumor activity (Dahut *et al*. 2013). There are ongoing phase II trials 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 that combines abiraterone with cabozantinib is also ongoing

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(NCT01574937), as are a phase II trial that combines bevacizumab, lenalidomide, docetaxel, and prednisone for the treatment of metastatic CRPC (NCT00942578). Given the immunosuppressive and pro-angiogenic actions of VEGF, new combination therapies that target VEGF signaling and promote immune function are likely to emerge (reviewed by Cheng & Fong (2014)). However, further studies are required to identify not only the optimal therapeutic combinations but also the sequencing of therapies with respect to cytotoxic chemotherapy use. This is of particular significance, given that the reduced tumor angiogenesis achieved by anti-VEGF therapies may impair optimal delivery of chemotherapeutics within tumor masses (Carmeliet & 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 the initial response to radiation therapy. Such recurrent diseases are noted to be more aggressive, to be more resistant to therapy, and to have poorer prognoses (Punnen *et al*. 2013). Recurrence has been partly attributed to subsequent improvements in the tumor vasculature being induced by radiation treatment. It has been reported that following radiation therapy, pro-angiogenic factors, including VEGF, are induced in the remaining malignant and stromal cells in the tumor. Mobilization of pro-angiogenic CD11b-positive myelomonocytic cells from the bone marrow to the tumor 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 revascularization (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 *et al*. (2012) examined long-term androgen suppression with bevacizumab and intensity-modulated radiation therapy in high-risk PCa with acute and late toxicity as endpoints. They reported that the addition of bevacizumab did not appear to worsen the effect of radiotherapy in PCa. A phase I trial that has recently completed recruitment is also studying the toxicity associated with the combination of sunitinib with hormone ablation and radiotherapy in patients with PCa (NCT00631529). More trials with overall survival as the endpoint are needed to assess the effect of combining anti-VEGFs with radiation therapy in prostate CRPC.

Conclusion

Tumors must exploit pro-angiogenesis pathways in order to metastasize. For this reason, targeting VEGF signaling 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 the regulation of *VEGF* in prostate cells (Kashyap *et al*. 2013) raises the potential to pharmacologically target the 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 the ongoing trials of epigenetic targeted therapies for PCa (Campbell & Tummino 2014), the simultaneous targeting of pro-androgenic, pro-estrogenic, and pro-angiogenic pathways with small molecular inhibitors of nuclear receptor coregulators is becoming an increasingly attractive approach.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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