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Regulation of Angiogenesis in Human Cancer via Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2)

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1. Introduction

Angiogenesis is the sprouting of new capillaries from pre-existing blood vessels that involves endothelial cell (EC) differentiation, proliferation, migration, cord formation and tubulogenesis (Risau 1997). This process was earlier recognized by the studies of Judah Folkman as a crucial step for tumor formation (Folkman 1971). Many endothelium-specific molecules can influence angiogenesis including members of the VEGF, angiopoietin and ephrin families. In addition, non vascular endothelium-specific factors contribute to blood vessel formation, i.e., platelet-derived growth factor, PDGF and transforming growth factor-β, TGF-β families (Yancopoulos et al. 2000). VEGF family members interact through some degree of specificity with receptor tyrosine kinases (RTK; VEGFR-1 to 3). VEGFR-1 (fms-like tyrosine kinase, Flt-1) (Shibuya et al. 1990), VEGFR-2 (fetal liver kinase, Flk-1 in mice or KDR in humans) (Matthews et al. 1991; Millauer et al. 1993; Terman et al. 1991), VEGFR-3 (Flt-4)(Pajusola et al. 1992) and a fourth receptor, Flt-3/Flk-2, belongs to the RTK family. The last receptor was identified but it does not bind to VEGF (Hannum et al. 1994). VEGF can also bind to a distinct type of high-affinity non-tyrosine kinase receptors: Neuropilin-1 and-2 (NRP-1/-2). These molecules are found in endothelial and neuronal cell surfaces (Jussila and Alitalo 2002), as well as in tumor cells (Bagri et al. 2009; Pellet-Many et al. 2008).

VEGFR-2 was discovered before the identification of its ligand, VEGF (Risau 1997; Yancopoulos et al. 2000). VEGFR-2 is a receptor-tyrosine kinase named KDR in human (Terman et al. 1992; Yancopoulos et al. 2000), Flk-1 (Matthews et al. 1991) or NYW/FLK-1 in mice (Oelrichs et al. 1993) and TKr-11 in rat (Sarzani et al. 1992) was earlier identified as a transducer of VEGF in EC (Waltenberger et al. 1994). VEGF-A is the major form that binds and signals through VEGFR-2 to develop blood vessels and to maintain the vascular network (Ferrara 1999). VEGFR-2 is thought to mediate the key effects of the endothelial-specific mitogen VEGF on cell proliferation and permeability. Therefore, the majority of VEGFR-2 actions are related to angiogenesis (Ferrara et al. 2003; Shibuya and Claesson-Welsh 2006). Homozygous deficient VEGFR-2 mice die in the second week of gestation as a consequence of insufficient development of hematopoietic and EC (Matthews et al. 1991), indicating that

VEGFR-2 is essential for life (Kabrun et al. 1997). Although, VEGF signals through VEGFR-1 (Flt-1) are required for the embryonic vasculature they are not essential for EC differentiation. Indeed, homozygous mice for targeted mutation of VEGFR-1 gene produce EC from angioblasts but develop non-functional blood vessels and die at around 10 days of gestation (Fong et al. 1995). It seems that VEGFR-1 and -2 have opposite roles in some biological contexts. VEGFR-2 mediates the major growth and permeability actions of VEGF (Shibuya 2006), whereas VEGFR-1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGFR-2 (Yancopoulos et al. 2000). On the other hand, regulation of lymphatic EC is mainly dependant on VEGFR-3/VEGF-C and VEGF-D actions (Jussila and Alitalo 2002). However, VEGFR-3 is also essential for early blood vessel development and plays a role in tumor angiogenesis (Laakkonen et al. 2007; Lohela et al. 2009). Although VEGFR-1, VEGFR-2 and VEGFR-3 have similar molecular structures, the last does not bind to VEGF-A (Achen et al. 2006; Lohela et al. 2009). Flt-3/Flk-2 is expressed on CD34+ hematopoietic stem cells, myelomonocytic progenitors, primitive B cell progenitors, and thymocytes and control differentiation of hematopoietic and non-hematopoietic cells (Hannum et al. 1994; Rappold et al. 1997).

2. Structure and function of VEGFR-2

VEGFR-2 is a transmembrane receptor that plays an important role in endothelial cell development (Risau 1997; Shalaby et al. 1995). VEGFR-2 consists of 1356 and 1345 amino acids in humans and mice, respectively. VEGFR-2 consists of 4 regions: the extracellular ligand-binding domain, transmembrane domain, tyrosine kinase domain, and downstream carboxy terminal region (Matthews et al. 1991; Millauer et al. 1993; Shibuya et al. 1990; Terman et al. 1991). The presence of seven immunoglobulin (Ig)-like domains characterizes the extracellular ligand-binding domain. The ligand-binding region is localized within the second and third Ig domains (Barleon et al. 1997b; Davis-Smyth et al. 1996; Shinkai et al. 1998; Tanaka et al. 1997). Although, the third Ig-like domain is critical for ligand binding, the second and fourth domains are important for ligand association, and the fifth and sixth domains are required for retention of the ligand bound to the receptor molecule (Shinkai et al. 1998). VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D and VEGF-E (Achen et al. 1998; Joukov et al. 1996b; Meyer et al. 1999; Ogawa et al. 1998; Shibuya 2003), whereas VEGFR-1 binds VEGF-A, VEGF-B and PIGF (Autiero et al. 2003; Makinen et al. 2001; Park et al. 1994). The classic interpretation of ligand-binding specificities is currently used for all aspects of vascular effects driven by VEGF-VEGFRs which have helped in elucidating their function. However, the potential formation of VEGFR heterodimers, i.e., VEGFR-2-VEGFR-1 (Huang et al. 2001) and VEGFR-2-VEGFR-3 (Dixelius et al. 2003) has generally been understated. It was noticed that substantial differences in signal transduction occur upon distinct VEGFR heterodimers complex formation because kinase domains and substrate specificities differ (Autiero et al. 2003; Dixelius et al. 2003; Huang et al. 2001; Mac Gabhann and Popel 2007; Neagoe et al. 2005). These complexes can also differentially interact with VEGF and PIGF (Autiero et al. 2003; Huang et al. 2001) resulting in unique signaling pattern and likely different feedback mechanisms (Mac Gabhann and Popel 2007).

VEGFR-2 is a type III receptor tyrosine kinase of the PDGFR family [PDGFRα/β, c-Kit, FLT3, and CSF-1 (cFMS)]. The majority of VEGFR-2 intracellular domains contain tyrosine residues (Tyr or Y) that are involved in redundant actions on vasculogenesis or angiogenesis. Major phosphorylation sites on VEGFR-2 and signaling partners have been reported as: Y^{951} (T-cell-specific adaptor, TSAd-Src); Y^{1054} and ^{1059} (kinase regulation); Y^{1175} [(PI-3K-Ras) and PI-3K-

AKT as well as phospholipase C gamma (PLCγ)-PKC-MEK)]; Y^{1214} (p38MAPK-HSP27) [(for Review see (Olsson et al. 2006; Rahimi 2006)]. pY^{951}VEGFR-2 (in the kinase insert domain) was recently found to increase angiogenesis (Tahir et al. 2009b) and seems to be also important for the interaction between VEGFR-2, PI-3K and PLCγ, which involves the adapter protein VEGF receptor-associated protein (VRAP also known as TSAd) (Wu et al. 2000). Other putatively important phosphorylated sites include Y^{996} in the kinase insert domain and Y^{1054} and Y^{1059} in the tyrosine kinase catalytic domain. These tyrosine residues, together with Y^{951}, when phosphorylated, act as docking sites to recruit molecules containing SH2, SH3 or PTB domains and to convey signals to downstream pathways (Petrova et al. 1999). Sequential activation of CDC42 and p38 MAPK by pY^{1214} (or Y^{1212} in mice) VEGFR-2 has been implicated in VEGF-induced actin remodeling (Lamalice et al. 2004). On the other hand, the upstream phosphorylation of Y^{801} within the juxtamembrane domain of VEGFR-2 seems to be required for the subsequent activation of the catalytic domain. However, the biological implications of these findings need to be further investigated (Solowiej et al. 2009). Moreover, human Y^{801} and ^{1175} VEGFR-2 (Y^{799} and ^{1173} mouse) are required for binding/activation of PI-3K and EC growth but no for migration or PLCγ1 activation (Dayanir et al. 2001). Additionally, pY^{1054} and ^{1059} in the activation loop are essential for VEGF-induced intracellular Ca^{2+} mobilization and the MAPK activation (Solowiej et al. 2009). However, the involvement of specific pY VEGFR-2 domains, activation of specific signaling pathways and driven functions is a point of controversial discussion (Guo et al. 2010). Recent data from Garonna et al., suggest that leptin (the major adipokine) treatment caused rapid phosphorylation of Y^{1175} VEGFR-2. These leptin effects positively impacted on EC cell proliferation, survival and migration. Leptin-VEGFR-2 actions in EC involved a crosstalk between p38 kinase/Akt1 and COX-2 (Garonna et al. 2011). These leptin effects were detected in absence of VEGF. Leptin induces rapid (5 min) phosphorylation of pY^{1175} VEGFR-2 in EC that was linked to proliferation, migration and tube formation in vitro (Garonna et al. 2011). We have also found leptin induces pY^{951} VEGFR-2 in 4T1 mouse breast cancer cells. However, the biological significance of this finding need to be further investigated (Guo and Gonzalez-Perez, unpublished). In addition, specific serine residues in the VEGFR-2 cytoplasmatic region are targeted by protein kinase C (PKC) and involved in regulatory mechanisms of receptor levels (ubiquitinylation and degradation) (Singh et al. 2005).

VEGF binding to VEGFR-2 triggers the specific activation of tyrosine amino acid residues within cytoplasmatic tail of the receptor inducing multiple signaling networks that result in EC survival, proliferation, migration, focal adhesion turnover, actin remodeling and vascular permeability (Kliche and Waltenberger 2001; Olsson et al. 2006; Zachary 2003). Signaling through mitogen-activated protein-kinase (MAP; including MEK-p42/p44MAPK and p38 MAPK; linked to proliferation) (Olsson et al. 2006; Takahashi and Shibuya 1997) and phosphatidylinositol 3′ kinase (PI-3K)/V-akt murine thymoma viral oncogene homolog 1 (Akt1; linked to survival) (Abedi and Zachary 1997) are common receptor tyrosine kinase activation patterns. Additional signaling pathways are also triggered upon VEGFR-2 activation (i.e., PLCγ1-PKC; linked to proliferation) (Veikkola et al. 2000), focal adhesion kinase (linked to migration) (Abedi and Zachary 1997) and T Cell–Specific Adapter-Src kinase (linked to migration and vascular permeability) (Matsumoto et al. 2005) (See Fig 1). Overexpression of caveolin-1 in prostate cancer cells specifically induce pY^{951} in the VEGFR-2 cytoplasmatic tail and increases angiogenesis (Tahir et al. 2009a). On the other hand, Y^{1175} mediates both PLCγ-1 and protein kinase A (PKA)-dependent signaling pathways required for VEGF-induced release of von Willebrand factor from EC (Xiong et al. 2009). Dayanir et al, found that activation of PI-3K/S6 but not Ras/MAPK kinase pathway is responsible for

VEGFR-2-mediated cell growth (Dayanir et al. 2001). Inhibition of p38 MAPK activity enhances VEGF-induced angiogenesis *in vitro* and *in vivo* and prolonged ERK1/2 activation and increased EC survival but abrogated VEGF-induced vascular permeability (Issbrucker et al. 2003). Intriguingly, VEGF-mediated proliferation of VEGFR-2 expressing fibroblasts was slower and weaker than in EC, suggesting the cell type-specific signaling mechanism(s) (Takahashi and Shibuya 1997). These results open the possibilities for differential signaling mechanisms/responses to VEGF via VEGFR-2 in cancer compared to EC. Inconsistent reports on VEGFR-2 signaling capabilities could be due to the complex interplay of signaling and inhibiting actions of other VEGF receptors. Albeit the activation and signaling of VEGFR-2 could also be modified by the formation of VEGFR-2 heterodimers exhibiting differential signaling potential as described above.

No available data on negative regulation of VEGFR-2 by phosphorylation of specific residues are available. However, de-phosphorylation of Y residues within VEGFR-2 cytoplasmatic tail contributes to its activity regulation in EC. T-cell protein tyrosine phosphatase (TCPTP, also known as PTN2) expressed by EC and several cell types could alter VEGF signalling by controlling phosphorylation of VEGFR-2. TCPTP was reported to dephosphorylate $Y^{1054/1059}$ and Y^{1214} as well as Y^{996} (this last Y dephosphorylation has not current defined functional significance). Y^{1175}, by contrast, remained phosphorylated. These actions of YCPTP inhibited VEGFR-2 kinase activity and prevented its internalization, which abrogated VEGF-mediated endothelial cell proliferation, angiogenic sprouting, chemokinesis and chemotaxis (Mattila et al. 2008).

3. The autocrine VEGF/VEGFR-2 loop: a cancer cell survival process

Intensive research has been done on VEGF/VEGFR-2 roles in vascular functions (Olsson et al. 2006). However, only a small number of reports highlight a less known function of VEGF signaling that can directly impact cancer cell survival: the autocrine loop in cancer cells. Some reports suggest that a strict molecular requirement for these autocrine actions of VEGF is the expression of VEGFR-1 as it was found in colon carcinoma (Andre et al. 2000). In line with these data, Wu et al, further reported that selective signaling through VEGFR-1 on breast cancer cells supports tumor growth through downstream activation of the p44/42 mitogen-activated protein kinase (MAPK) or Akt pathways (Wu et al. 2006). However, in breast cancer cells, VEGFR-2 isoform was not initially linked to cell survival (Andre et al. 2000; Mercurio et al. 2004).

The co-expression of NRP-1(Bachelder et al. 2001) and α6β4 integrin (Mercurio et al. 2004) but not VEGFR-2 was found essential for the binding of VEGF and activation of the PI-3K survival signaling pathway in breast cancer cells. Moreover, it was suggested that breast cancer cells do not express VEGFR-2 (Bachelder et al. 2001; Mercurio et al. 2004). In contrast, VEGFR-2 overexpression and phosphorylation could be linked to drug-resistance in breast cancer. VEGF/VEGFR-2 was found essential for cell survival in either estrogen receptor (ER) positive (MCF-7) (Aesoy et al. 2008; Svensson et al. 2005) or negative cells (MDA-MB-468) (Svensson et al. 2005) after tamoxifen treatment. A signaling cascade from VEGFR-2 via ERK1/2 to Ets-2 phosphorylation was correlated to better survival of untreated patients (Svensson et al. 2005). Moreover, a VEGF/VEGFR-2/p38MAPK kinase link was involved in poor outcome for tamoxifen-treated patients (Aesoy et al. 2008). VEGF stimulation of Akt phosphorylation and activation of ERK1/2 correlated to VEGFR-2 expression and activation in various breast carcinoma cell lines and primary culture of breast carcinoma cells (Weigand et al. 2005).

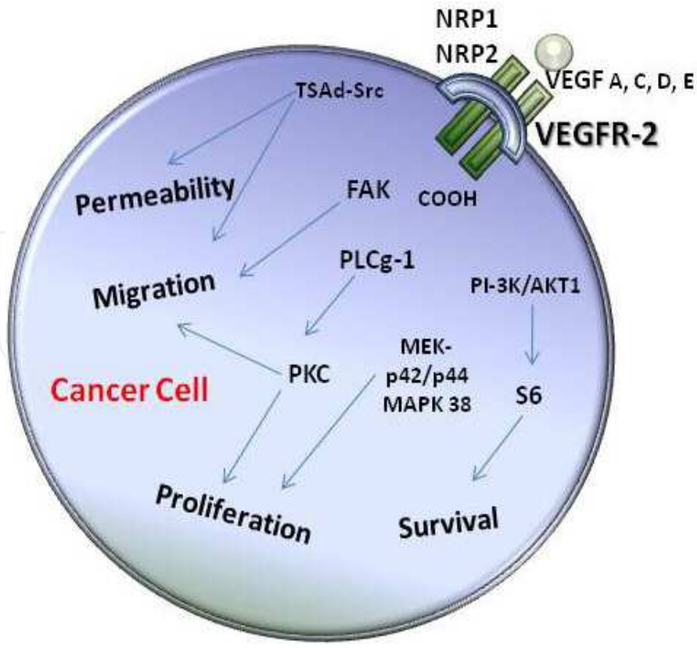

Fig. 1. VEGFR-2 signaling and biological functions.

Dimerization of VEGFR-2 occurs upon its binding to VEGF-A, -C, -D and –E. It was suggested that only mature forms of VEGF-C and VEGF-D bind with high affinity to VEGFR-2 or VEGFR-3 (Achen et al. 2005; Achen et al. 2006). NRP-1/-2 are co-receptors that stabilize the VEGFR-2 dimer. Upon ligand binding to VEGFR-2 several signaling pathways can be activated affecting diverse biological processes in endothelial and cancer cells.

Findings from our laboratory suggest that mouse (4T1, ER +) (Gonzalez et al. 2006) and human breast cancer cells (MCF-7, ER+ and MDA-MB-231, ER-) express VEGFR-2 in vitro and in vivo (Rene Gonzalez et al. 2009). Interestingly, in these cells the expression of VEGF and VEGFR-2 was linked to leptin signaling. White adipose tissue is the primary source of leptin in benign tissue but it is also expressed and secreted by cancer cells. Leptin is a known mitogenic, inflammatory and angiogenic factor for many tissues (Sierra-Honigmann et al. 1998) and increases the levels of VEGF/VEGFR-2 in cancer cells (Carino et al. 2008; Gonzalez et al. 2006; Rene Gonzalez et al. 2009). Therefore, leptin from corporal, mammary adipose tissue or cancer cells could affect cancer cells through autocrine and paracrine actions. Leptin increased the proliferation of 4T1 cells but treatment with anti-VEGFR-2 antibody resulted in a further increase in leptin-induced VEGF concentrations in the medium (Gonzalez et al 2006). This suggests a feedback loop between VEGF expression and VEGFR-2 that could be linked to cell survival/proliferation (Aesoy et al. 2008). Remarkably, inhibition of leptin signaling mediated an impressive reduction of tumor growth in mice that paralleled a significant reduction of VEGF and VEGFR-2 levels (Gonzalez et al. 2006; Rene Gonzalez et al. 2009). Moreover, leptin seems to overcome the effects of cisplatin on MCF-7 survival. A crosstalk between leptin/Notch and Wnt could be involved in the cisplatin resistance by MCF-7 (McGlothen and Gonzalez-Perez, unpublished results).

The endothelial-independent VEGF/VEGFR-2 autocrine loop was found essential for leukemia cell survival and migration in vivo (Dias et al. 2001). Results from these studies suggest that effective anti-angiogenic therapies to treat VEGF-producing and VEGFR-expressing leukemias may require blocking both paracrine and autocrine VEGF/VEGFR-2

angiogenic loops to achieve long term remission and cure (Dias et al. 2001). On the other hand, stimulation of the proliferation of prostate cancer cells by VEGF depends on the presence of VEGFR-2 through autocrine loops generated by IL-6 (H. Steiner et al. 2004a). An autocrine role for VEGF-A in chemoresistance has been demonstrated using the anti-VEGF-A antibody bevacizumab, which enhances antitumor activity of cytotoxic drugs. Moreover, combined bevacizumab and nab-paclitaxel treatment synergistically inhibited breast cancer growth, metastasis and increased the percentage of regression of well-established tumors (Volk et al. 2008). Furthermore, combination therapy using high-dosed nab-paclitaxel was found more efficient in eliminating advanced primary tumors and preexisting metastases (Volk et al. 2011). Overall these data suggest that targeting the VEGF/VEGFR-2 autocrine loop in cancer may be an innovative way to treat the disease. However, the relationships between activation of specific intracellular domains of VEGFR-2 and autocrine actions of VEGF in cancer cells remains to be studied in detail.

4. Expression of VEGFR-2 in human cancer

Because of the potential role of VEGFR-2 in tumor angiogenesis, its expression was started to investigate in malignancies soon after it was identified as a tyrosine kinase-receptor for VEGF (Waltenberger et al. 1994). VEGFR-2 was found weakly expressed in normal tissues or cells, but VEGFR-2 overexpression was reported in various cancers including lung, colon, uterus, ovarian cancer, as well as breast cancers (Giatromanolaki et al. 2007). The overexpression of VEGFR-2 not only occurs in cancer, but also the expression of VEGFR-2 relates to the disease stage, recurrence and worse outcome. Table I shows relative levels of expression of VEGFR-2 in different cancer types.

Tissue Subtype	Positive rate	Reference
Bladder Carcinoma	50%	(Xia et al. 2006)
Brain Glioma	71.4%	(Steiner et al. 2004b)
Breast Adenocarcinoma	64.5%	(Nakopoulou et al. 2002)
Cervix Adenosquamous carcinoma	73.3%	(Longatto-Filho et al. 2009)
Colon Carcinoma	71.4%	(Cheng et al. 2004)
Esophagus Carcinoma	100%	(Gockel et al. 2008)
Kidney Clear cell cancer	35%	(Badalian et al. 2007)
Lung Non-small cell carcinoma	54.2%	(Carrillo de Santa Pau et al. 2009)
Oral Carcinoma	↑↑	(Sato and Takeda 2009)
Ovary Carcinoma	100%	(Chen et al. 2004)
Pancreas Carcinoma	80%	(Itakura et al. 2000)
Prostate Cancer*	100%	(Stadler et al. 2004)
Skin Melanoma	↑↑	(Straume and Akslen 2003)

*Cell lines; ↑↑, overexpression of VEGFR-2

Table 1. VEGFR-2 expression in human tumors and malignant cell lines.

4.1 Breast cancer

A number of studies demonstrated that VEGFR-2 receptors are expressed in mouse and human breast cancer cell lines (Aesoy et al. 2008; Gonzalez et al. 2006; Rene Gonzalez et al. 2009; Svensson et al. 2005; Weigand et al. 2005). Upregulation of VEGFR-2 mRNA was

found earlier in invasive primary and metastatic breast cancers (Brown et al. 1995). Western blot and immunohistochemical analyses of endothelium and epithelium of mammary ducts in carcinomas, fibroadenomas and fibrocystic breast disease showed positive expression of VEGFR-2 that was also found in tumor stroma (Kranz et al. 1999). VEGFR-2 expression correlated with VEGF expression that suggested these molecules were co-expressed in breast cancer but this was not significantly associated with patient survival (Ryden et al. 2003). In addition, tumor-specific expression of VEGFR-2 correlated strongly with expression of VEGF-A and progesterone receptor (PR) negativity, whereas VEGF-A was not associated with hormone receptor status (Ryden et al. 2005). Invasive and in situ breast cancers express many angiogenic factors and this process was found throughout all tumor stages (Fox et al. 2007). Nakopoulou et al, detected VEGFR-2 in 64.5% (91/141) of invasive breast carcinomas showing a widespread cytoplasmic expression in most of the neoplastic cells (Nakopoulou et al. 2002). VEGFR-2 expression was well correlated with the nuclear grade of the invasive breast carcinoma (P = 0.003), but demonstrated no correlation with histologic grade, stage, and patient survival (Nakopoulou et al. 2002) as was previously noticed (Ryden et al. 2003). Interestingly, in breast cancer VEGFR-2 expression was significantly correlated with two well-established proliferation indices, Ki-67 (P = 0.037) and topo-IIα (P = 0.009). This suggests VEGF may exert growth factor activities on mammary cancer cells through its receptor VEGFR-2 (Nakopoulou et al. 2002). Linderholm et al recently reported that higher VEGFR-2 levels in tumor homogenates from primary breast cancer were correlated to higher levels of VEGF (P = 0.005), p38 MAPK (P = 0.018) and absence of ER (P = 0.008); larger tumors (P = 0.001), histopathological grade III (P = 0.018), distant metastasis (P = 0.044), shorter recurrence free survival (RFS) (P = 0.013), and shorter breast cancer corrected survival (BCCS) (P = 0.017) (Linderholm et al. 2011). Their results suggest that higher intratumoral levels of VEGFR-2 would be a candidate marker of intrinsic resistance for adjuvant endocrine therapy as it was earlier reported (Aesoy et al. 2008; Svensson et al. 2005).

4.2 Cervical and endometrial cancer

Cervical cancer, the malignant neoplasm of the cervix uteri or cervical area, it is the second most frequent malignancy in women worldwide, with one-third of patients dying from pharmacoresistant disease (Legge et al. 2010). In a study, VEGFR-2 positivity was observed in 22 of 30 cases (73.3%) cervical adenosquamous carcinoma but was significantly associated with lack of metastasis (p=0.038) (Longatto-Filho et al. 2009). In contrast, Kuemmel et al found that VEGFR-2 levels were increased in positive lymph node patients (p = 0.024) and in metastatic disease (p = 0.003). They further determined that circulating VEGFs and their soluble receptors were present in pre-invasive, invasive and recurrent cervical cancer (Kuemmel et al. 2009).

In a recent report, VEGFR-2 was not detected in normal cervical epithelium, but was positive in cervical intraepithelial neoplasia and cervical squamous cell carcinoma. Moreover, increasing expression of VEGFR-2 and VEGF were identified in cervical carcinoma indicating a correlation between their expression and carcinoma staging (Jach et al. 2010). In other report, positive immunostaining rate was 39% for VEGF, 65% for VEGFR-1 and 68% for VEGFR-2 in endometrial carcinomas (Talvensaari-Mattila et al. 2005). These results showed a significant correlation between VEGF and its receptors, but positive immunostaining was not related to poor prognosis (Talvensaari-Mattila et al. 2005). Using a novel monoclonal antibody recognizing the activated (phosphorylated) form of VEGFR-2, Giatromanolaki et al assessed strong and consistent cytoplasmic and nuclear pVEGFR-2

staining in the normally cycling endometrium, including epithelial, stromal and endothelial cells, suggesting a role of pVEGFR-2 in the normal menstrual cycle. Moreover, approximately one-third of the 70 stage I endometrioid adenocarcinomas analyzed exhibited an intense cytoplasmic and nuclear pVEGFR-2 in both cancer cells and peritumoral vessels. These authors also observed that pVEGFR-2 reactivity in cancer cells was directly related to VEGF, VEGF/VEGFR-2 complexes and HIF1α expression. Furthermore, pVEGFR-2 levels were significantly associated with poor prognosis (Giatromanolaki et al. 2006).

4.3 Colon cancer

The American Cancer Society estimates that there will be more than 140,000 new cases of colon cancer and more than 49,000 colon cancer deaths in the United States in 2011. Colon cancer is the third most common cancer and the third leading cause of cancer death among both men and women in the U.S. Colon cancer is unique among malignancies in that it is known to arise from distinct inflammatory conditions, namely, Crohn's disease and Ulcerative colitis. The link between inflammation, hypoxia and carcinogenesis continues to be elucidated. It is known that organ and cellular adaptation to hypoxia is mediated via HIF. Stabilization of HIF occurs via hypoxia-dependent inhibition of PHDs (prolyl hydroxylases).This stabilization and subsequent activation of HIF augments tumor vascularization, an important component of carcinogenesis and metastasis (Eltzschig and Carmeliet 2011). Patients with colitis associated cancer show activated VEGFR-2 on intestinal epithelial cells (IECs). In a murine model these IECs displayed increased VEGFR-2 mediated proliferation in response to VEGF stimulation. Functional studies then demonstrated that VEGFR signaling required STAT3 to promote IECs proliferation and tumor growth in vivo. This provides additional evidence linking inflammation to the development of colon cancer (Waldner et al. 2010). Giatromanolaki et al found increased levels of pVEGFR-2 in colon cancer cells and intratumoral vasculature as compared to normal epithelium. Additionally, the highest levels of pVEGFR-2 were seen in large tumors i.e. > 6 cm, those with poor histologic differentiation and high CEF1 (the nineteen complex, a non-snRNA containing protein complex, involved in splicing of nuclear RNAs via the spliceosome alpha expression). Increased VEGFR-2 expression also correlated with high VEGF and HIF expression (Giatromanolaki et al. 2007). In addition to chronic inflammatory states obesity can also predispose patients to the development of colon cancer. Padidar et al identified leptin-regulated genes localized in the colon by in situ hybridization. The pro-inflammatory cytokines, IL-6, IL-1β, and CXCL1 were upregulated by leptin and localized to cells in the gut epithelium, lamina propria, muscularis and at the peritoneal serosal surface (Padidar et al. 2011). This further establishes the role of inflammation in the pathogenesis of colon cancer. The role of activated oncogenes and loss of tumor suppressor genes and their effect on angiogenesis and subsequent tumor survival and metastasis was further reported (Zeng et al. 2010). Among those oncogenes most prominent in colon cancer is KRAS. Mutant KRAS alleles interact with hypoxia to induce VEGF in colon cancer. In addition, an adaptive mechanism in KRAS-wild type cells was dependent upon c-Src-mediated hypoxic activation of wild type KRAS through phosphorylation of AKT and induction of VEGF expression (Zeng et al. 2010). The expression of VEGF and its receptor, VEGFR-2, and the correlation with vascularity, metastasis and proliferation was also studied by Takahashi et al. These authors conducted immunohistochemical analysis on 52 human colon carcinomas and 10 adenomas. The expression of VEGF and VEGFR-2 was higher in metastatic compared to nonmetastatic neoplasms and directly correlated with extent of neovascularization and proliferation (Takahashi et al. 1995).

4.4 Esophageal cancer

Esophageal carcinoma is one of the most common malignancies in the world. Worse prognosis is linked to lymph node metastasis. Two main types of esophageal cancer are found in humans, squamous cell carcinoma and adenocarcinoma, both of them are related to VEGFR-2. In one study of squamous cell carcinoma, twenty-four (37.5%) of the tumors showed diffuse VEGF immunoreactivity significantly correlated with tumor status ($p < 0.05$) and poor prognosis (log-rank; $p < 0.05$). VEGFR-2 immunoreactivity was detected in the cancer cell cytoplasm in 26 patients (40.6%), but did not correlate with clinicopathological factors or prognosis (Kato et al. 2002). In a recent report (Gockel et al. 2008), adenocarcinoma samples revealed different levels of expression of VEGFR-1 (97%), VEGFR-2 (94%), VEGFR-3 (77%), PDGFRα (91%), PDGFRβ (85%) and EGFR-1 (97%). Similarly, squamous cell cancers revealed VEGFR-1 (100%), VEGFR-2 (100%), VEGFR-3 (53%), PDGFRα (100%), PDGFRβ (87%) and EGFR-1 (100%) expression. In the study, 94% and 100% of the esophageal adenocarcinomas and squamous cell carcinomas, respectively, expressed at least four out of these six RTKs. These results suggest VEGFR-2 may have a high rate co-expression with other RTK in both esophageal cancer types. Therefore, the application of multi-target RTK inhibitors could be a promising and novel approach for esophageal cancer (Gockel et al. 2008).

4.5 Prostate cancer

Prostate cancer (PCa) is the most prevalent noncutaneous malignancy and the second most frequent cause of cancer-related mortality in American men. VEGFR-1 and VEGFR-2 expression was earlier reported in prostate tumors and in LNCaP, PC3, and DU145 prostate cancer cell lines (Ferrer et al. 1997; Levine et al. 1998). Further, Jackson et al., showed that VEGFR-2 induced neovascularization and proliferation of LNCaP prostate cancer cells via autocrine/paracrine mechanisms. VEGFR-1 and VEGFR-2 receptors co-localize with VEGF in prostate tumor cells, prostatic intraepithelial neoplasia, and the basal cells of normal glands. Furthermore, specific staining for both receptors was decreased in poorly differentiated cancer (Jackson et al. 2002). In view of the importance of VEGFR-2 signaling in the growth of PCa several studies have been focused on the effects of anti-angiogenic therapies for reduction of tumor growth and metastasis (Bischof et al. 2004; Stadler et al. 2004).

4.6 Ovarian cancer

Ovarian cancer is the seventh most common cancer in women. It ranks fourth as the cause of cancer deaths in women. The American Cancer Society estimated the occurrence of 21,880 new cases of ovarian cancer in the United States of America in 2010. About 13,850 women were expected to die of the disease. Human epithelial ovarian cancer (EOC) is the most lethal malignancy involving the female genital tract. EOC patients most commonly are diagnosed at an advanced stage and often present with carcinomatosis and large volume ascites. The existence of an autocrine VEGF-A/VEGFR-2 loop in EOC has been suggested in several studies. The first study of the localization of VEGFR-2 expression in nonendothelial cells was reported by Boocock et al in 1995 (Boocock et al. 1995). Their research showed that mRNAs encoding VEGF, VEGFR-1 and VEGFR-2 were detected in primary ascitic cells and in ovarian carcinoma cell lines. Elevated expression of VEGF mRNA was found in all primary tumors and metastases, especially at the margins of tumor acini. VEGFR-2 mRNA was detected not only in vascular endothelial cells but also in tumor cells at primary malignant sites (Boocock et al. 1995). Chen et al examined 42 primary EOC, 29 benign ovarian tumors and 11 normal ovarian tissue samples to determine the expression of VEGF,

VEGFR-2, P-STAT1, P-STAT3, P-STAT5 and P-STAT6. Both VEGF and VEGFR expressions were significantly higher in EOC as compared to benign tumors and normal ovarian tissues ($p < 0.001$). Additionally, the expression of VEGFR-2 was significantly correlated with STAT3 and STAT5 [correlation coefficient (r) = 0.412 and 0.481, respectively] in EOC that suggested VEGF may activate STATs pathways via VEGFR (Chen et al. 2004). Paradoxically, targeted therapy designed to inhibit both VEGFR-2 and EGFR failed to show any clinical activity when used in a phase II trial (Annunziata et al. 2010). In this study, 12 patients with recurrent, persistent or refractory EOC were administered oral vandetanib every four weeks. There was no evidence of VEGFR-2 receptor inhibition in tissues analyzed after repeated biopsies (Annunziata et al. 2010). Conversely, Koukourakis et al examined the levels of pVEGFR-2 in both EOC cells and tumor associated vessels. High pVEGFR-2 levels were noted 55% (5/9) in EOC. Additionally, high pVEGFR-2 levels was linked to higher serum VEGF levels in patients with ovarian cancer (404 +/- 214 vs. 170 +/-143, $p = 0.10$) though this association was not statistically significant (Koukourakis et al. 2011).

4.7 CNS malignancies

Tumors of the central nervous system (CNS) include both non-malignant and malignant tumors of the brain and spinal cord. Primary brain tumors encompass a diverse spectrum of malignancies which are primarily derived from glial precursors. They are the most prevalent solid tumor in children and represent the leading cause of cancer death in children younger than age fifteen. Approximately 22,000 new cases of primary malignant brain and central-nervous system-tumors were diagnosed in the United States in 2010. VEGF plays a critical role in the angiogenesis and progression of malignant brain tumors. Knizetova et al demonstrated autocrine VEGF signaling mediated via VEGFR-2 that involved the co-activation of the c-Raf/MAPK, PIK3/Akt and PLC/PKC pathways using a panel of astrocytoma (grade III and IV/GBM) derived cell lines and clinical specimens of both high and low grade astrocytomas. The VEGF-VEGFR-2 interplay affected cancer cell cycle progression and viability and radioresistance in clinical specimens. Furthermore, the use of a selective inhibitor of VEGFR-2 (SU1498) limited the VEGF-mediated proliferation and viability of astrocytoma cells in culture (Knizetova et al. 2008).

KIT, PDGFR alpha and VEGFR-2 are important clinical targets for tyrosine kinase inhibitors. Puputti et al investigated the expression and amplification of KIT, PDGFRA, VEGFR-2, and EGFR in 87 gliomas which consisted of astrocytomas, anaplastic astrocytomas, oligodendrogliomas and oligoastrocytomas. VEGFR-2 amplifications occurred in 6-17% of the gliomas at diagnosis. KIT amplification was associated with KIT protein overexpression and with the presence of PDGFRA and EGFR amplifications both at time of first glioma diagnosis and at tumor recurrence. However, KIT amplification was associated with VEGFR-2 amplification only at time of tumor recurrence (Puputti et al. 2006). In another study, Joensuu et al investigated 43 primary glioblastomas for amplification of the genes encoding tyrosine kinases. VEGFR-2 was amplified in 39% of glioblastomas and was strongly associated with amplification of KIT and PDGFRA ($p < 0.0001$). These amplified kinases may well serve as potential targets for inhibitor therapy in these CNS malignancies (Joensuu et al. 2005). Amplification and overexpression of KIT, PDGFRA, and VEGFR-2 was also studied by Blom et al. in medulloblastomas (MB) and primitive neuroectodermal tumors (PNET), the most common malignant brain tumors in children (Blom et al. 2010). Using immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH) they found KIT, PDGFRA, and VEGFR-2 amplifications to be present in only 4% of MBs/PNETs.

KIT amplification was associated with concurrent PDGFRA and VEGFR-2 amplifications (p <or=0.001) but only increased copy number of PDGFRA was associated with poor overall survival (p=0.027) (Blom et al. 2010). A population based study conducted by Nobusawa et al of 390 glioblastomas using differential PCR demonstrated amplification of PDGFRA, KIT and VEGFR-2 genes in 33 (8.5%), 17 (4.4%) and 13 (3.3%) of glioblastomas respectively. None of these alterations were prognostic for overall survival (Nobusawa et al. 2011).

4.8 Kidney cancer

The two most common types of kidney cancer, reflecting their location within the kidney, are renal cell carcinoma (RCC) and urothelial cell carcinoma (UCC) of the renal pelvis. Tsuchiya et al investigated the expression of VEGF-related factor genes (VEGF, VEGF-B, and VEGF-C) and their receptors (VEGFR-1 and VEGFR-2) in RCC (Tsuchiya et al. 2001). They found significant differences in the expression level of VEGF, VEGFR-1 and VEGFR-2 between RCC and the corresponding normal renal tissue. Furthermore, the expression level of VEGF in the tumor tissue significantly correlated with those of VEGFR-1 and VEGFR-2. A moderate to high protein expression for VEGF, VEGFR-1, and VEGFR-2 was observed in both the tumor cells and the endothelial cells. These data suggest that VEGF and its receptors VEGFR-1 and VEGFR-2 cooperate and play a crucial role in the angiogenesis of RCC. Different RCC types may have dissimilar expression patterns of VEGF and receptor mRNA levels. In clear cell RCC, VEGFR-2 levels were higher in stage I-II than in more advanced stages, while in papillary RCC, VEGF and VEGFR-2 levels were higher in stage III than in stage I-II tumors (Ljungberg et al. 2006). In another report, the VEGFR-2 protein-positive phenotype of clear cell RCC was relatively frequent (7/20, 35%), but was lost in bone metastases (2/20, 10%) (Badalian et al. 2007). In line with the above observations in RCC, the expression of VEGF and VEGFR-2 was observed in 58% and 50%, respectively, of UCC cells (Xia et al. 2006). Moreover, VEGFR-2 expression correlated with disease stage (coefficient 0.23, p = 0.05). However, VEGF expression failed to correlate with clinical variables. Interestingly, VEGFR-2 expression increased with tumor invasion into the muscle (p <0.01). In addition, VEGFR-2 in positive bladder cancer cell lines was also increased in response to VEGF (Xia et al. 2006). Taken together, these data suggest increased VEGFR-2 expression correlates with several features that predict progression, disease stage and invasive phenotype of UCC. In summary, VEGFR-2 is associated with tumor stage and survival of both UCC and RCC. However, diverse subtypes of these cancers may have different expression patterns of VEGFR-2 levels. Therefore, a diversity of signaling pathways might be involved in regulating angiogenesis and cancer cell survival and proliferation in the specific UCC and RCC subtypes. Detailed knowledge of tumor angiogenesis in UCC and RCC is essential when designing new treatment trials where angiogenesis inhibition will be used.

4.9 Liver cancer

The American Cancer Society reports that while still quite rare in the United States, liver cancer is more prevalent in men (17,430 new diagnoses annually) than in women (6,690 annual diagnoses). There are several types of liver cancer, including hepatoblastoma, a rare type typically affecting children, cholangiocarcinoma – a cancer that affects the cells of the bile duct, angiosarcoma- a cancer that originates in the liver blood vessels, and hepatocellular carcinoma (HCC), which is by far the most common type of liver cancer (Altekruse et al. 2009). HCC accounts for 90% of all malignant liver tumors. This typically highly vascular tumor is the fifth most common type of cancer and ranks as the third leading cause of cancer

related deaths worldwide (Huang et al. 2011). It has been well established that VEGF plays a critical role in angiogenesis. If the known contributing factors to angiogenesis, namely VEGF and its receptors VEGFR-2, can be exploited, it may give rise to novel therapeutic targets for HCC (Ng et al. 2001). The hypoxic environment produced in HCC is a stimulant for the secretion of VEGF by the tumor that subsequently activates VEGFR-2 signals. Elevated expression of VEGFR-2 in HCC was correlated with worse outcome after liver transplantation. Vascular invasion was consistently associated with HCC recurrence ($p<0.01$) and overall mortality ($p<0.05$). Subjects with VEGFR-2 overexpression in tumor arterioles ($p<0.01$), venules ($p<0.05$) had worse overall survival (Kim 2008). A recent report from Huang et al showed higher expression of VEGFR-2 in HCC cells compared to normal hepatic cells ($p<0.001$). Moreover, the high expression of VEGFR-2 in HCC was related to large tumor diameter ($p=0.012$), poor differentiation ($p=0.007$), high serum α-fetoprotein ($p=0.029$), multifocal gross classification ($p=0.007$), and less than 5 years' survival ($p=0.029$). In addition, Kaplan-Meier survival and regression analyses showed that high VEGFR-2 expression ($p=0.009$) and stage grouping were independent prognostic factors (Huang et al. 2011). Anti-VEGFR-2 therapy is a promising novel potential therapy for HCC (Lang et al. 2008).

4.10 Lung cancer

Lung cancer accounts for 90% of deaths from cancer in men and approximately 80% of cancer deaths in women. Smoking increases lung cancer incidence and mortality by up to 20 times, compared to non-smokers. Additionally, lung cancer is responsible for more cancer related deaths than breast, prostate and colorectal cancer combined, as reported by the Centers for Disease Control (Tammemagi et al. 2011). Lung cancer is divided into two primary subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC makes up 80% of primary lung cancers and has a 10-15% overall survival rate. While resection is an effective treatment for early stages of lung cancer, most cases of NSCLC are enough advanced that resectioning is no longer an option (Jantus-Lewintre et al. 2011). The role of VEGFR-2 as a regulator of endothelial cell migration and proliferation has been previously established (Joukov et al. 1996a; Meyer et al. 1999). It has been proposed that VEGF and its receptors contribute to the proliferation and metastasis of tumor cells. A study from Kikuchi's laboratory reported the relevance of VEGF and VEGFRs in the progression of SCLC. VEGFR-2 was detectable in five SCLC cell lines (Tanno et al. 2004). Furthermore, VEGF/VEGFR-2 autocrine loop favoring the growth of SCLC has also been suggested (Kim et al. 2010). In a study by Tanno et al, VEGFR-2 was detected in several SCLC cells lines (Tanno et al. 2004). In addition, VEGFR-2 functions were investigated in SCLC using NCI-H82 SCLC cell line that overexpresses VEGFR-2. In these cells, VEGFR-2 increased cell proliferation, thus verifying the role of VEGFR-2 in SCLC (Tanno et al. 2004). While there is currently limited information regarding the mechanism of the prolific spread of SCLC, there is evidence to support the hypothesis that VEGFR-2 plays a role in the metastasis and proliferation of this cancer type. This supports the theory that anti-cancer drugs targeting the VEGF pathway are possible therapeutic means. The VEGF pathway has also recently been identified as a viable therapeutic target in NSCLC (Nikolinakos et al. 2010).

A study utilizing a mouse model to determine if initiation and maintenance of tumor angiogenesis in NSCLC could be associated with endothelial progenitor cells (EPCs CD133+) it was found these cells express high levels of VEGFR-2. The correlation between CD133 and VEGFR-2 implicates the receptor is associated with angiogenesis and neovascularization in NSCLC (Hilbe et al. 2004). Other report using pre-clinical studies suggested that VEGF-A

induced removal or shedding of VEGFR-2 in EC. VEGFR-2 shedding resulted in tumor shrinkage in NSCLC patients that was associated with expression of ADAM metalloprotease and IL-4 (Nikolinakos et al. 2010). Taken together, these results suggest that VEGFR-2 is not only a mediator of angiogenesis but a viable target. A common thread in treatment options for lung cancer seems to be the targeting of the VEGF pathway, including the VEGFR-2 receptor. Therefore, combinatorial therapies involving VEGFR-2 may prove useful in development of novel treatment options for this malignancy.

4.11 Other cancers

Some contradictory data on VEGFR-2 expression and survival of pancreatic cancer patients have been reported. A study reported that VEGFR-1 and VEGFR-2 mRNA were found in 17 and 15 of 24 pancreatic cancer samples, respectively. VEGF receptors were detected not only in blood vessels but also in pancreatic cancer cells. Remarkably, VEGFR-2 expression correlated with poor tumor differentiation and poorer survival, while VEGFR-1 expression did not correlate (Buchler et al. 2002). Accordingly, these data support the idea that VEGF/VEGFR-2 pathway regulates angiogenesis, local pancreatic tumor growth and metastasis and offers a potential new therapeutic option for this malignancy. In other report, VEGFR-2 mRNA was detected in several pancreatic cancer cell lines (Panc-1, AsPC-1, and MiaPaCa-2) (Higgins et al. 2006a). Conversely, Chung et al reported using Kaplan-Meier survival curves that VEGF and VEGFR-2 were not clearly associated with outcome in 76 tissue microarrays from pancreatic cancer. Moreover, the patients who had tumors with the lowest expression VEGFR-1 levels had the worst survival ($p = 0.0038$) (Chung et al. 2006). Causes of these opposed findings are not clearly understood.

In thyroid cancer, VEGF, VEGFR-1 and VEGFR-2 expression were analyzed on 34 papillary thyroid carcinomas (PTCs) and 18 follicular thyroid carcinomas (FTCs) and 8 poorly differentiated thyroid carcinomas (PDTCs). VEGFR-2 was found in 68% of PTCs, 56% of FTCs and 37% of PDTCs (Vieira et al. 2005). In addition, co-expression of VEGF with its receptors was observed in 50% of PTCs, 39% of FTCs and 12% of PDTCs, raising the possibility that VEGF/VEGFR-2 may signal in an autocrine loop in these neoplasias (Vieira et al. 2005). On the other hand, in metastatic medullary thyroid carcinoma (MTC) higher levels of VEGFR-2 were found in metastatic tumors as compared to primary tumors [p=2.8 x 10(-8)] (Rodriguez-Antona et al. 2010). In contrast, in a recent report, VEGFR-2 was detected in 91% (31/34) of MTC samples and had no correlation with tumor stage (tumor node metastasis) (Capp et al. 2010). Taken together these observations suggest that VEGF/VEGFR-2 expression is often found in diverse cancer types. Functions of the VEGF/VEGFR-2 appear to be expanded beyond to the development of tumor angiogenesis. Indeed, VEGF/VEGFR-2 autocrine/paracrine loop seems to play additional roles in cancer cell proliferation and survival.

5. Regulation of VEGFR-2 levels

Despite the essential role of VEGFR-2 in angiogenesis and carcinogenesis the molecular mechanisms controlling its expression are only partially known. Regulation of VEGFR-2 expression involves a series of complex mechanisms that include epigenetic changes, transcriptional regulation, cellular localization/trafficking, ligand binding, co-activator activity, adhesion molecule expression, constitutive-embryonic derived signaling pathways and cytokine-growth factor regulation. In addition, VEGFR-2 can assemble functional

complexes composed of homodimers and heterodimers with other VEGFR receptors and co-receptors that can bind VEGF or other angiogenic ligands thereby affecting VEGFR-2 signaling capabilities (Fig 2).

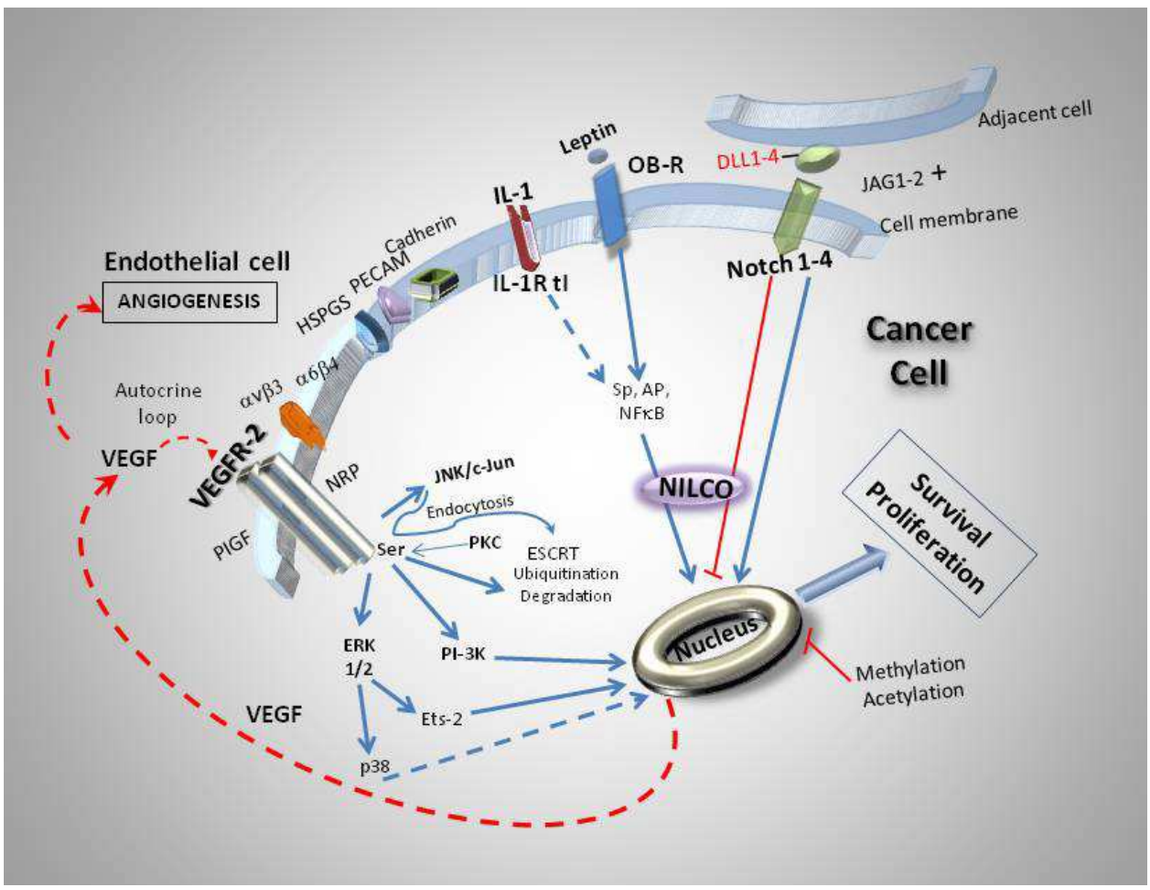

Fig. 2. Mechanisms of Regulation of VEGFR-2 levels in cancer cells

5.1 VEGFR-2 co-activators, cellular localization and trafficking

Heparin and heparan sulfates (components of proteoglycans, HSPGs) have affinity for VEGF165 (VEGF-A), the major isoform of VEGF, promoting enhanced phosphorylation of VEGFR-2. This probably occurs by enhancing ligand binding capabilities to VEGFR-2. Therefore, HSPGs affect the localization, extent and intensity of VEGFR signaling (Ashikari-Hada et al. 2005). NRP-1 can stabilize the VEGF/VEGFR-2 complex and particularly increase tumor angiogenesis (Miao et al. 2000). Blood flow could likely activate VEGFR-2 through the formation of mechanosensory complexes (Tzima et al. 2005). Adhesion molecules (PECAM-1 and VE-cadherin) (Carmeliet et al. 1999; Tzima et al. 2005) and αvβ3 integrin (Somanath et al. 2009) interplay with VEGFR-2 under diverse biological scenarios controlling VEGFR-2 expression and signaling. β3-integrin can limit the interaction of NRP-1 with VEGFR-2, thus negatively affecting VEGF-mediated angiogenesis (Robinson et al. 2009). Invasive ductal carcinoma has decreased expression of α6-integrin associated with higher tumor angiogenesis presumably linked to VEGFR-2 expression. Indeed, loss of α6-integrin correlates to overexpression of activated VEGFR-2 in murine melanoma and lung

carcinoma in endothelial-specific α6-knockout mice (Germain et al. 2009). However, the specific mechanisms involved in VEGFR-2 overexpression in tumors from these mice remains to be investigated. A negative regulator of angiogenesis is thrombospondin-1. This molecule negatively modulates VEGF actions through a complex with β1-integrin and VEGFR-2 (Zhang et al. 2009a). Inverse regulation of VEGFR-1 and VEGFR-2 could play an important role in controlling the growth and differentiation of tumor-associated EC. VEGF signaling through JNK/c-Jun pathway induces endocytosis, nuclear translocation and ubiquitin-mediated downregulation of VEGFR-2 in human squamous-cell carcinomas (Zhang et al. 2009b). A model recently proposed suggests that a negative feed-back loop regulates VEGFR-2 activities through the differential segregation/localization of VEGFR-1 and VEGFR-2 (Germain et al. 2009; Robinson et al. 2009). The higher affinity of VEGFR-1 for VEGF blocks VEGFR-2 activation. In the model, Ca (2+) induces the translocation of VEGFR-1 from the trans-Golgi network to the plasma membrane allowing preferential binding of VEGF. VEGFR-2 is degraded after activation by ubiquitin-mediated proteolysis that is linked to endosomal sorting complex required for transport and to Rab GTPases (Bruns et al. 2009). This could also be stimulated by PKC pathway that requires the removal of VEGFR-2 carboxyl terminus (Bruns et al.). Differential trafficking of VEGFR-2 is potentially due to the formation of complexes with diverse angiogenic regulators. These processes occur through the endosomal pathway controlling angiogenesis (Scott and Mellor 2009).

5.2 The Notch signaling-VEGFR-2 link

Notch is a family of mammalian transmembrane proteins that function as receptors for membrane bound ligands. There are four mammalian Notch genes, Notch1–Notch4, and five ligands, Jagged1 and Jagged2 (homologs of Drosophila Serrate-like proteins) and Delta-like 1 (DLL1), DLL3 and DLL4. Notch receptors have an extracellular domain made up of multiple epidermal growth factor domains (EGF), yet, Notch intracellular domain is made up of many domain types (Kovall 2008). The Notch proteins have been proven to affect diverse cell programs (proliferation, differentiation, and apoptosis) and as result Notch influences organogenesis and morphogenesis (Artavanis-Tsakonas et al. 1999). The activation of a Notch receptor is triggered by ligands expressed on adjacent Jagged and Delta cells.

Notch signaling and its crosstalks with many signaling pathways play an important role in cancer cell growth, migration, invasion, angiogenesis and metastasis. Entangled crosstalk between Notch and other developmental signaling (Hedgehog and Wnt), and signaling triggered by growth factors, estrogens and oncogenic kinases, could impact on Notch targeted genes [for review see (Guo et al. 2011)]. This evolutionarily conserved pathway in multicellular organisms regulates embryonic and stem cell fate (Bray 2006). It is generally believed that tumor angiogenesis will not occur in absence of Notch signaling. When a Notch decoy is introduced in place of a functional Notch receptor at a tumor site in the skin during angiogenesis, cell proliferation stops and the development of the new blood vessels ceases. This suggests that the Notch protein has some significant role in angiogenesis (Phng and Gerhardt 2009; Roca and Adams 2007). Indeed, active Notch1 (Stylianou et al. 2006) or Notch4 (Imatani and Callahan 2000) signaling are involved in breast cancer angiogenesis. Soares et al, first demonstrated that a cross talk between Notch and E2 signaling occurs in breast cancer and EC. Notch gene expression was required for tubule-like structure formation in EC. Notch gene expression clustered with HIF-1α and was upregulated by E2. Thus, Notch has significant role in human breast carcinogenesis and angiogenesis (Soares et al. 2004).

Neutralization of DLL4 greatly reduced EC-mediated activation of Notch 3 signaling in T-ALL cells and blocked tumorigenesis (Indraccolo et al. 2009). Moreover, silencing Notch3 by RNA interference had marked antiproliferative and proapoptotic effects on T-ALL cells in vitro and reduced tumorigenicity in vivo (Indraccolo et al. 2009). These results elucidate Notch3 and DLL4 interaction between endothelial and tumor cells, which promotes survival and triggers tumor growth. Unlike other Notch receptors, Notch2 may possibly play a tumor-suppressive role in human cancer (Guo et al. 2011), however, the role of Notch2 in angiogenesis is not well-understood.

It has been found that DLL4 and Jagged1-Notch signaling pathways have opposing effects on angiogenesis (Benedito et al. 2009). While Jagged1-Notch signaling serves as a proangiogenic regulator (Benedito et al. 2009), DLL4-Notch signaling has been shown to significantly decrease the expression of VEGFR-2 thus inhibiting the proliferation of angiogenic cells (Williams et al. 2006). These two signals operate in equilibrium with one another, so that as the concentration of one signal increases the other will decrease proportionally. As a result of the ligand signaling competitive nature towards one another, it is plausible to speculate that the two are used as antagonistic mechanisms to help regulate the processes of angiogenesis (Benedito et al. 2009; Williams et al. 2006). In vitro, the activation of Notch1 or Notch4 in EC induces the expression of the HESR-1 transcription factor (expressed in mature vasculature but reduced in proliferating EC) that in turns downregulates VEGFR-2. Notch-mediated reduction in VEGFR-2 levels results in decreased EC proliferation. This Notch mechanism may be involved in the phenotypic changes during EC proliferation and migration to network formation (Taylor et al. 2002). Overall, one could speculate that the two types of Notch ligands operate to signal the VEGFR-2 to either continue to promote cell proliferation or move onward in the angiogenic process to EC differentiation. Activation of Notch signaling in ER-negative breast cancer cells results in direct transcriptional up-regulation of the apoptosis inhibitor and cell cycle regulator survivin (baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5). Survivin is highly expressed by cancer cells and binds and inhibits caspase-3, controlling the checkpoint in the G2/M-phase of the cell cycle. Therefore, the Notch-survivin functional gene signature is a hallmark of basal breast cancer, and may contribute to disease pathogenesis (Lee et al. 2008b; Lee et al. 2008a).

In addition, Notch signaling modulates other pathways, such as PI-3K–Akt and NFκB, also activated by VEGFR-2 signaling as discussed above. Indeed, VEGF/VEGFR-2 signals activate and regulate Notch expression in EC and cancer cells (Liu et al. 2003). VEGF or ligand-induced Notch signaling up-regulates DLL4 through a positive feed-forward mechanism. By this mechanism, DLL4 could propagate its own expression and enable synchronization of Notch expression and signaling between ECs. This signaling pathway has been suggested to serve as a negative feedback loop for endothelial sprouting. Moreover, the feedback could be bidirectional as Notch reduces VEGF responsiveness through down-regulation of VEGFR-2 in EC (Caolo et al. 2010). In addition, DLL4 expressed in tumor cells, can function as a negative regulator of tumor angiogenesis by reducing the number of blood vessels. In vitro, DLL4 did not affect the growth of cancer cells: PC3 (prostate cancer), MDA-MB-231 (breast cancer) and B16 (mouse melanoma). However, DLL4 slightly but significantly inhibited the growth of HT1080 (human fibrosarcoma) and retarded the growth of U87 (human glioblastoma). In contrast, DLL4 acted as a positive driver for tumor growth in vivo of human glioblastoma and prostate cancer xenografts (Li et al. 2007). Moreover, Notch signaling from tumor cells is able to activate EC and trigger tumor angiogenesis in vitro and in a xenograft mouse tumor model (Zeng et al. 2005). Therefore, a crosstalk between Notch and VEGFR-2

signaling may be crucial for angiogenic processes. Notch-VEGFR-2 crosstalk could also be influenced by Wnt signaling. The Notch pathway serves not only to coordinate the effects of the VEGF pathway, but its crosstalk with the Wnt pathway mediates EC function in angiogenesis (Phng and Gerhardt 2009). Furthermore, vascular remodeling in cancer is dependent upon crosstalk between the VEGF, Notch and Wnt pathways (Katoh and Katoh 2006). Deregulation of these pathways has been implicated in many tumor types, including those of the lung (Daniel et al. 2006) and liver (Martinez Arias 2003).

The Wnt pathway is made up of a family of secreted glycoproteins, ranging in size from 39-46 kDa. There are 19 known Wnt homologues that that participate in three Wnt signaling pathways: the canonical pathway, the Wnt/Ca^{2+} pathway, and the planar cell polarity pathway. The canonical pathway participates in the stabilization of β–catenin and its subsequent translocation to the nucleus where it functions as an oncogene. Abnormal activation of the canonical Wnt/β-catenin pathway is one of the most frequent signaling abnormalities known in human cancer (Brennan and Brown 2004). Interestingly, in a study conducted by Nimmagadda et al, the quail homologue to VEGFR-2 (Quek1) was found to be upregulated by Wnt1 and Wnt3a (Nimmagadda et al. 2007). Wnt1 and Wnt3a were also shown to induce proliferation in EC necessary for angiogenesis. These results are consistent with those of Wang et al that showed Wnt signaling pathway activation was vital to the development of vascular structures in embryos (Wang et al. 2006). In addition, Wnt2 was identified as an autocrine growth factor for VEGFR-2 stimulation in hepatic sinusoidal EC (HSECs) (Klein et al. 2008). Investigations from Dr. Gerhardt's laboratory showed that the Notch-regulated ankyrin repeat protein (Nrarp) acts as a molecular link between Notch and Lef1 (a chaperone for distinct factors controlling transcription of Wnt target genes)-dependent Wnt signaling in EC to control stability of new vessel connections in mouse and zebrafish. DLL4/Notch-induced expression of Nrarp limits Notch signaling and promotes Wnt/Ctnnb1 (the β–catenin gene) signaling in endothelial stalk cells through interactions with Lef1 (Phng and Gerhardt 2009). Taken together, these data suggest that multi-directional targeting of VEGFR-2, Notch and Wnt pathway components could be a worthwhile endeavor in the search for novel therapeutic targets against cancer.

In human MCF-7 BC cells over-expression of the γ-secretase (the enzyme that catalyzes intramembrane cleavage of the Notch receptor upon ligand binding required for Notch activation) liberated Notch intercellular domain and increased HIF-1α protein levels by an unknown mechanism (W. Lee et al. 2008b; Lee et al. 2008a). Notch1 signaling can promote NFκB translocation to the nucleus and DNA binding by increasing both phosphorylation of the IκB kinase α/β complex (a repressor of NFκB activation) and the expression of some NFκB family members (Monsalve et al. 2009). We have found that leptin (an adipocytokine) activated NFκB, SP1 and HIF-1α (Gonzalez-Perez et al. 2010) and increased the expression of Notch mRNA and protein in breast cancer cells under normoxic conditions (Guo and Gonzalez-Perez 2011). Remarkably, leptin induced the expression of Notch1-4, Jagged1 and VEGFR-2 in these cells. Moreover, we have recently reported that a novel and unveiled crosstalk, NILCO (Notch, IL-1 and leptin crosstalk outcome) is essential for leptin-induced proliferation, migration and VEGF/VEGFR-2 expression by breast cancer cells (Guo and Gonzalez-Perez 2011). In particular, leptin-mediated activation of NFκB increased VEGFR-2 and Notch. Furthermore, leptin increased through several signaling pathways promoter activities of VEGFR-2-Luc transfected-cells. Interestingly, leptin effects on VEGFR-2 were abrogated by a γ–secretase inhibitor. Moreover, VEGFR-2 transcription and expression was heavily depending on VEGFR-2 gene methylation and histone acetylation that could be

linked to leptin and Notch effects (Guo and Gonzalez-Perez, unpublished data). However, the role of Notch-mediated regulation of VEGFR-2 and signaling crosstalk in cancer cells is so far unknown.

5.3 Estrogen-mediated regulation of VEGFR-2

Estrogens exert important regulatory functions on vessel wall components, which may contribute to the increased prevalence and severity of certain chronic inflammatory, autoimmune diseases, as well as tumor initiation, progression, particularly in tumors of the breast, endometrium, ovary and prostate (Ferreira et al. 2009; Russo and Russo 2006; Santen et al. 2009). EC have also been identified as targets for estrogens. ERs have been found in EC from various vascular beds. The regulatory functions of estrogen in EC responses are relevant to vessel inflammation, injury, and repair. In these cells estrogen affects nitric oxide production and release, modulates the expression of EC-adhesion molecules and regulates angiogenesis (Kim and Bender 2005; Rubanyi et al. 2002; Simoncini et al. 2004). The mechanisms through which estrogen regulates VEGFR-2 in angiogenesis are complex and may involve both genomic and non-genomic effects. It was earlier reported that, estrogen stimulates EC growth as well as VEGF-dependent angiogenesis by the receptor-mediated pathway, especially ERα (Suzuma et al. 1999; Tanemura et al. 2004). However, non-classical mechanisms through ERα/Sp3 and ERα/Sp4 complexes were found in some cancer cell lines, such as ZR-75 breast cancer cells. In these cells E2 activates GC-rich sites where Sp proteins but not ER-α bind to VEGFR-2 promoter to stimulate mRNA and protein expression (Higgins et al. 2006b). In contrast, in MCF-7 cells, the ERα/Sp protein-VEGFR-2 promoter interactions involve the recruitment of the co-repressors SMRT (silencing mediator of retinoid and thyroid hormone receptor) and NCoR (nuclear receptor corepressor) resulting in decreased VEGFR-2 mRNA levels (Higgins et al. 2008). Estrogens may also directly stimulate endothelial progenitor cells (EPCs). In a recently report (Baruscotti et al. 2010), physiological concentrations of E2 (10 nmol/L) was showed to increase EPC-induced capillary sprout and lumen formation in matrigel/fibrin/collagen systems. Whereas, heme oxygenase 1 (HO-1) and phosphorylation of ERK 1/2, Akt, and vVEGFR-2 were also increased, indicating that E2 via ERα promotes EPC-mediated capillary formation. The mechanism for these E2 actions probably involves non-genomic activation of RTKs and HO-1 activation.

5.4 Cytokine and growth factor regulation of VEGFR-2

VEGF-A is hypoxia-inducible showing a temporal expression pattern that generally parallels VEGFR-2 expression. Contradictory data on the direct role of hypoxia in the regulation of VEGFR-2 were reported. The differential and synergistic regulation of VEGF and VEGFR-2 by hypoxia in an organotypic cerebral slice culture system for EC was linked to a direct induction of VEGF that subsequently up-regulates VEGFR-2 in EC. VEGF-induced VEGFR-2 up-regulation was abrogated by a neutralizing anti-VEGF antibody (Kremer et al. 1997). In contrast, it was later reported that hypoxia up-regulates VEGFR-2 in cultured cells by a posttranscriptional mechanism (Gerber et al. 1997). Inflammation and angiogenesis are frequently coupled in pathological situations like breast cancer. One of the hallmarks of inflammation is an increase in vascular permeability, frequently driven by an excess of VEGF and other mediators. Inflammation induces EC activation and capillary sprouting (Arroyo and Iruela-Arispe 2010). Pathological angiogenesis is associated with the secretion of cytokines. However, the molecular and cellular mechanisms linking chronic inflammation to tumorigenesis remain largely unresolved. Many cytokines and growth factors are able to

increase VEGF expression (Hicklin and Ellis 2005). However, a reduced number of these factors have been confirmed to regulate VEGFR-2 expression. Majority of inflammatory cytokines exert inflammatory effects through the induction of NFκB. a hallmark of inflammatory responses. Activation of NFκB is essential for promoting inflammation-associated cancer (Pikarsky et al. 2004). VEGF (Pikarsky et al. 2004) and VEGFR-2 (Wu and Patterson 1999) promoters have NFκB responsive cis-elements. Therefore, cytokine-activated NFκB increases angiogenesis by direct upregulation of pro-angiogenic genes. VEGFR-2 is associated to inflammatory breast cancer and is therefore a target for cancer prevention.
Cytokines have diverse effects on VEGFR-2. PIGF, erythropoietin or PDGF were unable to up-regulate VEGFR-2 (Kremer et al. 1997). Transforming growth factor-beta (TGF-β) can down-regulate VEGFR-2 (Barleon et al. 1997a) but discordant results on TNF-α mediated down-regulation (Patterson et al. 1996) and up-regulation (Giraudo et al. 1998) of VEGFR-2 have been reported. Moreover, TNF showed contradictory effects on VEGFR-2 activity. TNF induced VEGFR-2 but blocked it signals, thus delaying the VEGF-driven angiogenic response (Sainson et al. 2008). Members of the chemokine family can also regulate VEGFR-2. CCL23 (also known as MPIF-1, MIP-3, or Ckb8) is a CC chemokine initially characterized as a chemoattractant for monocytes and dendritic cells. In HUVEC, CCL23 mainly induced VEGFR-2 expression at the transcriptional level. These effects were linked to CCL23-mediated phosphorylation of SAPK/JNK (Han et al. 2009). IL-1 is a known factor in cancer development and inducer of VEGF expression in different tissues (Carmi et al. 2009; Valdivia-Silva et al. 2009). Macrophages are recruited to tumors by chemokines, cytokines and growth factors, including VEGF, produced by tumor cells and other cell types in the tumor microenvironment. In turn macrophages and tumor cells secrete IL-1 that contributes to tumor progression by facilitating angiogenesis, matrix remodeling, invasion and metastasis (Chen et al. 2009). Inhibition of IL-1 signaling by exogenous IL-1Ra negatively impacted tumor angiogenesis in nude mice (Voronov et al. 2003). We have recently demonstrated that IL-1/IL-1R tI signaling upregulates VEGFR-2 in breast cancer cells (Zhou et al. 2011).

5.4.1 Leptin regulation of VEGFR-2

Recently, leptin was added to the list of factors that upregulate VEGF-A and VEGFR-2 (Carino et al. 2008; Gonzalez et al. 2006; Rene Gonzalez et al. 2009). Leptin is a small nonglycosilated protein (16 kD) product of the ob gene. Leptin is a pleitropic adipocytokine, with mitogenic and angiogenic effects, that promotes anchorage, proliferation of breast cancer cells, microvessel and hematopoiesis and increase the levels of several factors including cell cycle regulators (Gonzalez et al. 2006; Pischon et al. 2008; Rene Gonzalez et al. 2009). Leptin actions are more often than not related to energy balance. However, leptin is also recognized for its contributions to reproduction, angiogenesis, proliferation and inflammation. Leptin's actions are now being linked to the development and pathogenesis of cancer (Hu et al. 2002; Pischon et al. 2008). Higher levels of leptin are found in female, postmenopausal women and obese individuals. The leptin levels have been related to the incidence of various types of cancer, most notably breast cancer (Cleary and Maihle 1997; Ray and Cleary). Breast carcinoma cells express higher levels of leptin and leptin receptor, OB-R, than normal mammary cells and a significant correlation between leptin/OB-R levels with metastasis and lower survival of breast cancer patients has been found (Hu et al. 2002; Laud et al. 2002; Tessitore et al. 2000). We have previously found that leptin signaling plays an important role in the growth of breast cancer that is associated with the regulation of pro-

angiogenic, pro-inflammatory and pro-proliferative molecules (Rene Gonzalez et al. 2009). Leptin increases VEGFR-2 expression in endometrial cancer cells in vitro (Carino et al. 2008) and in breast cancer cells in vitro and in vivo (Gonzalez et al. 2006; Rene Gonzalez et al. 2009). Leptin upregulation of VEGF-A/VEGFR-2 was partially mediated by IL-1 system. Leptin upregulates IL-1 gene through activated SP1 and NF-κB. In addition, leptin-induced activation of PI-3K signaling pathway was related to increased levels of pmTOR, p70S6K1 and p4E-BP (Zhou et al. 2011). In human breast cancer cells ER-positive (MCF-7) or ER-negative (MDA-MB-231), leptin in a dose-response manner significantly increased the levels of VEGFR-2 protein and mRNA (Rene Gonzalez et al. 2009). However, the molecular mechanisms of how leptin signaling regulates VEGFR-2 are largely unknown (Beecken et al. 2000; Cirillo et al. 2008; Hausman and Richardson 2004). In addition, leptin induces transactivation of the HER2/*neu* proto-oncogene (*c-erbB-2*), and interacts with insulin like growth factor-1 to transactivate the EGF-receptor (EGFR) (Fiorio et al. 2008; Soma et al. 2008). Leptin stimulates aromatase expression and activation of ER (Catalano et al. 2003; Cirillo et al. 2008). A pegylated leptin peptide receptor antagonist (PEG-LPrA2) markedly reduced the growth of tumors and the expression of VEGF-A/VEGFR-2 in mouse models of syngeneic and human breast cancer xenografts (Gonzalez et al. 2006; Rene Gonzalez et al. 2009). The mice treated with PEG-LPrA2 had diminished expression of VEGF-A/VEGFR-2, OB-R, leptin, IL-1R tI, PCNA and cyclin D1 (Rene Gonzalez et al. 2009). PEG-LPrA2's effects were probably related to reduced NILCO (see Fig 2) (Guo and Gonzalez-Perez 2011). These data suggest that inhibition of leptin signaling may serve as a novel adjuvant for prevention and treatment of breast cancer. The alarming increase of incidence of obesity in the Western countries emphasizes the importance of these findings (Lee et al. 2008b; Lee et al. 2008a).

Leptin is likely linked to the growth of several cancer types and may influence the expression of VEGFR-2 in several malignancies. Results from xenografts from human ovarian cancer cells (OVCAR5 and IGROV1) subcutaneously inoculated on ovariectomized nude mice show correlations of VEGF-A/VEGFR-2 expression in all tumors. Interestingly, treatment of mice with PEG-LPrA2 induced a significant reduction of tumor growth. Moreover, incubation of ovarian cancer cells with leptin increased cell proliferation in a dose dependent fashion that was abrogated by pre-treatment with PEG-LPAr2. Furthermore, PEG-LPrA2 reduced leptin induced VEGF-A, VEGFR-2, Ki-67 as well as ERα levels. These data suggest elevated leptin would likely have pro-proliferative and pro-angiogenic effects on human ovarian cancer. Our data also suggests that leptin may be a link between obesity, estrogen and ovarian cancer (Rueda and Gonzalez-Perez, unpublished). Colon cancer development has also been linked to obesity (Frezza et al. 2006) and leptin signaling, which can orchestrate VEGF-A-driven angiogenesis and vascular development, thus providing a specific mechanism and potential target for obesity-associated cancer (Birmingham et al. 2009). A recent report stated that high levels of leptin in obese mice increased the growth of colorectal cancer induced by azoxymethane (a carcinogen) in normal mice in contrast to leptin deficient mice (*ob/ob* and *db/db*). These results suggested leptin is important for colon cancer growth in the context of obesity (Endo et al. 2011).

6. Clinical significance of targeting VEGFR-2

Solid tumor malignancies including breast, lung and prostate carcinomas are considered to be angiogenesis dependent. However, anti-angiogenic therapies have shown varying results partly because each tumor type secretes a distinct panel of angiogenic factors to sustain its

own microvascular network. Additionally, recent evidence has demonstrated that tumors develop resistance to anti-angiogenic therapy by turning on alternate angiogenic pathways when one pathway is therapeutically inhibited. The redundancy of these angiogenic pathways provides a plethora of targets for intervention. It is likely that successful complete inhibition of angiogenesis will rely on the use of combination and/or sequential therapies (Hayes et al. 2007; Roy and Perez 2009).

In this section we will briefly discuss experimental therapies for angiogenesis inhibition in breast cancer. As already discussed the role of angiogenesis in carcinogenesis is complex and mediated by many different factors. The central role of the Notch receptor and its attendant Jagged and Delta cell ligands, and their role in angiogenesis have led to development of Notch signaling inhibition as a therapeutic intervention. The GSIs or small molecule inhibitors of γ-secretase have shown inhibition of tumorigenesis. The main disadvantage with the use of GSIs is their non-specificity. Many physiological processes require Notch signaling, therefore, toxicity profiles may be profound. Additionally, subsequent development of antibodies directed specifically against the Notch receptor or its ligands would offer another therapeutic alternative (Shi and Harris 2006).

Tumor hypoxia occurs as tumors grow subsequently leading to an increase in HIF-1α. Acetylation and deacetylation post-translational modifications are critical to HIF-1α signaling. As such, HDAC inhibitors can be considered as a possible therapeutic intervention in breast cancer treatment (Ellis et al. 2009). The use of HDAC inhibitors in mouse models in combination with VEGF receptor tyrosine kinase inhibitor decreases the expression of angiogenesis related genes such as angiopoietin-2 and its receptor Tie-2, and survivin in EC. HDAC inhibitors are already in clinical use in treating hematologic malignanices such as myelodisplastic syndrome (MDS) and acute myeloid leukemia (AML) (Qian et al. 2004). Regulation of VEGFR-2 expression by Sp proteins has been previously discussed in this paper. It has been suggested that VEGFR-2 can be targeted by drugs that down regulate Sp proteins or block Sp-dependent trans-activation (Higgins et al. 2006a). Activation of VEGFR-2 via binding of Sp3 and Sp4 with ERα to promoter region of VEGFR-2 is enhanced by E2. It could be hypothesized that blocking secretion of E2 would downregulate this effect and hence inhibit angiogenesis (Higgins et al. 2006b; Liang et al. 2006).

Some anti-angiogenic treatment strategies have entered the clinic to date. These agents include a humanized monoclonal antibody directed to VEGF-A (Bevacizumab; Avastin; Genentech Inc, South San Francisco, CA), a chimeric monoclonal antibody directed to the VEGFR-2 (IMC-1C11), several small molecule inhibitors of the VEGFR-2 tyrosine kinase, and a nuclease-stabilized ribozyme (Angiozyme) that specific cleaves both VEGFR-1 and VEGFR-2 mRNA (Weng and Usman 2001). Preclinical models have shown regression of solid tumor growth and angiogenesis with anti-VEGF monoclonal antibodies alone or in combination with chemotherapy (Borgstrom et al. 1999; Kim et al. 1993; Millauer et al. 1994). Clinical benefit with bevacizumab has been reported from clinically trials in metastatic colon, renal cell, and breast cancer (Hurwitz et al. 2004; Miller et al. 2005; Wedam et al. 2006; Yang et al. 2003). Bevacizumab was able to significant decrease (66.7%) phosphorylated VEGFR-2 (Y951) in tumor cells and increase in tumor apoptosis after one cycle of bevacizumab alone (Wedam et al. 2006). However, it has been reported that treatment with bevacizumab may induce alterations in human brain and tumor endothelial cells, leading to escape mechanisms from anti-VEGF-A therapy. Therefore, it is probable that VEGF-C and –D might act as alternative pro-angiogenic factors in these cirscunstances (Grau et al. 2011). Applications of bevacizumab therapy are not confined to cancer. Macular degeneration, a

disorder of the retina that is enhanced by age, is the most common cause of irreversible vision loss in older people. The disease is characterized by abnormal blood vessels grow beneath the retina. Therefore, several anti-angiogenic treatments are being tested in the clinic. Among these, anti-VEGF therapies have potential successful applications. Investigations from intravitreal bevacizumab (Avastin) therapy showed promising 6-month results in patients with neovascular macular degeneration (Weigert et al. 2008).

IMC-1C11, a chimeric monoclonal antibody, binds specifically to the EC-surface extracellular domain of VEGFR-2, blocks VEGFR-VEGFR interaction and prevents VEGFR activation of the intracellular tyrosine kinase pathway (Lu et al. 2000; Zhu et al. 1998). The initial Phase I trial of IMC-1C11 was carried out in patients with metastatic colorectal carcinoma. This has provided evidence of the safety and low toxicity for an antibody blockade of VEGFR-2, as well as insight into dose and schedule requirements (Posey et al. 2003). A fully human anti-VEGFR-2 agent has been produced as a second-generation agent, which is anticipated to be nonimmunogenic for chronic administration as a single agent and in combination with chemotherapy or radiation. Semaxanib (SU5416) was the first specific synthesized potent and selective inhibitor of the VEGFR tyrosine kinase that is presently under evaluation in Phase I clinical studies for the treatment of human cancers (Fong et al. 1999). SU5416 was showed to induce growth inhibition in mouse xenotransplants of human tumors. But in several phase II trials, results were disappointing, albeit providing a good security profile (Fury et al. 2007). Other additional inhibitors of the VEGFR-2 tyrosine kinase are currently being examined in clinical trials, such as PTK 787, ZD6474, and CP547632 which have been selected on the basis of relatively selective inhibition of the VEGFR-2 ATP binding site (Ansiaux et al. 2009; Banerjee et al. 2009; Beebe et al. 2003). SCC-S2, a novel antiapoptotic molecule, has shown to decrease the proliferation and tumorigenicity of MDA-MB-435 human breast cancer cells (Kumar et al. 2004). Treatment of these cells with a cationic liposomal formulation of SCC-S2 antisense oligo correlated with decreased expression of VEGFR-2 in tumor cells as well as human lung microvascular EC and loss of cell viability (Zhang et al. 2006). Targeted therapies with the introduction of adenoviral vector expressing inducible Caspase-9, (iCaspase-9) under transcriptional regulation with EC-specific VEGFR-2 promoter induced apoptosis of proliferating human dermal microvascular EC (HDMECs) (Song et al. 2008).

Numerous studies using novel anti-angiogenic inhibitors have lent additional support to the critical role of antiangiogenesis in colon cancer pathogenesis. Sakurai et al examined the effect of a novel angiogenesis inhibitor, Ki23057, on HUVEC tube formation in colon cancer (Sakurai et al. 2007). Immunoprecipitation revealed the inhibition of tyrosine phosphorylation of VEGFR-2 in HUVECs. However, no inhibitory effect was seen on the proliferation of the colon cancer cell lines: LM-H3, LoVo and LS174T. Conversely, Ki23057 showed a significant inhibitory effect on the growth of xenografted LM-H3 tumors as well as on the spread of cancer cells to the liver. Anti-CD31 antibody staining was significantly reduced in microvessels compared to controls (Sakurai et al. 2007). Other studies demonstrated advantages of combined therapy against EGFR and VEGFR-2. Tonra et al used anti-EGFR (cetuximab) and VEGFR-2 (DC101) antibodies in pancreatic (BxPc-3) and colon cancer (GEO) cell lines. The dose response of the combined treatment revealed synergism for both cell lines (CI=0.1, $P<0.01$ for BxPC-3 and CI=0.1, $P<0.01$ for GEO) (Tonra et al. 2006). Several studies have been conducted using VEGFR-2 inhibitors for treatment highlighting the role of this pathway in the pathogenesis, proliferation and survival of PCa. The use of these agents provides another therapeutic option beyond chemotherapy for those

patients who have become hormone refractory. A preclinical trial conducted by Bischof et al using a combination of irradiation, VEGFR-2 inhibition (SU5416) and chemotherapy (premetrexed) in human endothelial and tumor cells. The PCa cell line, PC3, exhibited a significant increase in antiproliferative effects ($p<0.05$) (Bischof et al. 2004). Conversely, a phase II trial employing VEGFR-2 inhibitor, SU5416, in 36 chemotherapy naive patients did not produce any significant effect on PCa growth or on in vivo PSA kinetics (Stadler et al. 2004). Additional studies are needed to fully elucidate the role of VEGFR-2 in this particular malignancy. Anti-angiogenesis treatment has been used for HCC. Combined inhibition of Raf and VEGFR-2 with the small molecule inhibitor NVP-AAL881 (Novartis, USA) was reported to efficiently disrupt oncogenic signaling and reduce tumor growth and vascularization of HCC. Hence, this strategy could prove valuable for therapy of HCC (Lang et al. 2008). New compounds/technologies are being developed to target VEGFR-2. Mice treated with VEGFR-2-based DNA vaccine showed significant reduction of renal carcinomas (Yan et al. 2009). A series of dual c-Met/VEGFR-2 kinase inhibitors were found to significantly affect growth of human xenografts (Mannion et al. 2009). Medicinal plants could be new sources for anti-VEGFR-2 drugs. Acetyl-11-keto-beta-boswellic acid (AKBA) derived from Bowawellia serrata inhibits prostate tumor growth by blocking VEGFR-2 signaling (Pang et al. 2009). Previous studies in our laboratory have demonstrated that leptin-signal inhibition resulted in decreased growth of mammary tumors derived from mouse and humans. The expression of VEGF-A and VEGFR-2 was increased under leptin signaling in cell culture and decreased by the actions of leptin peptide antagonists (PEG-LPrA2) in vitro and in vivo (Rene Gonzalez et al. 2009). Moreover, PEG-LPrA2 actions on carcinogenic (DMBA)-induced breast tumors in DIO (diet-induced-obesity) mice negatively affected VEGFR-2 and NILCO (Gonzalez-Perez, unpublished). Thus, our data strongly suggest that leptin signaling inhibition could serve as an additional preventative and/or therapeutic modality for breast cancer.

The question of which would be a more suitable target: VEGF or VEGFR-2, for tumor anti-angiogenic therapy is still unsolved. Furthermore, experimental data show inconsistent results from anti-VEGF or anti-VEGFR-2 therapies in animal models as opposed to clinical trials. Reasons for these discordant results are unknown, but most likely rely on the complexity of human cancer and redundant actions of many pro-angiogenic factors. One proposed mechanisms of tumor escape from anti-VEGF-A therapy is the upregulation of FGF2, a pleiotropic angiogenic inducer, which is expressed by several tumor types (Alessi et al. 2009). Preclinical studies investigating anti-angiogenic drug resistance by tumors show that at least 4 factors could be involved in the failure of these therapies: (1) upregulation of bFGF; (2) overexpression of MMP-9; (3) increased levels of SDF-1α (stromal-cell derived-factor 1α) and (4) HIF1α -induced recruitment of bone-marrow-derived CD45+ myeloid cells (Dempke and Heinemann 2009). In addition, results from our investigations suggest that leptin, derived either from adipose tissue or cancer cells could increase tumor angiogenesis by directly affecting EC angiogenic features by promoting proliferation, survival and secretion of VEGF-A and activating NILCO in in malignant cells (Garonna et al. 2011; Gonzalez et al. 2006; Gonzalez-Perez et al. 2010; Guo and Gonzalez-Perez 2011; Rene Gonzalez et al. 2009; Zhou et al. 2011). Probably, the more effective means to abolish VEGF-A/VEGFR-2 autocrine/paracrine effects affecting cancer cells and angiogenic features is to use combined therapies against kinase activity and crosstalk to several signaling partners, including co-receptors, adhesion molecules, developmental signaling, growth factors and cytokines (i.e., NRP-1/-2, integrins, EGF, Notch, leptin, etc). However,

tumor-developed resistance to anti-angiogenic therapies would be a latent issue. Moreover, current data suggest that these combined therapies would probably show differential impact on diverse cancer/stage types.

7. Conclusions

Aberrant VEGFR-2 expression/signaling, found in majority of cancer types, are closely related to worse prognostic. Therefore, this evidence supports the idea that targeting VEGFR-2 overexpression in endothelial/malignant cells could be an effective way to treat several cancers. Many factors have been identified as potential regulators of VEGFR-2 expression and function in diverse biological scenarios. Paracrine effects of VEGF, the NILCO crosstalk and diverse cytokines/growth factors secreted by cancer cells up-regulated VEGFR-2 in EC as well as in cancer. These molecules also orchestrate autocrine/paracrine upregulation of VEGFR-2 that is essential for the survival/proliferation actions of VEGF/VEGFR-2 loop in cancer cells. Specificity of activated tyrosine amino acid residues within cytoplasmatic tail of VEGFR-2 in inducing signaling networks and biological effects in cancer require more investigation. Complexity of signals derived from activated heterodimers of VEGFR-2 and VEGFR-1 or VEGFR-3 in cancer need to be further unraveled. Additional studies are needed to advance testing and developing specific anti-VEGFR-2 therapies. Deeper understanding of VEGFR-2 regulation and signaling crosstalk mechanisms in cancer and EC cells will likely lead to the development of new therapeutic modalities. Translational studies are needed to test these agents for efficacy and toxicity in diverse cancers.

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9. Glossary

4T1 cells: mouse mammary cancer cell line; AhR: aryl hydrocarbon receptor; Akt: protein kinase B; BAECs: bovine aortic endothelial cells; ChiPs: chromatin immunoprecipitation assays; Cyclin D1: kinase and regulator of cell cycle D1; DLL1-3: Delta-like 1-3; DMBA: 7,12 dimethylbenz[A]antracene; EC: endothelial cells; EGFR: epidermal growth factor receptor; Elf-1: Ets domain transcription factor; ER: estrogen receptor; ERK 1/2: extracellular regulated kinase 1 and 2; Ets: E-twenty six family of transcription factor; FGFR: fibroblast growth factor receptor; HAT: histone acetyltransferases; HDAC: histone deacetylases; HIF-1α: hypoxia regulated factor-1 alpha; HUVECs: human umbilical vein endothelial cells; JNK: c-Jun N-terminal kinase or SAPK (stress activated protein kinase); JunD: Transcription factor jun-D; MAPK: mitogen activated protein kinase; MCF-7: ER positive human breast cancer cell line; MDA-MB-231: ER negative human breast cancer cell line; MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase; mTOR: mammalian target of rapamycin; NFκB: eukaryotic nuclear transcription factor kappa B; NILCO: Notch, IL-1 and leptin crosstalk outcome; NRP-1/-2: Neuropilin-1 and-2 receptors; OB-R: leptin receptor;

P38 kinase: extracellular regulated kinase 38; PDGF: platelet-derived growth factor; PIGF: placental growth factor or PGF; PI-3K: phosphoinositide 3-kinase; PKC: protein kinase C; RTK: receptor tyrosine kinase; SDF1α: stromal-cell derived-factor 1α; Sp1-3: Specificity protein 1-3; STAT3: signal transducer and activator of transcription 3; TGF-β: transforming growth factor beta; TNF-α: tumor necrosis factor alpha; VEGF: Vascular endothelial growth factor; VEGFR-1: Vascular endothelial growth factor receptor 1 or Flt-1; VEGFR-2: Vascular endothelial growth factor receptor 2 or KDR or Flk-1; VEGFR-3: Vascular endothelial growth factor receptor 3 or Flt-4.

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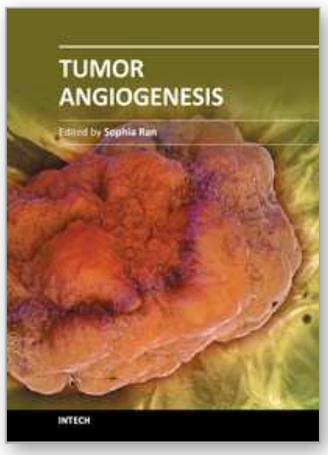

Tumor Angiogenesis

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

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