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Neovascularization in Ewing's sarcoma

Minireview

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Ewing's sarcoma is the second most common bone malignancy in adolescents and young adults after osteosarcoma. Similar to other solid tumors, Ewing's sarcomas require an adequate vascular supply to grow and survive. The development and maintenance of vascular supply is accomplished via three main mechanisms; angiogenesis, vasculogenesis, and tumor cell vasculogenic mimicry. In addition, growth factors, parallel biochemical pathways and the tumor microenvironment are implicated in the initiation and maintenance of neovascularization. This article summarizes the different mechanisms and factors that contribute to neovascularization in Ewing's sarcoma, and discusses the significance of this phenomenon for current treatment options.

Key words: sarcoma, Ewing, angiogenesis, vasculogenesis

Ewing's sarcoma is the second most common bone malignancy in adolescents and young adults after osteosarcoma. Most often it is characterized by a chromosomal translocation between chromosome 11 and 22, generating the EWS-FLI1 fusion gene [1–4]. The protein encoded by this and other related *EWS* translocations acts as an aberrant transcription factor driving the malignant behavior of the transformed cell. Current treatments for Ewing's sarcoma are directed toward both the primary tumor and presumed microscopic metastasis. The intensive multimodal treatment with combination chemotherapy, surgery and radiation therapy has increased the 5-year event-free survival from less than 10% to over 70% [5].

Similar to other solid tumors, Ewing's sarcoma requires a viable vascular supply for tumor cells to grow beyond the limits of oxygen and nutrient diffusion into tissues [6], utilizing three main strategies to develop and maintain its supply: angiogenesis, vasculogenesis, and tumor cell vasculogenic mimicry [3, 5–9]. These processes occur as a result of stimulation of the cells in the immediate tumor area [3, 5–14]. Additionally, multiple growth factors including vascular endothelial growth factor (VEGF) affect tumor development [12–14]. Understanding the biology and mechanisms involved in Ewing's sarcoma tumor growth and progression may lead to the identification and consideration of novel therapeutic targets and approaches [14]. Combining VEGF receptor 2 (VEGFR-2)-targeted agents with chemotherapy may improve the efficacy of treatments. Such approaches may be effective in Ewing's sarcoma because the importance of neovascularization during tumor growth has been reported previously [3, 5–14].

Neovascularization is not yet standardized and references in literature are sparse. This article is an attempt to put essential information in one place, creating a comprehensive review to explore the current status of neovascularization research in Ewing's sarcoma, to highlight recent evidence that strengthens the hypothesis for this unusual ability of tumor cells, and to discuss the significance of this phenomenon for current treatment approaches.

Neovascularization – angiogenesis, vasculogenesis and vasculogenic mimicry

Neoplastic blood vessel formation (neovascularization) provides a local network for tumor growth and a systemic network for tumor metastasis. Without the formation of supporting vasculature, tumor cells would be unable to

obtain the nutrients and oxygen necessary for proliferation, and would not be able to mediate metastatic spread. The process of neovascularization is comprised of angiogenesis, vasculogenesis and vasculogenic mimicry [7–9]. Angiogenesis refers to the proliferation of fully differentiated endothelial cells and extension of blood vessels from preexisting vascular structures. Vasculogenesis refers to the *de novo* formation of vessel networks through the recruitment of bone marrow-derived precursor cells. Vasculogenic mimicry refers to direct lining of periodic acid-Schiff (PAS) positive, matrix-associated vascular channels by Ewing's sarcoma cells [9].

Angiogenesis. Angiogenesis is a rate-limiting factor for local and distant tumor growth [10]. Tumors of 1 to 2 mm^3 obtain oxygen and nutrients by passive diffusion from neighboring blood vessels. To grow beyond this size, tumors must recruit blood vessels to nourish and oxygenate their cells, meaning that angiogenesis must occur. Angiogenesis in Ewing's sarcoma is regulated by angiogenic and non-angiogenic factors of the microenvironment. The angiogenic factors include the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF); the non-angiogenic factors include hypoxia, necrosis and metabolic rate of the tumor [3, 11, 12]. In general, angiogenesis is regulated by a delicately controlled balance between the angiogenic and non-angiogenic factors. Dysfunction of this balance by environmental stressors or genetic changes such as hypoxia, acidosis, oncogene activation and loss of tumor suppressor genes result in increased angiogenesis [9].

Vasculogenesis. Vasculogenesis was originally thought to occur only in embryonic development. However, a vasculogenesis-like mechanism that recruits non-local cells to the area of new vessel formation has been shown to occur in postnatal life in both physiological and pathological conditions when new vascular networks are formed or expanded, such as in wound healing, revascularization of ischemic tissue, or tumor growth [13, 14].

The contribution of vasculogenesis to angiogenesis varies considerably depending on the tumor's histology. In Ewing's sarcoma, angiogenesis and vasculogenesis contribute to the expansion of the tumor vascular network that supports the growth of the tumor. Vasculogenesis can also be defined as the recruitment of bone marrow-derived precursor cells into the tumor area with subsequent differentiation into endothelial cells. In Ewing's sarcoma, 10% of the neo-vessels contain bone marrow-derived as opposed to locally derived cells. Bone marrow cells contribute to the endothelial and peri-endothelial components that form the Ewing's sarcoma vasculature, as has also been observed in other tumor models [15, 16]. Inhibiting this process suppresses the ability of Ewing's sarcoma cells to grow. Additionally, inhibition of bone marrow cell chemotaxis into the tumor area results in the formation of significantly smaller and less vascular tumors. Furthermore, stimulating the migration of bone marrow cells into VEGF-inhibited Ewing's sarcoma tumors *in vivo* enhances the neovascularization and rescues tumor growth [17].

Vasculogenic mimicry. Vasculogenic mimicry was initially described in aggressive, uveal forms of melanoma as a process of "dedifferentiation" of tumor cells into an "endothelial-like" phenotype [18]. In these tumors, vasculogenic mimicry was identified as periodic acid-Schiff (PAS) positive, matrix-associated channels devoid of bona fide endothelium. Vasculogenic mimicry channels appeared to contain erythrocytes and were therefore hypothesized to connect with the existing vasculature. Notably, patients displaying the presence of PAS-positive networks in their tumors had an increased mortality compared to those patients which did not [18]. Following this observation, it was suggested that some tumors were capable of forming their own vascular channels which could carry blood, oxygen and nutrients in collaboration with blood vessels formed by conventional routes of tumor angiogenesis (i.e. sprouting). The clinical implications for vasculogenic mimicry included that vasculogenic mimicry-forming tumors were more virulent than their non-vasculogenic mimicry counterparts and vasculogenic mimicry-lined channels might not respond predictably to conventional anti-angiogenic therapies [19].

Ewing's sarcoma cells appear capable of direct lining vascular channels (vasculogenic mimicry) [5–9]. Vasculogenic mimicry in Ewing's sarcoma is not driven by VEGF, neither are the other factors affecting neovascularization described above. Using immunohistochemistry, pools of blood ("blood lakes") are found in 92% of human Ewing's sarcoma tumor samples; their cells lining are CD31 and CD34 negative, but CD99 positive [5]. Their appearance in electron microscopy is characteristic of tumor and not endothelial cell origin. The lining tumor cells also express TFPI-1/2, VE-cadherin, and EphA2, proteins that are important for vasculogenic mimicry [5]. Additionally, Ewing's sarcoma cell lines can form vascular structures to a variable extent when grown in a collagen matrix, without being enhanced or inhibited by VEGF factor or blocking antibodies. The cell lines with a greater propensity for vascular structures formation present greater expression of genes identified in other malignancies as markers of vasculogenic mimicry such as integrin α3, VE-cadherin, TFPI-1, EphA2, laminin5γ2, Tie-1, neuropilin, and endoglin. Perfusion and intravital microscopy in Ewing xenografts also showed that these tumor cell-lined blood lakes appear to be functional and in continuity with the systemic circulation [5].

Biochemical pathways of neovascularization in Ewing's sarcoma

Growth factors, parallel biochemical pathways and the tumor microenvironment are implicated in the initiation and maintenance of neovascularization in Ewing's sarcoma (Figure 1).

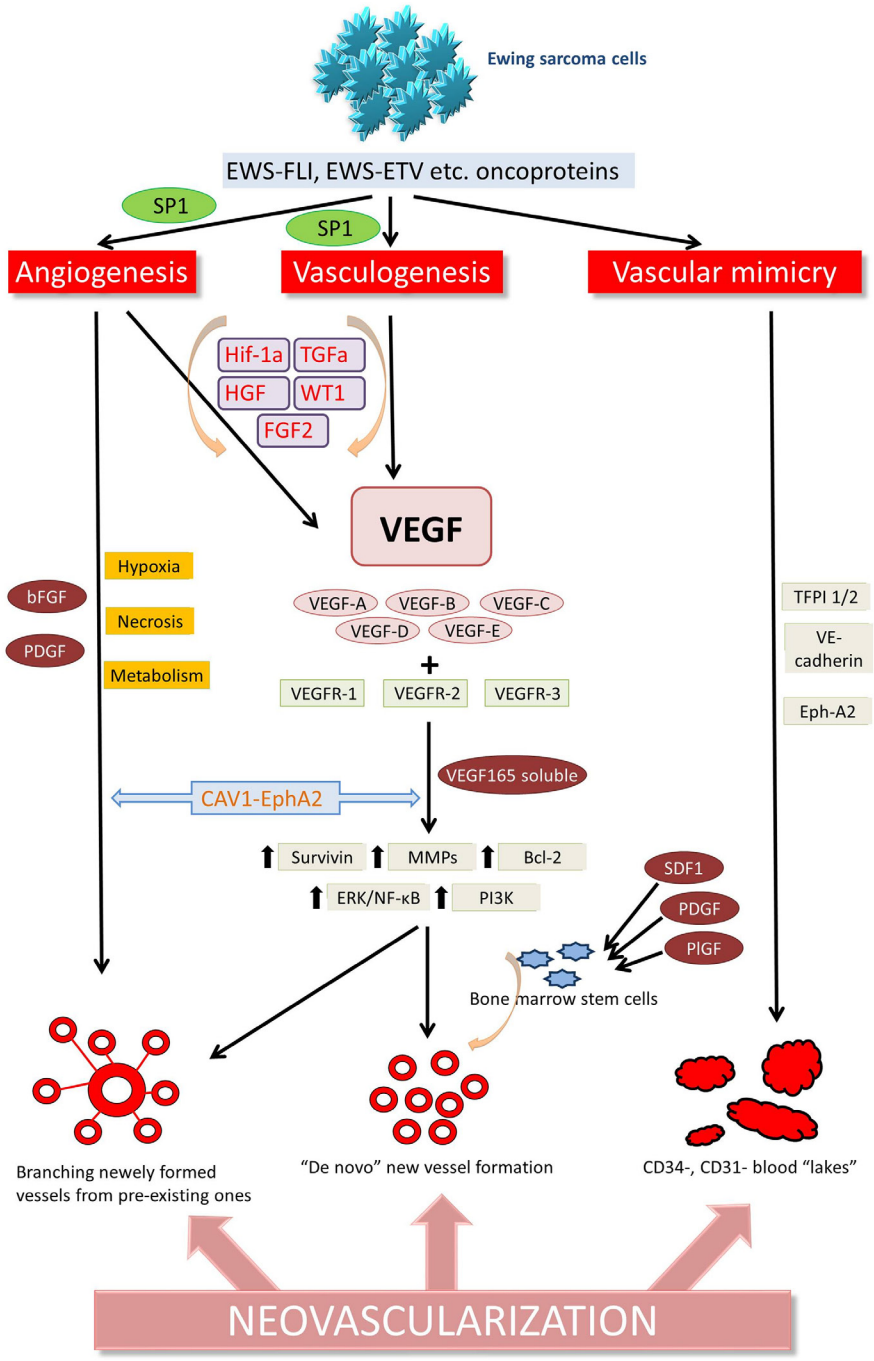

Figure 1. Schematic illustration of the events that lead to neovascularization in Ewing's sarcoma. The chimeric proteins (EWS-FLI, EWS-ETV) produced by neoplastic cells seem to be the triggering fact for the three main neovascularization pathways through the transcription factor SP1. Angiogenesis results in branching newly formed vessels from pre-existing vessels either directly controlled by bFGF and PDGF, or through the VEGF pathway. Hypoxia, necrosis and metabolic stress also mediate angiogenesis, while EphA2-CAV1 axis participates in the promotion of endothelial cell migration and angiogenesis. Vasculogenesis refers to the cascade of events that lead to "*de novo*" vessel formation mainly through the enhancement of VEGF. There are a number of different VEGF molecules (VEGFA through VEGFE) that bind to VEGF receptors (VEGFR1-3) through the upregulation induced by HIF-1, TGF-α, FGF-2, HGF and WT1. Metalloproteinases (MMPs) are upregulated; they act on the vascular network by breaking down the extracellular matrix (ECM) and allow for tumor cell invasion, as well as the migration of the precursor cells that give rise to vascular structures (pericytes and endothelial cells). VEGF signaling also induces the expression of the anti-apoptotic factors Bcl-2 and survivin, as well as the ERK/NF-kB and PI3K pathways. Cell migration is also assisted by the SDF1, PlGF and PDGF factors. In the process of vasculogenic mimicry, the lining tumor cells of the formed vascular channels express TFPI-1/2, VE-cadherin, and EphA2 proteins that are also important for this process.

Vascular endothelial growth factor (VEGF). VEGF is the best characterized pro-angiogenic factor. It is considered the most important factor involved in neovascularization of tumors; it mediates the mobilization and differentiation of endothelial progenitor cells (EPCs), and stimulates the formation of functional tumor vessels [7]. Inhibition of VEGF induces tumor regression largely by impacting the tumor vessels.

There are a number of different VEGF molecules (VEGFA through VEGFE) that bind to VEGF receptors (VEGFR1–3). VEGFA binds to VEGFR2 and initiates a number of divergent signaling pathways. Among the proteins that are upregulated after VEGF activation are the matrix metalloproteinases (MMPs) and plasmin proteases, which act on the vascular network by breaking down the extracellular matrix (ECM) and allow for tumor cell invasion, as well as the migration of the precursor cells that give rise to vascular structures (pericytes and endothelial cells). VEGF signaling also induces the expression of the anti-apoptotic factors Bcl-2 and survivin, as well as the ERK/NF-kB and PI3K pathways. These effectors promote tumor cell proliferation and survival.

Several lines of evidence have highlighted the importance of the VEGF in the neovascularization process of Ewing's sarcoma [4–8]. Elevated VEGF levels have been detected in Ewing's sarcoma cell lines, and in the serum of Ewing's sarcoma patients compared to controls [8, 9, 20]. VEGF expression has been correlated with increased Ewing's tumor microvessel density (MVD) as well as poor patient outcome [21]. Interestingly, *EWS-ETS* fusion oncoproteins drive the expression of VEGF in a transcription factor Sp1 dependent manner and may contribute to the increased VEGF levels observed in these patients [9].

Ewing's sarcoma cells produce one or more VEGF isoforms. These isoforms bind to their receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), and stimulate both proliferation and survival of the associated tumor endothelium. VEGFR-2 is considered to be more dominant [22]; treatment with an antibody directed against VEGFR-2 seems to result in the inhibition of tumor growth as well as decreasing tumor vessel density [22]. The recruitment of bone marrow-derived cells into the tumor area is also severely decreased in the tumors treated with anti-VEGFR-2 antibody.

Experimental studies showed overexpression of the soluble VEGF165 isoform and chemotaxis of bone marrow-derived cells to VEGF165-containing Matrigel plugs *in vivo* Ewing's sarcoma cells. These bone marrow-derived cells contribute to the expansion of the growing tumor vascular network [14, 23]. It is also proven that VEGF165 is critical for the growth of Ewing's sarcoma *in vivo* [14]. Specifically inhibiting the VEGF165 isoform decreases bone marrow-derived cell infiltration, tumor neovascularization and thus, tumor growth. Ewing's sarcoma cells deficient in VEGF165-isoform expression form smaller tumors with reduced MVD [8]; while VEGF165-isoform re-expression in these cells restores MVD [5]. VEGF165 also appears to be important in the recruitment of bone marrow-derived cells into the vascular network of Ewing's sarcoma (vasculogenesis). These bone marrow-derived cells can differentiate into both tumor-associated ECs and pericytes [24, 25]. Only CD34-positive bone marrow-derived cells contribute to this process [25]. VEGF165-deficient Ewing's sarcoma cells recruited significantly fewer bone marrow-derived cells into xenograft tumors compared with VEGF165-expressing cells [8, 23]. Forced expression of the VEGF189 isoform into VEGF165-deficient cells does not enhance bone marrow cell recruitment, indicating a specific role of VEGF165 in driving this process. Inhibiting VEGFR-2 attenuates the recruitment of bone marrow cells into growing tumors [13]. Although VEGF and specifically VEGF165 stimulate vasculogenesis, other cytokines such as stromal cell-derived factor-1 (SDF-1) may also promote bone marrow-derived cell recruitment into tumors and provide an alternate pathway to support vasculogenesis [26].

VEGF has been shown to be a potent chemotactic factor for bone marrow cells and to induce colony formation by endothelial progenitor cells. Ewing's sarcoma cells express high levels of VEGF and there seems to be a switch from the ECM-bound 189 isoform to the more soluble VEGF165 isoform [14]. Probably, this switch favors an increase in secreted VEGF, which in turn results in the recruitment of bone marrow-derived cells to the tumor area. At the same time, interference with VEGFR-2 appears to block the chemotaxis of bone marrow-derived cells in addition to inhibiting tumor growth and tumor vessel development. This clearly represents a link between the recruitment of bone marrow-derived cells, tumor vessel development and tumor growth, strengthening the hypothesis that in addition to angiogenesis, a postnatal vasculogenesis-like mechanism plays an important role in the growth of Ewing's sarcoma [13].

Interesting data support the hypothesis that the VEGF pathway may be the target of the EWS/ETS fusion oncoproteins of Ewing's sarcoma. In a study [27], plasmids that expressed EWS/Friend leukemia virus integration (EWS/FLI) or EWS/ETS translocation variant (EWS/ETV) were transfected into RK13 cells and the levels of VEGF expression were recorded. Transfection of either EWS/FLI or EWS/ETV resulted in increased activation of the VEGF promoter. Follow-up experiments demonstrated that this effect does not appear to be caused by direct DNA binding by either EWS/FLI or EWS/ETV and does not require the presence of the hypoxia response element of the VEGF promoter [27].

VEGF has been found to be upregulated by a number of other factors that are released in response to the rapid proliferation of tumor cells. These factors include hypoxia-inducible factor-1 (HIF-1), transforming growth factor-α (TGF-α), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), and transcription factor Wilms tumor protein 1 (WT1) [28, 29]. The activation of growth factor receptors such as the epidermal growth factor receptor (EGFR) and integrin lead to Src activation, which in turn initiates Ras/MAPK signaling and activation of the transcription

factor STAT3 allowing for cell cycle progression and proliferation [30, 31]. STAT3 signaling is necessary for VEGF production; therefore, STAT3 activation results in a positive feed-back loop that further increases the production of FGF and VEGF, leading to an increased induction of vascular permeability and neovascularization [32].

The insulin-like growth factor (IGF) pathway may positively regulate VEGF expression [9]. Conversely, Ewing's sarcoma cells may also stimulate angiogenesis through down-regulation of the endogenous antiangiogenic protein thrombospondin (TSP). TSP represents a family of secreted glycoproteins that have been implicated in mediating cell-to-cell and cell-to-matrix signaling pathways. Two members of this family, TSP1 and TSP2, negatively regulate angiogenesis by inhibiting EC migration, promoting apoptosis, and inhibiting accessibility of endothelial cell surface receptors to mobilized growth factors. The biological functions of TSP1 and TSP2 are largely overlapping but their tissue expression patterns differ, particularly during normal development. TSP1 and TSP2 have been shown to exert their antiangiogenic effects both by decreasing vascular endothelial growth and by inhibiting activation of growth factors in the ECM.

Unlike VEGFR-1 and VEGFR-2 that can bind any of the VEGF isoforms, the neuropilin family of receptors demonstrate VEGF isoform-specific binding [33]. Neuropilin-2 binds VEGF145 and VEGF165, but not VEGF121. Neuropilin receptors are thought to modulate VEGF signaling through VEGFR-1 and VEGFR-2 [12].

Migration of pericytes and vascular mural cells that fortify endothelial tubes in developing tissues or tumors selectively protect vessels against apoptosis when VEGF levels decline. PDGF-b secreted by the endothelium and signaling through its receptor (PDGFR-b) have been identified as key mediators of this process [34, 35]. Moreover, it has been shown that bone microvascular endothelial cells themselves express PDGFR-b *in vitro*. In this setting, ligand binding induces rapid phosphorylation and subsequent activation of protein kinase B (Akt) and extracellular signal-related kinase 1 (ERK1/ERK2) that increases EC division and survival. Functional PDGFR-b expression has also been documented in Ewing's sarcoma cell lines and tumor specimens, suggesting an important role for the PDGF signaling pathway for vascular stability and tumor proliferation in Ewing's sarcoma [9]. However, the PDGF-B or PDGF-D ligands do not appear to be derived from the tumor cells; these ligands may in fact derive from the vascular endothelium. Finally, in experimental Ewing's sarcoma tumors that recur after exposure to VEGF blockade, vessels are characterized by significant increase in diameter and proliferation of vascular mural cells with increased expression of factors that promote endothelial integrity (angiopoietin-1, Ang-1) and PDGF-B [13].

Tumor VEGF levels have also been shown to correlate with prognosis more than the tumor's microvessel density. Results like these may reflect methodological difficulties in determining accurate microvessel counts. Alternatively, these results raise the possibility that VEGF impacts prognosis through a mechanism other than driving tumor vessel growth. For example, VEGF may facilitate tumor metastasis by increasing vascular permeability. Increased vascular permeability may also stimulate tumor growth by increasing nutrient availability. VEGF levels could serve as a surrogate marker of other cytokines involved in tumor growth or VEGF could play a role in directly stimulating tumor growth [36].

Hypoxia-inducible factor-1 (HIF-1). HIF-1 is a key transcription factor that regulates the expression of genes responsible for the survival and adaptation of cells as they move from normoxia (~21% pO_2) to hypoxia (~1% pO_2). HIF-1a is stabilized in the extreme hypoxic conditions within a tumor, it binds to the promoter region of VEGF and mediates its upregulation [20].

HIF-1a and HIF-2a are expressed in Ewing's sarcoma [37]. HIF-1 is made up of an oxygen related α subunit (HIF-1α) and a constitutive β subunit (HIF-1β) [38]. The stability of HIF-1α is regulated by prolyl-hydroxylase domain proteins (PHDs), while its transcription is regulated by factor inhibiting HIF (FIH). In normoxic and mildly hypoxic conditions, PHDs hydroxylate HIF-1α, resulting in its association with von Hipper-Lindau (pVHL) ubiquitin E3 ligase complex allowing for rapid proteasomal degradation of HIF-1α [39–41]. In the extreme hypoxic conditions within a tumor, HIF-1α is stabilized and binds to the promoter region of VEGF where it mediates its upregulation. This signaling cascade can take place in both tumor cells and the non-malignant tumor associated endothelial cells, which are found in the hypoxic center of tumors [9].

HIF-1a is predominantly localized in the nucleus of Ewing's sarcoma cells, whereas HIF-2a expression was mainly cytoplasmic. Ewing's sarcoma cells show strong induction of HIF-2a protein and a moderate increase in HIF-1a in low glucose conditions [37]. However, with the clinical data available, no correlation was observed between HIF expression and clinical parameters including tumor volume, metastasis and survival [3, 37].

Wilms tumor protein 1 (WT-1). WT1 upregulates VEGF transcription, resulting in increased angiogenic activity. In Ewing's sarcoma cell lines, there has been found a correlation between endogenous WT1 expression and VEGF expression. WT1 expression causes increased VEGF transcription, which in turn results in increased expression of bioactive VEGF protein and a proangiogenic phenotype. Upregulation of WT1 in the low WT1-expressing cell lines led to a corresponding increase in VEGF expression (both mRNA and protein), whereas silencing of WT1 in the high WT1-expressing cell lines led to a corresponding decrease in VEGF expression. Results from promoter-reporter, chromatin immunoprecipitation, and site-directed mutagenesis assays demonstrate that VEGF is a direct target of the transcriptional regulatory activity of WT1 and identifies a specific sequence within the VEGF promoter that is essential for the effect of WT1 on promoter activity [42].

Endogenous WT1 expression is essential for an optimal response to hypoxia. After demonstrating that WT1 expression is induced by hypoxia in Ewing's sarcoma cell lines, it has been shown that hypoxia-mediated upregulation of VEGF is attenuated by a WT1-specific shRNA that blocks induction of WT1 by hypoxia. Clearly, VEGF is upregulated by hypoxia in WT1-null cells by a direct effect of HIF-1 on the activity of the VEGF promoter. This data obviously places WT1 as a key mediator of the maximal VEGF induction in response to hypoxia [42].

Ewing's sarcoma/Friend leukemia integration 1 transcription factor (EWS/FLI1). Even Ewing's sarcoma's main pathological gene seems to play a direct role in tumor's angiogenesis. Ewing's sarcoma cell lines showed direct reduction of the production and antagonistic effect of EWS/FLI1 to TSP1 and TSP2 *in vivo* and *in vitro*. In a study [43], NIH3T3 cells transfected with the Ewing's sarcoma specific fusion oncogenes EWS/FLI1, EWS/ERG, and EWS/ETV1 had reduced expression of TSP2. This effect may be mediated by binding of the fusion oncoprotein to the TSP promoter. In Ewing's sarcoma cell lines, TSP1 expression is repressed. With short hairpin RNA treatment intended to block EWS/FLI1 expression, TSP1 expression is restored in these cells [43]. EWS/FLI1 has significant effects towards neovascularization: (1) EWS/ETS fusions down-regulate TSP2 expression in NIH3T3 cells; (2) EWS/FLI1 inhibits TSP2 transcription in a DNA binding-dependent manner; (3) EWS/FLI1 binds to the endogenous TSP2 promoter in NIH3T3 cells; and (4) EWS/FLI1 down-regulates TSP1 in human Ewing's sarcoma cell lines [43].

Stromal cell-derived factor-1α (SDF-1a). SDF-1α stimulates the migration of bone marrow cells similar to VEGF. The effects of SDF-1α on tumor neovascularization include augmented chemotaxis of bone marrow cells, retainment of bone marrow-derived pericytes in close association with the vessel endothelial lining, enhanced overall pericyte coverage of tumor neovessels, and remodeling of vascular endothelium into larger functional structures. These processes promote the growth of Ewing's sarcoma tumors, even if VEGF165 is markedly reduced. This data support the hypothesis that bone marrow-derived cells play a critical role in the expansion of Ewing's sarcoma neovascularization, and that vasculogenesis may be the mechanism by which tumors can circumvent the effects of anti-angiogenic VEGF-targeted therapy [26].

Placenta derived growth factor (P1GF). PlGF may induce angiogenesis by increasing endothelial cell survival and enhancing their response to VEGF, as well as increasing vessel density, size, and permeability [44–47]. Additionally, PlGF may also modulate VEGF activity by forming functional heterodimers with VEGF [47, 48]. Following activation of Flt-1 by PlGF, Flt-1 may amplify VEGF signaling by intermolecular transphosphorylation of Flk-1/KDR [47]. Together, VEGF and PlGF may recruit bone marrow-derived endothelial cells, a process that has been shown to potentiate the neovascularization of Ewing's sarcoma tumors [12, 49, 50].

Caveolin1-Ephrin A2 (CAV1-EphA2) signaling. EphA2 promotes angiogenesis. Lack of CAV1 results in a significant reduction in MVD *in vivo*. *In vitro*, this phenomenon correlates with EphA2 receptor inactivation, lack of AKT response and bFGF downregulation. Furthermore, interaction between EphA2 and CAV1 is necessary for the right localization and signaling of the receptor to produce bFGF through AKT and to promote migration of endothelial cells. Finally, introduction of a dominant-negative form of EphA2 into Ewing's sarcoma cells mostly reproduced the effects occurred by CAV1 silencing, strongly suggesting that the EphA2-CAV1 axis participates in the promotion of endothelial cell migration and angiogenesis [6].

Bone marrow cells and blood vessel pericytes. Bone marrow-driven vasculogenesis is essential for Ewing's sarcoma growth [51]. The upregulation of pro-angiogenic factors such as VEGF, FGF, TGF-α, HGF, PDGF, angiopoietin 1 (Ang1), and ephrin-B2 combined with the downregulation of anti-angiogenic proteins such as TSP1, TGF-β, troponin I, pigment epithelial-derived factor (PEDF) and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) allow for rapid neovascularization [9, 28, 52–55]. Ewing's sarcoma recruits bone marrow-derived progenitor cells to participate in tumor angiogenesis. Endothelial progenitor cells from the bone marrow migrate and incorporate into areas of physiological and pathological neovascularization to form new blood vessels. CD341 stem cells from bone marrow migrate to the tumor vascular bed, differentiate into endothelial progenitor cells and participate in neovascularization.

Blood vessel pericytes also play a critical role in tumor vessel formation. The bone marrow is a reservoir of pericyte progenitors. However, whether bone marrow cells migrated into the tumor and then differentiated into pericytes or had already differentiated before arriving in the tumor is unclear. The tumor-associated pericytes express VEGFR-2 and DC101 treatment not only inhibits bone marrow-derived cell chemotaxis and tumor vessel formation, but also pericyte formation on the tumor vessels [13, 56].

Angiogenesis and treatment options in Ewing's sarcoma

Understanding the biological processes required for Ewing's sarcoma neovascularization seems to be of great significance, as chemical agents against the tumor's neovascularization present themselves as the most hopeful solution that will describe the pathway towards new therapeutical methods. Moreover, neovascularization is essential for sustained tumor growth and provides systemic network that stimulates metastasis [9]. The development of a functional vascular system is a hallmark of solid tumors [7]. Ewing's sarcoma, like other solid tumors, are reliant on a functional vascular network for the delivery of nutrients and oxygen and for the removal of waste [8]. Therefore, one possible way to inhibit tumor growth and promote tumor regression is by preventing the tumor from developing a vascular supply,

essentially starving the tumor of nutrients and oxygen. Defining the molecular mechanisms that direct blood vessel formation in Ewing's sarcoma is key to identifying therapeutic targets that might prevent vascular development [51].

Bevacizumab, a monoclonal antibody that inhibits VEGF-A signaling, has been the most tested agent. It is already FDA approved for use in patients with a variety of cancer types, including colorectal cancer and glioblastoma multiforme. In a recent phase I clinical trial conducted by the Children's Oncology Group (COG) examining the effects of the use of bevacizumab against ES, the antibody was administered every two weeks in 28-day courses to children with solid refractory tumors, including 5 patients with Ewing's sarcoma. Two of the 5 had stable disease for greater than 4 months, and one had stable disease for two months, while no dose-limiting toxicities occurred [56]. In addition to bevacizumab, REGN421 is currently being evaluated as a therapeutic method against solid tumors, including ES. REGN421 is a neutralizing antibody of DLL4.

However, it is almost certain that anti-vascular therapies are not going to be efficient as single agents against bulk disease. Therefore, a lot of clinical trials are yet to be planned and conducted, using therapeutic protocols including anti-angiogenesis factors, in order to examine the possible synergistic effects of each different drug.

Conclusions

Ewing's sarcoma utilizes three main strategies to develop neovascularization for tumor cells to grow: angiogenesis, vasculogenesis, and vasculogenic mimicry. Several biochemical pathways playing a critical role in the process of neovascularization have been discovered, such as VEGF, HIF-1, WT-1, SDF 1-a, P1GF, EWS/FLI1 and CAV1-EphA2 signaling.

Ewing's sarcoma neovascularization is a research field with many landscapes awaiting to be discovered and explored. Maybe even more biochemical pathways are yet to be discovered. The deep knowledge of all the biochemical processes resulting in neovascularization promises new therapeutic agents and strategies that may change the existent protocols and provide better outcomes for children or adults with this tumor.

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