MODULATION OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS BY VASCULAR ENDOTHELIAL GROWTH INHIBITOR (VEGI)

by

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MODULATION OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS BY VASUCLAR ENDOTHELIAL GROWTH INHIBITOR (VEGI)

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Bone marrow (BM)-derived endothelial progenitor cells (EPCs) have a critical role in tumor vasculogenesis, mobilizing to tumors and supporting *de novo* formation of blood vessels essential for tumor growth and metastasis. Vascular endothelial growth inhibitor (VEGI; TL1A) is a member of the tumor necrosis superfamily (TNFSF15) and is produced predominantly by endothelial cells (ECs). VEGI has been shown to act in an autocrine manner by specifically targeting ECs to inhibit their proliferation and induce apoptosis, resulting in elimination of ECs in established tumor vasculature and inhibition of angiogenesis. However, it remains unclear whether VEGI exerts its function solely on fully differentiated ECs or if it is able to modulate BM-derived EPCs as well. Here, the effect of recombinant VEGI on BM-derived EPC function is evaluated in an effort to establish the potential therapeutic value of VEGI. We found that VEGI inhibits the differentiation of EPCs from murine BM under EC stimulating culture conditions. Consistently, VEGI treatment decreases the capability of the cells to adhere, migrate and form capillary-like structures necessary for vascular formation. Additionally, differentiated BM-derived EPCs in cultures underwent apoptosis in response to VEGI treatment. To investigate the impact of VEGI on BM-derived EPC-supported tumor vasculogenesis, mice bearing Lewis lung carcinoma (LLC) tumors were treated with intraperitoneal injection of recombinant VEGI. VEGI treatment significantly decreased the population of BM-derived EPCs found in the tumors while increasing their population in the bone marrow. Furthermore, an

overall increase in apoptosis of BM-derived cells at the tumor site was observed after VEGI treatment. Our results indicate VEGI prevents incorporation of BM-derived EPCs into LLC tumors, resulting in the inhibition of EPC-supported tumor vasculogenesis and tumor growth. Together, these findings suggest that VEGI takes part in the modulation of tumor vasculogenesis by inhibiting BM-derived EPC differentiation and mobilization as well as inducing apoptosis. These studies yield important insights into the function of VEGI in postnatal vasculogenesis, helping to facilitate the development of therapeutic uses of VEGI in cancer.

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PREFACE

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1.0 INTRODUCTION

Anti-angiogenesis is an important avenue and approach to cancer therapy. The development of anti-angiogenic drugs has focused on endogenous angiogenesis inhibitors that innately modulate endothelial cell (EC) growth. Increasing evidence points to tumor vasculogenesis as a principle mechanism for the support and growth of tumors. Therefore, the discovery of endogenous inhibitors which are able to not only suppress tumor angiogenesis, but tumor vasculogenesis as well, is of high therapeutic value.

1.1 NEOVASCULARIZATION

1.1.1 Angiogenesis

The vascular endothelium is an essential component of the cardiovascular system and provides a dynamic barrier between circulating blood and surrounding tissues in the body. The endothelium monolayer plays a vital role in regulating vascular homeostasis, coagulation and inflammation, and as such, restoration of the monolayer is essential following damage or cell death [1, 2]. Under normal conditions, the endothelium undergoes a low turnover rate, retaining ECs in a quiescent state in order to maintain vascular homeostasis [3]. However, the number of

circulating ECs found in a healthy subject can be stimulated to proliferate during postnatal neovascularization, such that occurs following acute stress or injury of the vascular endothelium. It has been commonly believed that postnatal neovascularization, the vascular growth and remodeling in newborns and adults, is mainly attributed to angiogenesis, a process of capillary sprouting from pre-existing capillary vessels (Figure 1) [4]. The process of angiogenesis can occur during normal physiological conditions such as embryonic development and the female reproductive cycle. Angiogenesis can also be engaged under pathological conditions such as tumor growth, macular degeneration, ischemia and rheumatoid arthritis [1, 5].

The angiogenic response is a complex process that has been well studied and it involves extensive cellular adhesive interactions between ECs and surrounding extracellular matrix components. The initial step in angiogenesis begins with vasodilation and increased permeability of pre-existing capillaries. Next, pericytes surrounding the capillaries detach and the EC basement membrane is degraded by proteases. This allows for the migration of ECs into the interstitial space for sprouting. During migration, ECs interact with the extracellular matrix via interactions with cell surface integrin such as $\alpha v\beta 3$ and $\alpha v\beta 5$. ECs then undergo proliferation and elongation at the migrating tip and adhere to begin lumen formation. The newly formed vessel then generates a new basement membrane by recruiting pericytes to stabilize the vessel wall, leading to formation of intact microvessels and blood flow [6, 7].

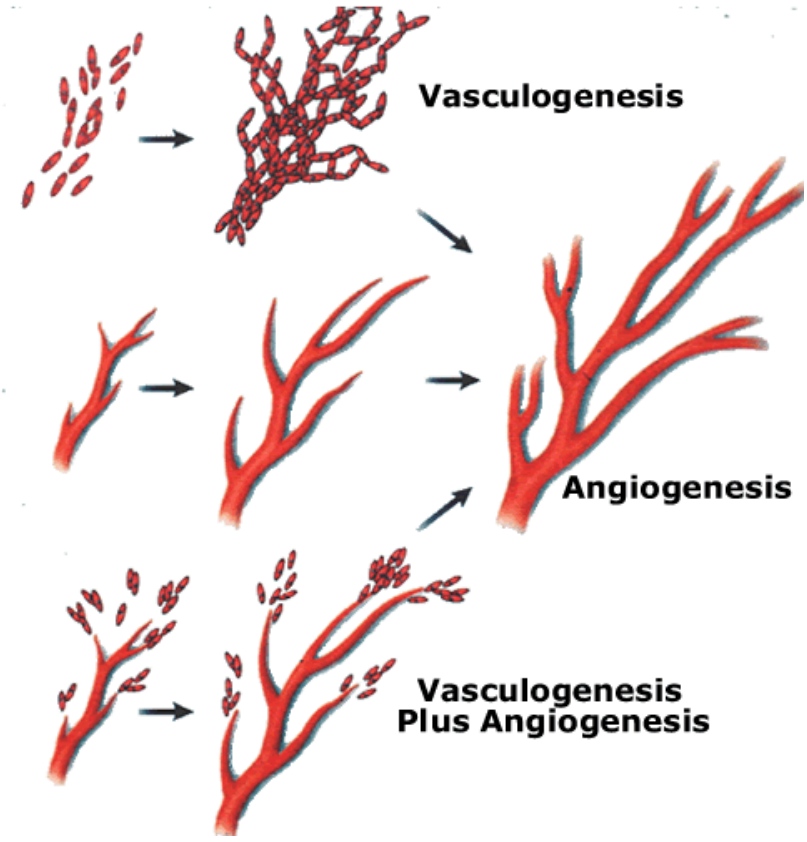


Figure 1. Schematic representation of angiogenesis and vasculogenesis.
Vasculogenesis is the aggregation of EPCs, which migrate from distant sites, to form blood vessels. Angiogenesis is the formation of new vessels via sprouting from pre-existing vessels. Both processes can occur simultaneously during formation of a new vasculature. Figure taken from [8].

1.1.2 Vasculogenesis

The proliferation, migration, and stabilization of blood vessels formed during neovascularization is necessary for repairing injured tissues or meeting increased metabolic demands [9]. Until a decade ago, it was thought that postnatal neovascularization occurred primarily via angiogenesis. However, the capacity of mature ECs to create new vessels is limited since they are terminally differentiated cells with a low proliferative potential. The discovery of progenitor cells of

endothelial lineage in the circulation of adults that can differentiate into mature ECs has suggested an alternate paradigm for neovascularization [10, 11]. A growing body of data demonstrates that these endothelial progenitor cells (EPCs) contribute to postnatal neovascularization by participating in vasculogenesis, the *de novo* formation of new blood vessels, a process originally thought to occur only during embryogenesis (Figure 1) [1, 12].

During embryonic development, the process of vasculogenesis involves the growth and fusion of multiple blood islands that eventually result in the formation of a capillary network within a previously avascular yolk sac [13]. This capillary network differentiates into an arteriovenous vascular system after blood circulation begins [14]. The embryonic blood islands are composed of cells destined to become either the blood cells that circulate in the vascular system or the ECs responsible for the construction of blood vessels. The center of the embryonic blood island consists of hematopoietic stem cells (HSCs) which are destined to generate hematopoietic blood cells. Situated on the periphery of the blood islands are EPCs [15]. HSCs and EPCs not only have a spacial association, but they are also considered to be derived from a common precursor, known as a hemangioblast [12, 16].

In recent years, accumulating evidence points to EPCs playing a critical role in vasculogenesis, which is essential for normal physiological neovascularization during tissue growth, wound healing, and organ regeneration [17, 18]. Postnatal vasculogenesis also contributes to endogenous neovascularization of developing tumors, severe hindlimb ischemia, and myocardial ischemia [18]. In the adult, EPCs are believed to be recruited from the bone marrow, migrate to sites requiring neovascularization, and participate in the assembly of newly-forming blood vessels [11, 19].

1.1.3 Tumor Neovascularization

The development of a single cancerous cell into a malignant tumor mass is a multistep process [20]. Malignant transformation of tumor cells requires the generation of a fertile microenvironment in which tumor cells can proliferate and interact with a complex network of stromal cells and neovessels (Figure 2). During the premalignant stages of carcinogenesis, tumor cells manifest altered responses to factors in the extracellular microenvironment, resulting in uncontrolled cell proliferation. There have been numerous reports that indicate the growth of tumors depends on the rapid recruitment of ECs to contribute to a functional neovasculature. Vascular formation within a tumor also allows for the distribution of cancerous cells to secondary sites [21].

Unlike a normal vasculature, the vasculature of a tumor is dilated [22], leaky [23], and disorganized [24]. These characteristics cause tumor vasculatures to lack stability and can occasionally lead to micro-hemorrhages or vessel collapse [25]. The development of blood vessels within a tumor occurs in response to the demand for nutrients and oxygen by the growing tumor mass. There is a physical limit, about 100 μm, to the distance small molecules are able to diffuse across the stroma between a nearby capillary and the tumor mass [21, 26]. Once that limit is surpassed, hypoxic conditions occur in the center of the tumor, inducing angiogenesis via a step termed the “angiogenic switch,” in which the balance between activators and inhibitors of angiogenesis tips in favor of pro-angiogenesis [26, 27].

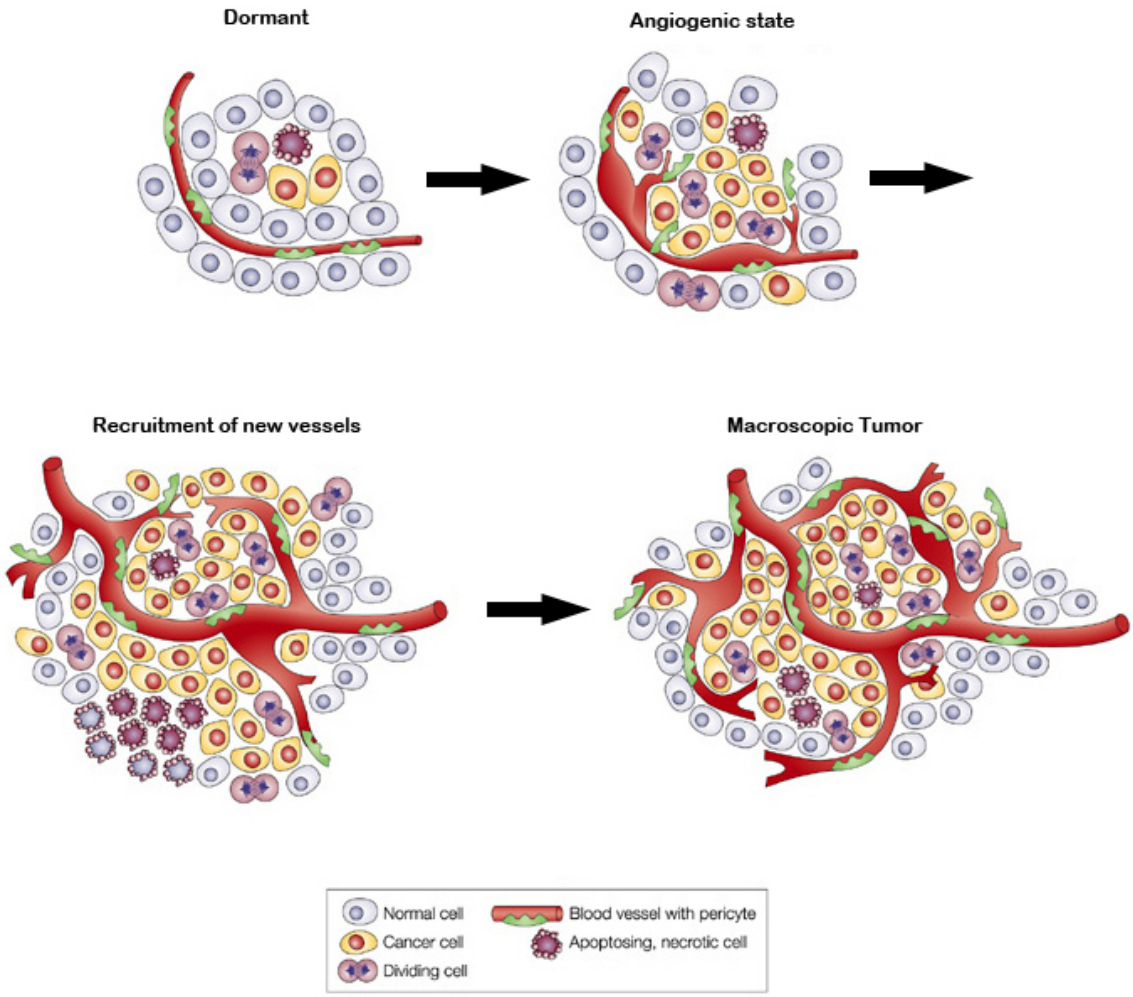


Figure 2. The progression of a tumor to a malignant phenotype.

Genetic abnormalities lead to the growth of a small microscopic tumor to a highly vascularized tumor mass. Tumor progression is marked by the ability of the tumor to secrete pro-angiogenic growth factors that induce an angiogenic state, resulting in the recruitment and formation of new of blood vessels from the surrounding stroma, which allows for the growth of a macroscopic tumor. The new vasculature then permits metastasis, leading to the eventual death of the host. Figure modified from [6].

Early theories of tumor neovascularization revolved solely around angiogenesis being the source of a tumor's vascular supply [4, 22]. It was suggested that vessels can be either co-opted from adjacent remodeled pre-existing capillaries or that ECs from surrounding established vessels can proliferate and migrate to cause capillary sprouting [22, 27-29]. However, abnormal neovascularization under disease conditions, such as that in cancer development, also involves postnatal vasculogenesis [4, 18, 30]. Recent evidence shows that EPCs migrate from the bone marrow to the tumor site and differentiate into a new endothelium in the tumor bed, providing an alternative method of tumor vascularization [31].

EPCs play an important role in tumor growth by contributing to tumor neovasculature, as shown with increased EPC markers in the tumors of cancer patients [30, 32, 33]. It has been shown that EPCs control the angiogenesis switch, a critical step in the transition of an avascular, dormant tumor to a vascularized, rapidly growing tumor [34]. It has also been shown that EPCs are subject to stimulation by vascular endothelial growth factor (VEGF), and that targeting VEGF or modifying the bone marrow to express an angiogenesis inhibitor blocks tumor growth [35].

1.2 ENDOTHELIAL PROGENITOR CELLS

1.2.1 Discovery

EPCs are defined as cells that are capable of differentiating into ECs by showing characteristics of stemness (proliferative capacity and resistance to stress) and clonal expression (the ability of a single cell to multiply) [36]. Prior to 1997, EPCs were thought to exist only in embryos and were initially known as angioblasts. These cells were derived from extra-embryonic mesenchyme cells and differentiated into mature ECs to aid in the formation of primitive blood vessels [37]. However in 1997, Asahara et al. reported the first detailed description of the isolation of adult bone marrow (BM)-derived EPCs from human peripheral blood [11]. Since then, there has been a vast amount of research interest in EPCs.

Research on avian embryonic development has suggested that embryonic EPCs and hematopoietic cells are derived from one common precursor cell, putatively termed the hemangioblast [15]. The hemangioblast originates from the mesoderm germ layer of epiblasts and symmetrically divides to form angioblasts and pluripotent HSCs [37]. Available accumulating evidence points to the existence of hemangioblasts in adults [37, 38]. The identification of HSCs in peripheral blood and bone marrow that could sustain hematopoietic reconstitution provided evidence for HSCs in adult tissues [39, 40]. Their related descendants, EPCs, have been isolated along with HSCs in hematopoietic organs [15]. The existence of adult hemangioblasts strongly suggests EPCs contribute to the maintenance and repair of the vascular and hematopoietic systems. Moreover, adult hemangioblasts will aid in the development of new therapies in the treatment of disease, and to the further understanding of EPCs.

1.2.2 Location

EPCs can be found in bone marrow, peripheral blood and umbilical cord blood. It is proposed that EPCs originate from the Lin^{-}-c-Kit^{+}-$Sca\text{-}1^{+}$ population of mouse bone marrow and it has been shown that $Sca\text{-}1^{+}$ cells can function as EPCs [41]. Similar to the peripheral blood in adults, umbilical cord blood is also a rich source of EPCs and contains high levels of $CD133^{+}$ and $CD34^{+}$ HSCs that can differentiate into ECs *ex vivo* [42, 43]. Additionally, it has been demonstrated that the human embryonic aorta contains EPCs capable of differentiating into mature ECs under culture conditions [44].

More recently, EPCs have also been identified within the walls of adult blood vessels. Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) derived from vessel walls are normally considered to be fully mature ECs. However, it was shown that HUVECs and HAECs could be passaged for at least 40 population doublings, indicating a hierarchy of EPCs within HUVEC and HAEC monolayers that can be discriminated by their clonogenic potential [42]. In line with these data, the existence of EPCs in a distinct zone of the vascular wall between the smooth muscle and connective tissue layers was found. Moreover, it was demonstrated that EPCs exist in the wall of adult human blood vessels of several organs, among them the liver [45].

Other studies have shown that EPCs can be derived from myeloid cells. These cells are located inside the peripheral blood and they have the ability to cross-differentiate into the EC lineage. Current studies have suggested that these monocyte-derived EPCs are less able to proliferate compared to EPCs derived from cord blood or the adult bone marrow, however they still have a comparable ability to contribute to neovascularization. It is thought

that either the proliferation capacity of EPCs is not important *in vivo*, or that these monocyte-derived EPCs produce growth factors that help balance their inability to undergo ample proliferation [36, 46].

1.2.3 Isolation and Characterization

EPCs were initially identified by the co-expression of CD34 (an antigen expressed by all HSCs that is lost as it differentiates) and Flk-1 (VEGFR2, a receptor for vascular endothelial growth factor) on the surface of these cells [11]. Asahara et al. used a polyclonal antibody to show that $CD34^+$ and $Flk\text{-}1^+$ isolated cells can differentiate into ECs *in vitro*. When $CD34^+$, $Flk\text{-}1^+$, $CD34^-$, or $Flk\text{-}1^-$ cells were injected into mice, rats and rabbits undergoing neovascularization due to hindlimb ischemia, $CD34^+$ and $Flk\text{-}1^+$ cells, but rarely $CD34^-$ or $Flk\text{-}1^-$ cells, incorporate into the vasculature in a manner consistent with vasculogenesis, a paradigm otherwise restricted to embryogenesis. After administration to mice, EPCs were shown to differentiate *in vivo* into ECs that associate with vessels and express PECAM-1 (CD31, an EC marker). Intravenous administration of mature ECs failed to result in incorporation of these cells into the foci of neovascularization [11]. In this manner, BM-derived EPCs were shown to be functionally distinct from mature ECs.

EPCs can be isolated using magnetic micro beads, adherence culture and flow cytometry. Once isolated, the cells are cultured on fibronectin coated plates containing medium with specific growth factors, such as VEGF and epidermal growth factors, to facilitate the growth of endothelial-like cells. After initial adhesion *in vitro*, EPCs begin to lose their progenitor characteristics and start to differentiate [11, 47-50]. Since the initial isolation of EPCs, a number of groups have set out to better define this cell population by identifying their expression pattern

of cell surface markers [19, 47, 49, 51]. EPCs were shown to express combinations of surface markers including Flk-1, E-selectin, fibroblast growth factor (FGF) receptor, CD34, c-Kit, CD31, von Willebrand factor (vWF), vascular endothelial cadherin (VE-cadherin), Tie-2 (angiopoietin-1 receptor precursor), and CD133 (AC133; prominin-1) [48, 52]. However, to date there is neither an exclusive EPC marker nor a simple definition of EPC characteristics. EPCs may therefore encompass a group of cells existing in a variety of stages ranging from primitive hemangioblasts to fully differentiated ECs.

EPCs can be roughly subdivided into three inter-related classes. One class of EPCs is represented by cells that are likely to be hemangioblasts. In mice, this includes Lin^{-}-c-Kit^{+}-$Sca\text{-}1^{+}$ cells and $Sca\text{-}1^{+}$-c-kit^{+}-$CD34^{-}$ cells from the bone marrow [53, 54]. In humans, this includes $CD113^{+}$, $CD34^{+}$, and $Flk\text{-}1^{+}$ cells from the bone marrow and blood [11, 49]. Two subpopulation classes of EPCs with distinct patterns of cell growth and ability to secrete angiogenic factors have been described. The two populations are termed early and late EPCs. Early and late EPC populations differ in their morphology. Early EPCs are spindle-shaped cells that have a peak growth in culture at 2–3 weeks and die around week 4 [46]. Late EPCs are cobblestone shaped and usually appear after 2–3 weeks in culture and can be maintained for up to 12 weeks [46, 55]. Although their putative precursors and the exact differentiation lineage of EPCs remain to be determined, it is believed that early EPCs which are localized in the bone marrow or found immediately after migration into the circulation are $CD133^{+}$-$CD34^{+}$-$Flk\text{-}1^{+}$ cells, whereas later stage circulating EPCs are still positive for $CD34^{+}$-$Flk\text{-}1^{+}$ but lose CD133 and begin to express other cell surface markers typical to mature ECs [31].

Another method of EPC evaluation is to determine their ability to support vasculogenesis. Evaluation of the angiogenic properties of EPCs utilizes assays that test the migration and tube formation capabilities of the cells to generate capillary-like EC networks. The migration assay is based on the chemotactic ability of EPCs. Cultured EPCs are harvested, counted, resuspended in fresh medium, and then moved to a modified Boyden chamber placed in a culture dish containing VEGF. The EPCs will migrate to the lower side of the filter toward the stimulating factor. The filter can then be washed and EPCs that have migrated can be counted [56]. To analyze tube formation, an assay is used in which EPCs obtained after culture are placed on a basement membrane matrix gel (Matrigel). After additional days of culture on the Matrigel, the number of EC networks, designated as tubes, can be detected and counted [57].

Although surface markers are the primary indicator of EPCs, they can be downregulated, making a sole antigen unreliable as the definitive marker of adult EPCs [58]. For example, EPCs found in the peripheral blood are positive for CD133, but can also be negative for CD133, a benchmark indicator for EPCs maturing into ECs [59]. CD133, a five transmembrane glycoprotein whose function is unknown, is a novel HSC marker and its expression is rapidly downregulated as hematopoietic progenitors and EPCs differentiate [60]. However, in conjunction with other protein markers, it is possible to depict a cell that is likely an EPC. It was demonstrated that a subset of $CD133^+$ cells mobilized from the peripheral blood can differentiate into EC when cultured in the presence of VEGF, fibroblast growth factor-2 (FGF-2), and collagen. Maturation and *in vitro* differentiation of these cells abolished CD133 expression [49]. Phenotypic analysis revealed that most of these cells displayed endothelial features, including the expression of Flk-1, Tie-2, and vWF [61-64]. All these data indicate that CD133 is a major candidate as the selective marker for identifying early EPCs with angioblast potential. Cells that

are $CD34^+$, Flk-1^+, and $CD133^+$ constitute a phenotypically and functionally distinct population of EPCs that may play a role in postnatal vasculogenesis. However, because CD133 is expressed by HSCs as well [65], the method for differentiating between immature EPCs, committed hematopoietic cells, and their putative common precursor, the hemangioblast, has yet to be developed further. Identification of their differences is further complicated by the fact that hematopoietic cell subsets express markers similar to those of ECs [33, 66].

1.2.4 Mobilization

To support vascularization, EPCs must mobilize to the site of neovascularization and differentiate into mature ECs. Within the bone marrow, EPCs are in a quiescent state. When the body is developing new blood vessels (such as during the developmental stage), or affected by injury, EPCs are activated and migrate into the vascular zone of the bone marrow where proliferation is increased. Various injuries such as ischemia, atherosclerotic lesions, traumatic wound, tumor angiogenesis [67-70], and heart infarction [71] cause the frequency of EPCs in the peripheral blood to increase up to 50-fold [68]. Although the molecular pathways involved in EPC mobilization are in the early stage of definition, the up-regulation of VEGF is thought to be a significant contributor to this mechanism [72]. VEGF is thought to be a key cytokine that effectively induces the mobilization of EPCs and HSCs into the circulation by interaction with its receptors (VEGFR-2 and VEGFR-1) [73].

VEGF can activate matrix metalloproteinase-9 (MMP-9) on EPCs. MMP-9 cleaves the membrane-bound kit ligand, a stem cell cytokine, in bone marrow stromal cells to release soluble kit ligand, enabling c-Kit^+ EPCs to migrate from a quiescent bone marrow niche toward the vascular region of the bone marrow, called the vascular zone. This translocation activates EPCs

from a quiescent to a proliferative state [74]. Besides activating MMP-9, VEGF also up-regulates stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR-4 [75, 76]. SDF-1 is chemotactic for EPCs and recruits EPCs to sites of neovascularization (Figure 3) [77]. In animal studies, local injection of SDF-1 into target organs increases EPC homing to the organ site, but only in the presence of VEGF. In the absence of VEGF, SDF-1 fails to enhance BM-derived cell recruitment. Blocking CXCR-4 abolished progenitor homing, regardless of the presence or absence of VEGF. Therefore, it appears that SDF-1 by itself is not sufficient enough to mobilize EPCs without an additional signal, such as VEGF [78]. Because additional studies have demonstrated that SDF-1 is essential for the adhesion of BM-derived cells, SDF-1 may significantly help to sequester EPCs at the site of vessel formation [76]. These studies indicate that VEGF, through interaction with MMP-9 and SDF-1, rapidly triggers the release of EPCs into the bloodstream. Accordingly, VEGF induces the mobilization of EPCs from the bone marrow of cancer patients [79, 80] and EPC levels in the circulation rise within 24 hours following VEGF treatment [81].

Cytokines that induce release of leukocytes or erythrocytes may also influence EPC mobilization. Increased numbers of EPCs were reported in animals following exogenous granulocyte macrophage colony stimulating factor (GM-CSF) treatment, and accelerated blood vessel growth was observed [82]. In another report, granulocyte colony-stimulating factor (G-CSF) increased incorporation of BM-derived cells into new tumor blood vessels, which markedly promoted growth of colon cancer cells that were inoculated subcutaneously in mice [83]. Furthermore, levels of erythropoietin (EPO) were found to be significantly associated with the number and function of EPCs [84]. Similarly, administration of recombinant human EPO increased the number of functionally active EPCs in peripheral blood [85].

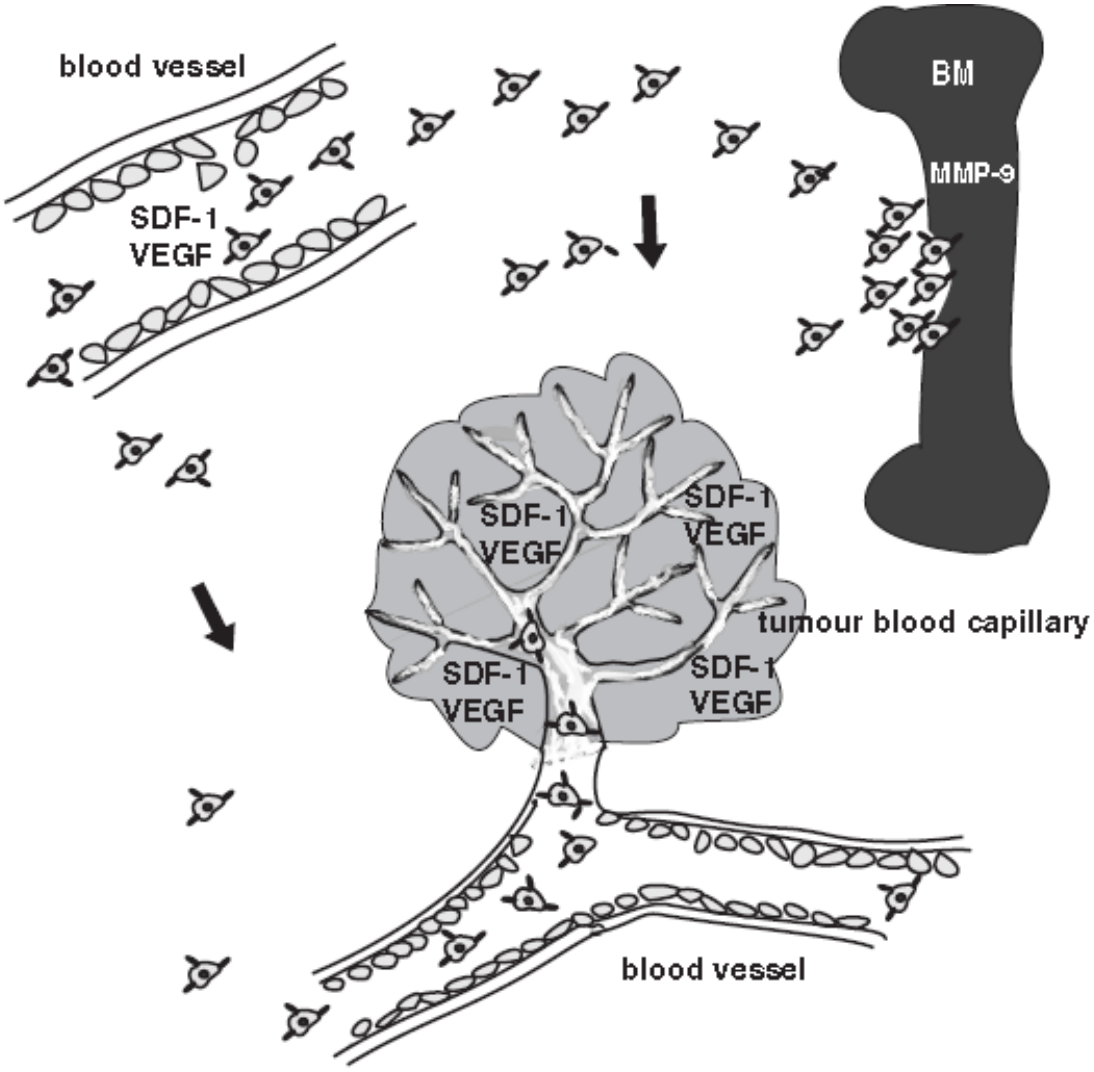


Figure 3. **EPCs are involved in blood vessel repair and tumor angiogenesis.**

Increases in the expressions of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and stromal-derived factor-1 (SDF-1) are responsible for the mobilization of EPCs from the bone marrow (BM) into the blood vessels. Figure adapted from [58].

Other molecules that have been shown to facilitate EPC mobilization from the bone marrow to the periphery are: Tie 2, FGF, hydroxymethylglutaryl–coenzyme A synthase inhibitor [86], 17ß-Estradiol together with FGF-2 [87], and reactive oxygen species (ROS) such as nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and Rac1 [88]. In addition to the above factors, there is also data indicating that placental growth factor [89], platelet-derived growth factor-CC [90], nitric oxide [91], physical training [92], and estrogens [93] stimulate EPC mobilization as well. Additionally, EPCs express protease cathepsin L [94], plasminogen activators, heparinases, chymases, and tryptases [95-97]. These types of proteases are important for matrix degradation and invasion by EPCs.

After EPCs are activated and begin to migrate, a guidance mechanism is required to help direct their interaction with the extracellular matrix at the site of vasculogenesis. One study shows that ephB2 and ephB4 enhances SDF-1/CXCR4's signaling and chemotaxis, which orchestrates the movement of EPCs to the specific site [98]. A key signaling molecule, Akt, is thought to regulate EPC homing and migration by modulating the expression of adhesion molecules [99]. When the EPCs interact with the extracellular matrix, they do so by interaction with integrins present on the extracellular matrix, such as αvβ3 and αvβ5 [100]. In contrast to enhancing EPC migration, there are also additional soluble factors that may inhibit EPC vasculogenesis. C-reactive protein (CRP), which has been shown to be an indicator of vascular death, and tumor necrosis factor-α (TNF- α) promote apoptosis, attenuate the function, and reduce the number of EPCs [101, 102]. The presence of CRP has yielded decreases in the angiogenic potential of EPCs and up-regulates EPC apoptosis by negatively affecting VEGF expression.

1.3 ROLE OF EPC IN TUMOR VASCULOGENESIS

Tumors require blood and nutrients in order to grow, proliferate, and metastasize. Research has demonstrated that BM-derived EPCs participate in neovascularization and contribute to tumor vascularization [18, 81]. Tumors release various cytokines and chemokines, most notably VEGF, angiopoeitin [103], G-CSF[83], and SDF-1 [104]. Because EPCs have receptors for these factors, including Flk-1 for VEGF and CXCR-4 for SDF-1, EPCs will hone to the tumor site to initiate blood vessel formation. Once they arrive at the site, EPCs will not only differentiate and form cellular clusters to begin developing a functional microvasculature, they will also attract more EPCs to the site by causing the tumor to release more VEGF [105, 106]. Hence, the vicious cycle of tumor growth, proliferation, and metastasis continues.

The study of tumor neovascularization has focused primarily on the process of angiogenesis but has begun to shift with the growing body of data that suggests vasculogenesis is an important mechanism for activating the angiogenic switch [34]. The availability of transgenic mice where β-galactosidase (Lac-Z) or green fluorescent protein (GFP) are expressed in ECs or in all tissues has helped with the understading of EPC-supported tumor vasculogenesis. Asahara et al. subcutaneously injected murine colon cancer cells into mice engrafted with bone marrow of transgenic mice constitutively expressing Lac-Z from EC-specific promoters. Three weeks after tumor implantation, histological examination of the tumors showed multiple Lac-Z^+ cells at sites of tumor xenotransplants, both within the tumor stroma and incorporated into the endothelial layer of tumor blood vessels [11].

Lyden et al. further studied the role of BM-derived EPCs in tumor growth using inhibitor of differentiation (Id)-mutant mice that were unable to support growth of implanted tumors because of defects in angiogenesis [33]. BM transplantation from wild-type mice, not from Id-

mutant mice, restored the tumor neovascularization and growth in Id-mutant mice. Examination of the tumors revealed that BM-derived EPCs contributed to more than 90% of the endothelium, suggesting that vasculogenesis played an overwhelming role in tumor neovascularization [33]. In subsequent animal transplantation models, EPCs were incorporated into neovessels, sometimes by as much as 50% [107], whereas other authors reported lower but significant levels between 10% and 20% [108].

Gao et al. examined whether BM-derived EPCs directly contribute to vasculogenesis and progression of micrometastisis to macrometastasis *in vivo* [34]. To facilitate identification of tumor cells and BM-derived EPCs, the authors inoculated GFP^+ chimeric mice with Lewis lung carcinoma (LLC) cells expressing red fluorescent protein. They observed that during tumor growth, there was a window during which tumor cells undergo an angiogenic switch and significant vessel infiltration can then be seen. Nearly 13% of the ECs in the tumor vessels expressed GFP and CD31. In addition, blocking EPC mobilization causes severe angiogenesis inhibition and significantly impairs tumor progression, leading the authors to determine that EPCs are critical regulators of the angiogenic switch [34].

Other studies have investigated the role of vasculogenesis in human tumors by assessing patients who developed malignancies after bone marrow transplantation from donors of the opposite sex. By colocalizing sex chromosome–specific probes with ECs markers, it was found that the percentage of BM-derived ECs in the tumor vasculatures ranged from 1% (head and neck sarcoma) to 12% (lymphoma), with a mean of 4.8% [109]. Furthermore, EPCs have been detected at increased frequency in the peripheral blood of patients with various malignancies including lung [110], hepatocellular [80], breast [78], and colorectal [111] cancers, as well as

myeloma multiplex [112], myelofibrosis [113], non-Hodgkin's lymphoma [114], acute myeloid leukemia [115], and malignant gliomas [116].

These observations have been challenged by some studies in which EPCs had no measurable contribution to tumor neovessels. In a bone marrow transplantation model in which donor bone marrow was transduced with a lentiviral vector encoding GFP driven by the endothelial-specific Tie-2 promoter, it was estimated that only 0.05% of blood vessels in tumor xenografts were derived from BM-derived EPCs [107]. Similar reports were shown in transplantation models using bone marrow cells with GFP ubiquitously expressed, suggesting no contribution of BM-derived EPCs to the endothelial lining of tumor vessels. These results conflict with multiple prior reports on EPC contribution to tumor vasculogenesis and may be due to differences in methodology. It appears the contribution of BM-derived EPCs to tumor vasculature depends heavily on the experimental model.

Efforts to quantify tumor reliance on vasculogenesis have suggested a wide variation in EPC-supported vasculogenesis in mouse models, ranging from 0% to more than 90% [33, 117, 118]. The wide variation in the degree of tumor vasculogenesis may be the result of different surface markers used for detection, different model systems (e.g., different reporter constructs and types of tumor) or the method of tissue sampling (e.g., vasculogenic vessels were not uniformly distributed but occurred in “hotspots”) [118-120]. Data suggest that BM-derived EPC involvement in tumor vascularization might also vary depending on the tumor stage [121, 122]. In one study, it was shown that only advanced tumors incorporate BM-derived EPCs into neovessels, perhaps in response to the increasing demand for a larger blood supply by the tumor [122]. Another study by Nolan et al. suggests BM-derived EPCs are incorporated at a high efficiency (20-30%) into the vasculature for a brief period during the early phase of tumor

growth (days 4-6 following tumor implantations). In the weeks following, BM-derived EPC becomes diluted by local non-BM-derived EPCs, resulting in only 1% of EPCS to be detected in the tumor after 4 weeks of growth [123].

Future studies to examine location and numbers of BM-derived cells during tumor progression are necessary to further our understanding of their importance in tumor vasculogenesis. Although angiogenesis is overwhelmingly the most widely studied process, it is not the only mechanism of blood vessel formation within a tumor. Tumor vascularization may be supported by the mobilization and functional incorporation of BM-derived EPCs. As such, EPCs have been detected at increased frequency in the circulation of cancer patients and lymphoma bearing mice [124, 125]. Additionally, tumor volume and tumor secretion of VEGF were found to be correlated with EPC mobilization [124, 125]. Because vascularization is seen as a fundamental step in tumor progression, there is value to identifying anti-angiogenic therapies that target both angiogenesis and vasculogenesis as an approach to treat cancer.

1.4 VASCULAR ENDOTHELIAL GROWTH INHIBITOR

1.4.1 Identification

Although the mechanism of postnatal neovascularization modulation is not fully understood, it is clear that there is a balance between stimulators and inhibitors acting as an angiogenic switch [27]. One of these inhibitors, vascular endothelial growth inihibitor (VEGI; TNFSF15; TL1A), is a member of the tumor necrosis factor (TNF) superfamily and can be found in the vasculature of many normal tissues, suggesting it plays a role in vascular homeostasis [126]. VEGI was discovered during an attempt to identify an autocrine inhibitor of angiogenesis. A search of cDNA databases identified a novel protein specifically expressed by ECs that had sequence homology to the TNF superfamily (TNFSF), whose members have a diverse array of biological functions, including regulating the growth of normal cells by inducing apoptosis or enhancing cell survival and proliferation. Hydrophobicity analysis of the protein revealed a 13-amino acid hydrophobic region that follows the amino terminal segment of 12 residues, with a carboxyl terminus on the exterior cell surface (residues 26-174), a single transmembrane domain, and a short cytoplasmic tail. These features are consistent with characteristics of a type II transmembrane protein. The protein, with a molecular mass of 22 kD, exhibited a 20–30% sequence homology to other TNF family members [127], similar to the degree of conservation found among other members of the TNF family. This protein was subsequently found to inhibit EC growth, thus, it was named VEGI. The protein is also termed TNF-like Ligand 1 (TL1) by some groups [128] and has received the HGMW-approved designation of TNFSF15.

1.4.2 Structure and Isoforms

In 2007, Jin et al. determined the crystal structure of VEGI [129, 130]. VEGI has self-rotation functions with three monomers in the asymmetric unit and they are related by a 3-fold non-crystallographic symmetry. The three monomers in the asymmetric unit form a homotrimer and assume a jellyroll β-sandwich, resembling the trimer structure typical of other members of the TNF family of ligands. Structure alignment between VEGI and other members of the TNFSF reveals similar length and overall structure, revealing their resemblance as predicted by their sequence homology [129, 130].

To date, there are three isoforms of VEGI. The initial isoform of VEGI that was characterized was designated VEGI-174. This isoform is comprised of 174 amino acids, with 1-25 AA residues at the N-terminus forming an intracellular and transmembrane domain, while residues 26-174 at the C-terminus form an extracellular domain. Two other isoforms, VEGI-251 and VEGI-192, were subsequently determined and these isoforms exhibit the same conserved carboxyl terminal domain of 151 acid residues as VEGI-174 [126, 131, 132]. However, the three isoforms differ in their N-terminal regions (Figure 4) [126].

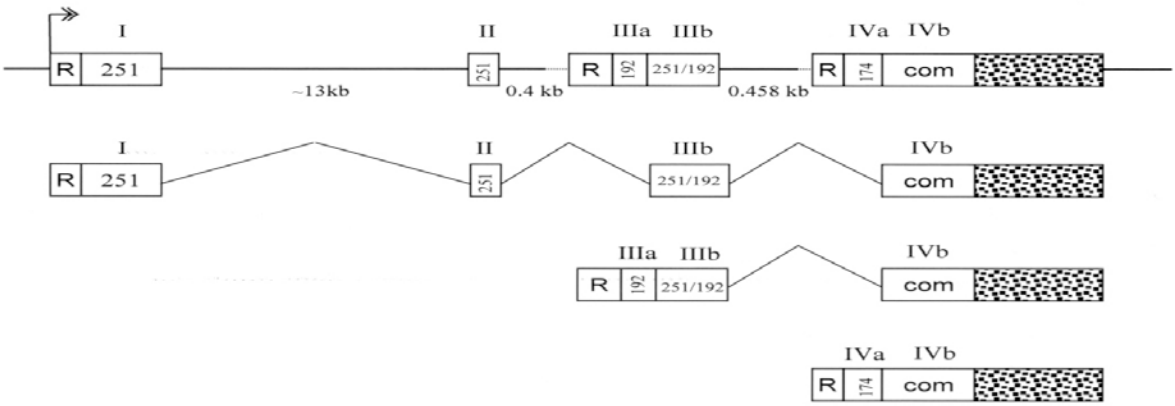


Figure 4. **Gene structure of human VEGI and proposed generation of isoforms.**
Boxes with roman numerals represent exons. The putative transcription start site is indicated by a double arrowhead. R denotes the 5′ untranslated sequence unique to each respective transcript. Stippled boxes represent the common 3′ untranslated region. Approximate sizes of the introns are indicated. VEGI-251, VEGI-192, or VEGI-174 specific sequences are labeled '251', '192', or '174'. Exon IIIb encodes residues shared by both VEGI-251 and VEGI-192. The introns 5' of exons III and IV are dashed because the 5' ends or initiation sites of VEGI-192 and VEGI-174 transcripts have not yet been determined. 'COM' denotes the coding region of the last exon that is common to all three isoforms. Figure taken from [126].

VEGI-251 is also known as TL1A [133] and it is the longest and most abundant isoform of VEGI [130]. After the extracellular domain of VEGI-251 is cleaved off from the cell membrane by unidentified proteases, VEGI-251 possesses a putative secretory signal peptide that exists in soluble form and its overexpression causes apoptosis of ECs and inhibition of tumor growth [129]. In contrast, there was no effect of full-length VEGI-174 on tumor growth when overexpressed. However, a secretable fusion protein comprising a secretion signal peptide and the putative extracellular domain of VEGI-174 was able to inhibit tumor growth [134, 135]. Further studies have shown that the solubilized extracellular domain of the three isoforms of VEGI is responsible for its biological activity [126, 131, 136, 137]. VEGI has also been detected in media conditioned by EC and in human circulation. Together, these findings support the view that secretion of VEGI is necessary to mediate its effect in inhibiting EC growth.

1.4.3 Expression

VEGI mRNA expression is detected predominantly in ECs and VEGI gene transcripts are found in many adult human tissues, including placenta, lung, kidney, skeletal muscle, pancreas, spleen, prostate, small intestine and colon, suggesting the gene product plays a role in the function of a normal vasculature. All three isoforms of VEGI have been found in human ECs, including HAECs, HUVECs, and human microvascular ECs. Low levels are detected in adult bovine aortic endothelial (ABAE) cells and human coronary artery smooth muscle [126, 127, 138].

VEGI protein expression has been found at high levels in the placenta, kidney, lung and liver, skeletal muscle, and heart. The 7.5 kb transcript encoding VEGI-251 is expressed at high levels in the placenta, kidney, lung, and liver, whereas the 2 kb transcript corresponding to VEGI-174 is detected in liver, kidney, skeletal muscle, and heart. VEGI-174 mRNA is more abundant in heart, skeletal muscle, pancreas, adrenal gland, and liver, while VEGI-251 is more abundant in fetal kidney and fetal lung. Both VEGI-251 and VEGI-174 were detected in prostate, salivary gland, and placenta [126]. The failure to detect the transcripts of this VEGI in other tissues may be due to a relatively small proportion of ECs in these tissues. The expression pattern of VEGI suggests a tissue specific function of this novel protein.

Although VEGI is primarily expressed in ECs, it was found that VEGI is also highly expressed in dendritic cells after *in vitro* activation and is up-regulated in Crohn's disease, rheumatoid arthritis, and mouse models of inflammatory bowel disease [139-141]. Furthermore, VEGI expression was also found in activated lymphocytes, plasma cells, and monocytes [133, 140, 142].

1.4.4 Anti-angiogenic Activity

VEGI is largely produced by ECs and it specifically induces apoptosis in ECs via an autocrine pathway [126, 127, 138]. Since VEGI is found in many normal and tumor tissues, it is thought that there is a physiological and pathological role for this unique molecule in regulating neovasularization. The anti-angiogenesis activity of VEGI was assessed using recombinant VEGI consisting of the putative extracellular domain in two angiogenesis models. In one angiogenesis model, ABAE cells grown on a layer of collagen gel are stimulated with FGF-2 to form a network of capillary-like structures. Addition of the VEGI inhibited the proliferation of ABAE cells and completely inhibited the growth of capillary-like tubes [137]. In another experiment, human vascular ECs transfected with a VEGI-expressing plasmid exhibited a decreased ability to form microtubules in Matrigel [143]. The anti-angiogenic activity of VEGI was also examined using a modified chick embryo chorioallantoic membrane (CAM) assay in which new capillary vessels will grow into a collagen gel pellet placed on the CAM. Recombinant VEGI inhibited about 50% of the new capillary growth into the gel pellet induced by either FGF or VEGF [137]. Importantly, these results suggest that VEGI can inhibit angiogenesis regardless of the types of angiogenic stimuli.

Interestingly, as increasing evidence points to VEGI being an endogenous inhibitor of EC growth, it is important to note that no inhibitory effect is detected thus far in other cell types using similar concentrations of VEGI [137]. VEGI has no effect on the growth of human breast cancer cells (MDA-MB-231 or MDA-MB-435) under similar experimental conditions performed on ECs. It has been shown, however, that a VEGI preparation was able to inhibit human breast cancer MCF7 cell growth in culture [144], although a substantially higher concentration of VEGI was required. VEGI also did not inhibit the proliferation of human T-cell leukemia cells

(Jurkat), human Burkitts lymphoma cells (Raji), human monocytic leukemia cells (THP-1), or human promyelocytic leukemia cells (HL60), cells that are often responsive to the cytotoxic activity of the TNFs [127].

Studies have shown that the mechanism by which VEGI exerts its inhibitory effect on angiogenesis involves modulation of the EC cycle by enforcing growth arrest of quiescent ECs while causing proliferating ECs to undergo apoptosis [138]. By analyzing proliferating ECs, Yue et al. demonstrated that VEGI can induce ECs to shrink and induced blebbing of the plasma membrane associated with apoptosis. Further studies demonstrated that VEGI, in addition to inducing apoptosis, can elicit growth arrest of ABAE cells in the G0/G1stage of the growth cycle. VEGI prevents G0/G1 cells from re-entering the cell cycle in response to growth stimuli and early G1-growth arrest occurs. Importantly, it was shown that cells in this VEGI-arrested state lacked all the hallmarks of apoptosis, such as DNA fragmentation, caspase-3 activation, and Annexin-V staining, which is normally seen in VEGI-treated cells that have entered the growth cycle[138]. Moreover, VEGI induces apoptosis via activation of the stress protein kinases, SAPK/JNK and p38 MAPK (SAPK, stress-activated protein kinase, JNK, c-Jun N-terminal protein kinase, p38 MAPK, p38 mitogen-activated protein kinase), and the caspases, mainly caspase-3- like protease [138].

The activity of VEGI has been postulated to arise from its interaction with members of the TNF receptor superfamily. VEGI is a ligand for two TNF receptors, namely death receptor 3 (DR3) and decoy receptor 3 (DcR3). Previously, there has been evidence indicating DR3 is the functional receptor of VEGI 251 [133]. It contains a death domain in its cytoplasmic tail and VEGI induces apoptosis in DR3 expressing cell lines, such as in HUVECs, reducing angiogenesis. In contrast, it has been indicated that DcR3 enhances angiogenesis by blocking the

autocrine function of VEGI [142]. DcR3 is over-expressed in malignant tumors arising from oesophagus, stomach, glioma, lung, colon, and rectum [130, 140-142]. Direct interactions between VEGI and both DR3 and DcR3 has been detected either by co-immunoprecipitation or a flow cytometry based assay [133, 145]. Consistently, the use of anti-VEGI or anti-DR3 antibodies lead to increases of cell proliferation and motility, resulting in induced formation of a capillary network [146]. The angiogenic effect of VEGI antibody and DR3 antibody is similar to that induced by DcR3.

1.4.5 Anticancer Activity

The unique characteristics of VEGI suggest it may be an ideal candidate for the treatment of pathological angiogenesis. Pathological angiogenesis, as seen in growing tumors, is prolonged and uncontrolled once initiated, and can be due to the absence or suppression of a physiological inhibitor like VEGI. Treatment of tumors with VEGI may allow proliferating vasculature to undergo apoptosis while leaving normal quiescent vessels unaffected. In parallel with this thought, recombinant VEGI has been shown to be a highly potent inhibitor of EC proliferation in tumors, resulting in specific elimination of ECs in tumor vasculature and inhibition of angiogenesis [131]. More importantly, VEGI treatment of tumor cells showed no overt toxicity to ECs of the normal vasculature in other tissues [131]. Furthermore, it was found that VEGI was able to markedly inhibit tumor formation and growth in a variety of angiogenesis and cancer models [127, 137].

Xenograft tumor models were used to assess the anti-angiogenic ability of VEGI. Highly tumorigenic breast cancer cells (MDA-MB-231 or MDA-MB-435) were mixed with VEGI-overexpressing Chinese hamster ovary (CHO) cells and injected into the mammary fat pads of

athymic nude mice. Growth of the xenograft tumors was monitored and despite the high tumorigenicity of the breast cancer cell lines used, marked inhibition of tumor growth was observed. A repeat experiment with MDA-MB-435 revealed a complete inhibition of tumor formation. Vector-transfected CHO cells had no effect on tumor growth in either case [137]. Since VEGI does not inhibit the growth of these breast cancer cells in culture, the anticancer activity of the protein most likely derives from its anti-angiogenic activity. The anticancer activity of VEGI-overexpressing CHO cells was also studied in a prostate cancer animal model. The CHO cells were mixed with prostate cancer cells (PC3) at various ratios (10:1, 5:1, 1:1 and 0:1) before being subcutaneously injected into athymic nude mice. Initially, the tumors grew, but then disappeared in the groups with higher ratios of CHO:PC3. PC3 cells implanted alone exhibited progressive tumor growth [137]. The results indicate that higher levels of VEGI may cause the elimination of established tumors.

While VEGI is normally expressed in the vascular ECs of many adult tissues, it has been reported that VEGI is absent or expressed at low levels in tumors vasculatures of breast cancer [134], prostate cancer [147], and urothelial cancer [148], supporting the idea that VEGI is a negative regulator of neovascularization. It was also reported that patients with breast cancer that expressed reduced levels of VEGI had a higher local recurrence, shorter survival time and an overall poorer prognosis compared to patients expressing higher levels of VEGI [134]. The lowered expression of VEGI in tumors and the ability of recombinant VEGI to specifically suppress tumor angiogenesis make VEGI a promising anticancer agent. However, it is yet unknown whether VEGI is able to inhibit tumor vasculogenesis.

1.5 PURPOSE

This research will be a first step in understanding the role of VEGI in modulating tumor vasculogenesis. While VEGI inhibits angiogenesis by specifically inhibiting EC proliferation and inducing apoptosis, it is unknown whether VEGI can inhibit EPC-supported vasculogenesis. Given that tumor vasculature arises from both angiogenesis and vasculogenesis, with EPCs being the activator of the angiogenic switch, it is highly significant to determine whether VEGI can also inhibit EPC-supported vasculogenesis. If VEGI is able to exert an inhibitory function not only on mature differentiated ECs but BM-derived EPCs as well, it may prevent EPCs from participating in tumor vasculogenesis. This could lead to further elimination of the tumor vasculature resulting in profound inhibition of tumor formation, growth, or metastasis. Our goal is to determine the changes that occur in BM-derived EPC-supported tumor vasculogenesis in response to VEGI treatment. In addition, the proposed research could yield important insights into the function of VEGI in postnatal vasculogenesis, advancing the understanding of the pathobiology of tumor neovascularization and helping to facilitate the development of therapeutic uses of VEGI in cancer.

2.0 VEGI INHIBITS BM-DERIVED EPC DIFFERENTIATION AND FUNCTION *IN VIVO*

2.1 ABSTRACT

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) have been shown to have a critical role in supporting *de novo* formation of blood vessels during postnatal and tumor vasculogenesis. Vascular endothelial growth inhibitor (VEGI; TNFSF15; TL1A) is an endogenous inhibitor of angiogenesis that specifically induces apoptosis of proliferating endothelial cells (ECs). However, it is unknown whether VEGI is able to modulate BM-derived EPCs as well. We report here that VEGI inhibits the differentiation of BM-derived cells into EPCs under EC stimulating culture conditions. Bone marrow cells cultured in the presence of VEGI showed a significant decrease in expression of EC surface markers that was coupled with a diminished ability of the cells to migrate, adhere, and form capillary-like structures critical for neovascularization. Additionally, VEGI was found to activate cell growth signaling and inhibit cell differentiation signaling in early stage EPCs. In contrast, VEGI treatment decreased the number of adhered cells in culture by inducing apoptosis in late stage differentiated EPCs. Moreover, the apoptosis of BM-derived EPCs in response to VEGI treatment is mediated by

death receptor-3 (DR3), which is expressed only in late stage EPCs, and antibodies that neutralize DR3 inhibits VEGI induced apoptosis. These findings indicate that VEGI plays a biologically significant role in postnatal vasculogenesis by negatively modulating differentiation of BM-derived cells into EPCs.

2.2 INTRODUCTION

Until a decade ago, it was thought that postnatal neovascularization, the proliferation, migration and stabilization of new blood vessels, occurred solely via angiogenesis, the process of capillary sprouting from pre-existing blood vessels [22]. Since then, bone marrow (BM)-derived endothelial progenitor cells (EPCs) have been discovered and found to contribute to postnatal neovascularization via vasculogenesis, the *de novo* formation of new vessels [11, 19, 82, 149, 150]. There are two populations of EPCs in culture, termed early and late EPCs, which differ in their morphology and have distinct patterns of cell growth. As EPCs differentiate from early to late stage EPCs, they lose their progenitor characteristics and gain endothelial characteristics. To date, there is no exclusive EPC marker, however EPCs have been commonly identified by the co-expression of stem and endothelial cell (EC) surface markers, including CD133, Sca-1, Flk-1, Tie-2, E-selectin and VE-cadherin [48, 151].

Under normal conditions, EPCs are in a quiescent state within the bone marrow niche and their numbers in the circulating blood are very low. However, trauma to the endothelium during occurrences such as tumor development, wound injury or ischemia, induces EPCs migration from the bone marrow into the periphery, resulting in increased EPC numbers in the blood [68, 72]. Many stimulating growth factors and cytokines have been shown to activate signaling

molecules which induce the migration and differentiation of EPCs [81, 152, 153]. One such signaling factor, Akt, modulates the expression of adhesion molecules and controls EPC homing and migration [99]. Akt also plays a role in modulating EPC differentiation into mature ECs [154]. However, cytokines that negatively regulate EPC differentiation are not as well known.

Vascular endothelial growth inhibitor (VEGI; TNFSF15; TL1A) is a member of the tumor necrosis factor (TNF) superfamily and plays a vital role in maintaining vascular homeostasis. VEGI is an endogenous inhibitor that is primarily expressed by vascular ECs to inhibit their proliferation [127]. Specifically, VEGI enforces growth arrest of quiescent ECs in G0 and G1 phases of the cell cycle, while inducing apoptosis of proliferating ECs, thereby inhibiting angiogenesis [137, 138, 155]. It has also been demonstrated that VEGI activates T-cells [133, 156, 157] and stimulates the maturation of dendritic cells [158], indicating VEGI helps modulate the interaction between the endothelium and the immune system. The inhibitory activity of VEGI has been shown to occur via mitogen-activated protein kinases (MAPKs) and the caspases [138]. Furthermore, the activity of VEGI has been thought to be due to its interaction with death receptor 3 (DR3), a member of the TNF receptor superfamily [133, 159].

In this study, we examine whether VEGI has an inhibitory effect on BM-derived EPC. We found that VEGI can inhibit the differentiation of bone marrow stem cells into EPCs, resulting in obstructed migration and adhesion of these cells in culture. Additionally, we found that VEGI induces apoptosis of differentiated BM-derived EPCs. These findings provide important insights into the modulation of BM-derived EPC differentiation and function.

2.3 MATERIALS AND METHODS

2.3.1 Antibodies and reagents

Anti-DR3 antibody, fluorochrome-conjugated anti-mouse Sca-1, Flk-1, Tie-2, E-selectin, VE-cadherin, CD31, CD117 and AC133 antibodies were purchased from eBioscience (San Diego, CA). The antibodies for total or phosphorylated p38, Akt and Erk were purchased from Cell Signaling Technology (Danvers, MA). Integrin α5, integrin αv, Flk-1, Tie-2, E-selectin, VE-cadherin, AC133, CD117, and DR3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor Dyes conjugated secondary antibody, Calcein Acetoxymethyl and fluorescent phallotoxin were purchased from Invitrogen Corporation (Carlsbad, CA). VEGF, fibronectin, and Matrigel were purchased from R&D Systems (Minneapolis, MN). ECM Cell adhesion array kit and Chemicon QCMTM cell migration assay kit were purchased from Millipore (Billerica, MA). Caspase-3/CPP32 Colorimetric Assay Kit was purchased from BioVision Research (Mountain View, CA). VEGI isoform VEGI-192 was prepared as described [131]. One unit of VEGI activity equals the concentration of VEGI required for half-maximum inhibition of bovine aortic EC growth in culture, known as the IC50 [155]. The endotoxin level in the VEGI preparation is 25 ng/mg, with the final endotoxin concentration in cell cultures of 0.025 ng/ml.

2.3.2 Mice

Eight-week old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments involving animal subjects were performed in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee approved protocols.

2.3.3 Cell Preparation

Whole bone marrow stem cells were isolated from the femurs and tibia of adult mice via bone marrow flush. Cells were first lysed with ACK cell lysis buffer (Lonza, Walkersville, MD) and then purified for the Sca-1^+ population using biotin conjugated Sca-1 antibody and a biotin magnetic selection kit (StemCell Technologies). 6 x 10^6 cells/well were placed in 10 μg/ml fibronectin-coated 6-well plates and cultured in endothelial growth medium (EGM-2) supplemented with a bulletkit containing EGF, hydrocortisone, VEGF, FGF-B, heparin, IGF, gentamicin and 5% heat-inactivated fetal bovine serum (all from Lonza, Walkersville, MD).

2.3.4 Flow cytometry

5 x 10^5 cells were collected per sample and washed with 2 ml of fluorescence-activated cell sorter (FACS) buffer (1% BSA and 0.05% sodium azide in PBS). After washing, the cells were resuspended in 100 μl of FACS buffer containing 1 μg of the indicated antibody, dispensed in a minimum of 1 x 10^5 cells per sample, gently mixed, and incubated on ice for 30 minutes. The cells were washed with FACS buffer, resuspended in PBS and analyzed within 1 hour using Coulter FACS equipment and EXPO analysis software (Beckman Coulter, Fullerton, CA).

2.3.5 Cell adhesion assay

One-week cultured EPCs treated in the absence or presence of VEGI were collected and re-plated (0.2 x 10^6 cells/100 µl) on 96-well plates coated with extracellular matrix proteins (Chemicon ECM adhesion Kit), then cultured for 2 hours. The wells were gently washed with PBS and any adherent cells remaining in the wells were stained with crystal violet for 10 minutes. After washing and extraction, the absorbance at 560nm was determined by a spectrophotometer.

2.3.6 Cell migration assay

Cultured cells with or without VEGI treatment were reseeded into the upper chamber of a Modified Boyden Chamber (Chemicon QCM Cell migration kit) containing serum and growth factor free culture media (0.2 x 10^6 cells/100 µl). Stimuli were added into the lower chamber and the cells were allowed to migrate for 8 hours. Migrated cells were collected, lysed, and dyed with CyQunat GR to analyze absorbance at 480/520nm using a fluorescence plate reader. In the wound-healing assay, a strip of cells was removed with a sterile pipette tip from the cell monolayer of 10 day cultured EPCs. The migration of cells into the empty space was recorded for 48 hours.

2.3.7 Tube formation assay

A Matrigel tube formation assay was performed as described [160]. Cultured EPCs with or without VEGI treatment were re-plated in a 48-well culture plate coated with Matrigel

containing 100 ng/ml of VEGF ($5x10^4$ cells/well). After 20 hours, microscopic images of vascular structural tubes formed by the cells were taken. Total tube length was determined using Image J software (NIH).

2.3.8 Cellomics Array Scan

Using 96-well black plates, 3×10^3 cells/well were cultured in the absence or presence of VEGI, and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, washed with PBS, then blocked with 3% BSA for 30 minutes. Cell surface markers and adhesion signal proteins were labeled with primary antibody and fluorescent dye conjugated secondary antibody. Nuclei were stained with Hoechst. Images of the cells were taken and analyzed for fluorescence density in the cytoplasm and nucleus by Cellomics array scan (ArrayScan VTI HCS Reader, Cellomics Inc., Pittsburgh, PA).

2.3.9 Cell proliferation and viability assay

Purified Sca-1^+ bone marrow cells were cultured with or without VEGI. Adherent and suspended cell populations were collected separately on various days of culture. To evaluate cell proliferation and viability, cells were dyed with Calcein Acetoxymethyl (Molecular Probes) at 2 μM and incubated for 1 hour. The cells were washed, and absorbance was read at 480/520nm with a fluorescence plate reader.

2.3.10 Cell apoptosis assay

Adherent and suspended cells were collected separately, and then centrifuged onto slides using a Cytocentrifuge (Shandon Cytospin 3 Centrifuge). The cells were washed and fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton X-100 in PBS for 2 minutes on ice. After blocking with 3% BSA, the cells were labeled for DNA strand breaks by a TUNEL assay kit (Roche). The cells were also labeled with anti-E-selectin primary antibody and fluorochrome-conjugated secondary antibody. Nuclei were stained with Hoechst and the cells were then sealed with anti-fading agent for 24 hours and analyzed with a fluorescent microscope (Nikon Eclipse E800, Japan).

2.3.11 Caspase-3 activity assay

Caspase-3/CPP32 Colorimetric Assay Kit (Mountain View, CA) was used to analyze caspase-3 activity. Cells were first lysed using cell lysis buffer for 2 hours at 4ºC on a shaker. Plates were then centrifuged and cleared lysates were transferred to a new 96-well plate. Cleared cell lysates were incubated with caspase-3 reaction buffer and substrate DEVD-pNA. The chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA was detected at 405 nm by Benchmarked Plus spectrophotometer (BioRad, Hercules, CA).

2.3.12 Immunofluorescence staining

Cells were washed and fixed with 4% paraformaldehyde in PBS at room temperature for 20 minutes, then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Samples were washed with PBS, blocked for non-specific antibody binding with 3% BSA for 30 minutes, followed by incubation with primary antibodies for 1 hour at room temperature. After 5 washes with PBS, cells were incubated with fluorochrome-conjugated secondary antibodies for 1 hour. After nuclei staining with Hoechst, the cells were sealed with anti-fading agent for 24 hours and analyzed with a fluorescent (Nikon Eclipse E800, Japan) or confocal (Olympus Fluoview 1000, Japan) microscope.

2.3.13 Western blot analysis

Cells were collected and lysed. Proteins in the cell homogenate were then resolved using SDS-PAGE. Next, the proteins were transferred onto a Hybond-ECL nitrocellulose membrane and blocked with 5% nonfat dry milk powder in TBS-T buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween) for 1 hour at room temperature. After blocking, the membrane was incubated with primary antibodies against the targeted protein and stored overnight at 4°C. The membrane was washed with TBS-T and incubated with the corresponding HRP-conjugated secondary antibody for 1 hour at room temperature. After another wash with TBS-T, the membrane was developed using the ECL System (Amersham Pharmacia Biotech, Arlington Heights, IL).

2.3.14 Statistical analysis

Results shown are the mean ± SE of a minimum of three separate experiments. Student's t-test for independent samples was used to compare data between experimental groups. Values of $P<0.05$ were considered statistically significant and are indicated with an asterisk.

2.4 RESULTS

2.4.1 Characterization of mouse BM-derived EPC

Whole bone marrow from C57BL/6J mice was purified for the Sca-1^+ cell population. Once purified, the bone marrow cells were cultured in EC growth media to facilitate the differentiation of EPCs. After two weeks of culture, the cells had adhered to the culture plates, become confluent, and elongated in shape, mirroring EC morphology (Figure 5).

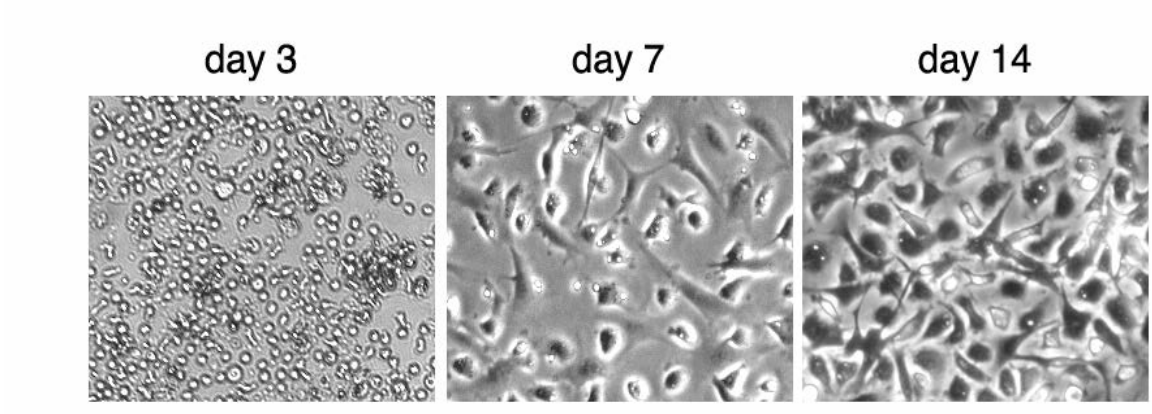
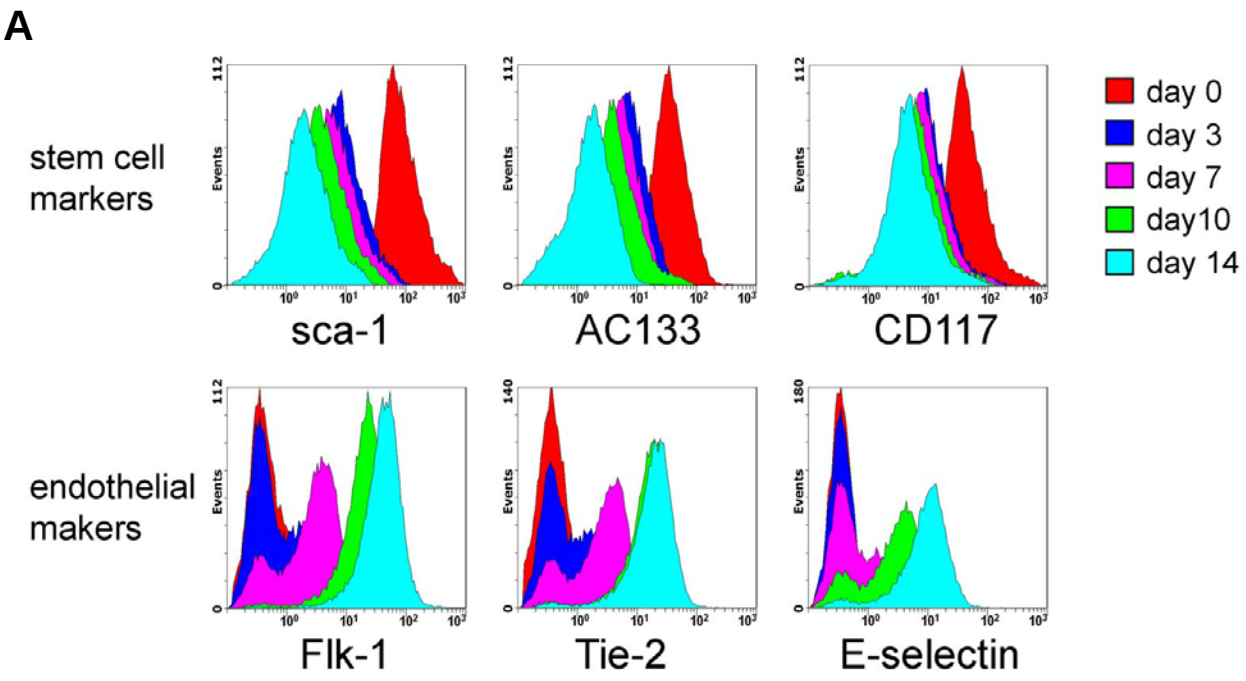


Figure 5. **Morphology changes of BM-derived EPCs in culture.**
Phase-contrast images of cultured Sca-1^+ bone marrow cells over two weeks show progressive differentiation of BM-derived EPCs (20x objective lens).

Flow cytometric analysis revealed the cells expressed a concurrent change in the expression pattern of cell surface markers. Day 0 to day 14 cells were stained for expression of stem cell markers Sca-1, CD117 and AC133 and EC markers Flk-1, Tie-2 and E-selectin. Consistent with the initial morphology change that bone marrow cells undergo within 7 days, it was seen that one-week cultured cells possessed both EC markers and stem cell markers (Figure 6A). We found that over the continued course of culture, stem cell marker expression decreased. However, EC marker expression increased in the same two-week cell cultures, indicating cells were differentiating toward ECs (Figure 6A). Looking at protein expression levels, it is also observed that EC markers begin to be expressed in later cultures (Figure 6B). Immunofluorescence staining confirmed EC marker expression on day 14 cultured cells (Figure 6C). These changes in cell morphology and surface marker expressions indicate bone marrow cells were able to differentiate into EPCs around day 7 of cell culture.



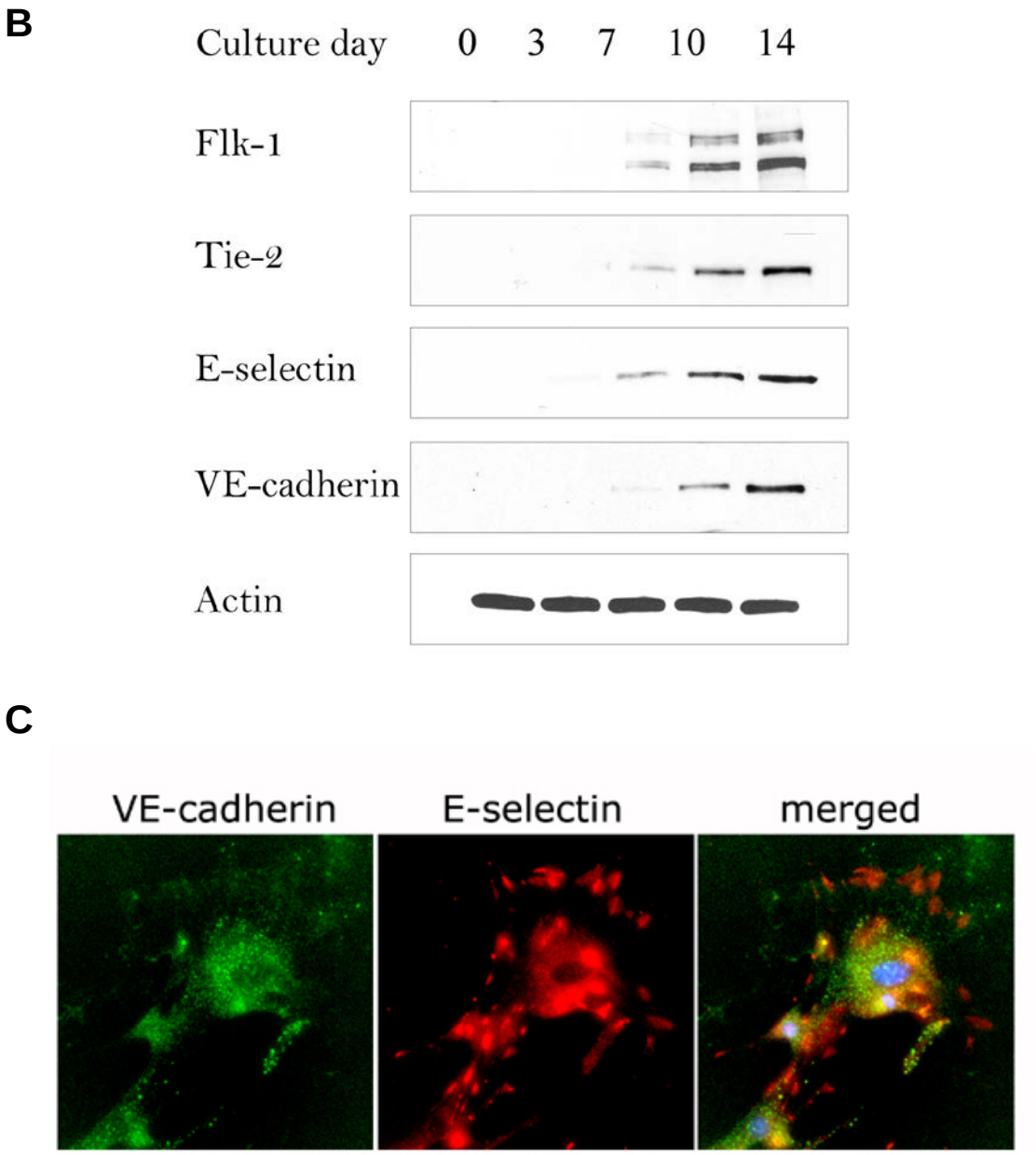


Figure 6. **Expression pattern of BM-derived EPC surface markers.**

A. Histograms of stem and EC marker expressions on day 0, 3, 7, 10 and 14 cultured cells. B. Protein level of EC markers in EPC cultures shown by Western blot analysis. C. Immunofluorescence staining for EC markers on day 14 cultured cells. Green, VE-cadherin; Red, E-selectin; Blue, cell nuclei.

2.4.2 VEGI inhibits EC marker expression on BM-derived EPC

To determine the effect of VEGI on the differentiation of BM-derived EPCs, purified Sca-1^+ bone marrow cells were cultured in the presence or absence of VEGI. After 10 days of culture, control cells differentiated into elongated spindle shaped cells reminiscent of ECs and adhered to the culture plates. Meanwhile, the majority of VEGI-treated cells maintained a small circular appearance, still resembling bone marrow cells (Figure 7). Accordingly, VEGI treatment impacted the expression of EPC surface markers. In control cell cultures, Flk-1 and E-selectin expression significantly increased from day 3 to day 10. However, cells treated with VEGI did not display the same increased expression of Flk-1 and E-selectin. There was no difference in the expression of stem cell marker, Sca-1, between the two groups (Figure 8).

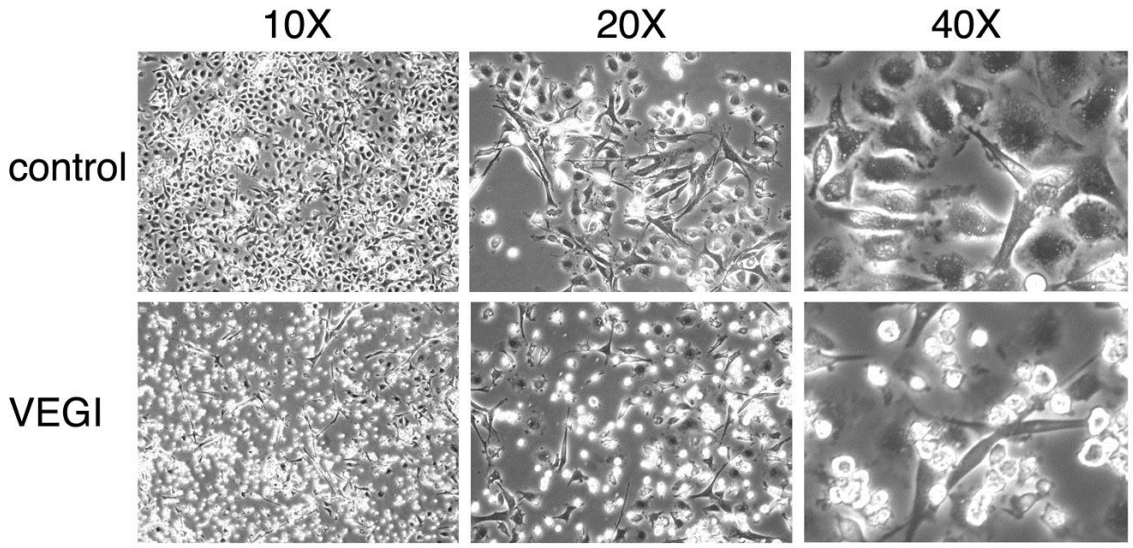


Figure 7. **VEGI inhibits differentiation of BM-derived EPC in culture.**
Phase-contrast images show a morphological difference between cultured EPCs in the absence or presence of VEGI for 10 days.

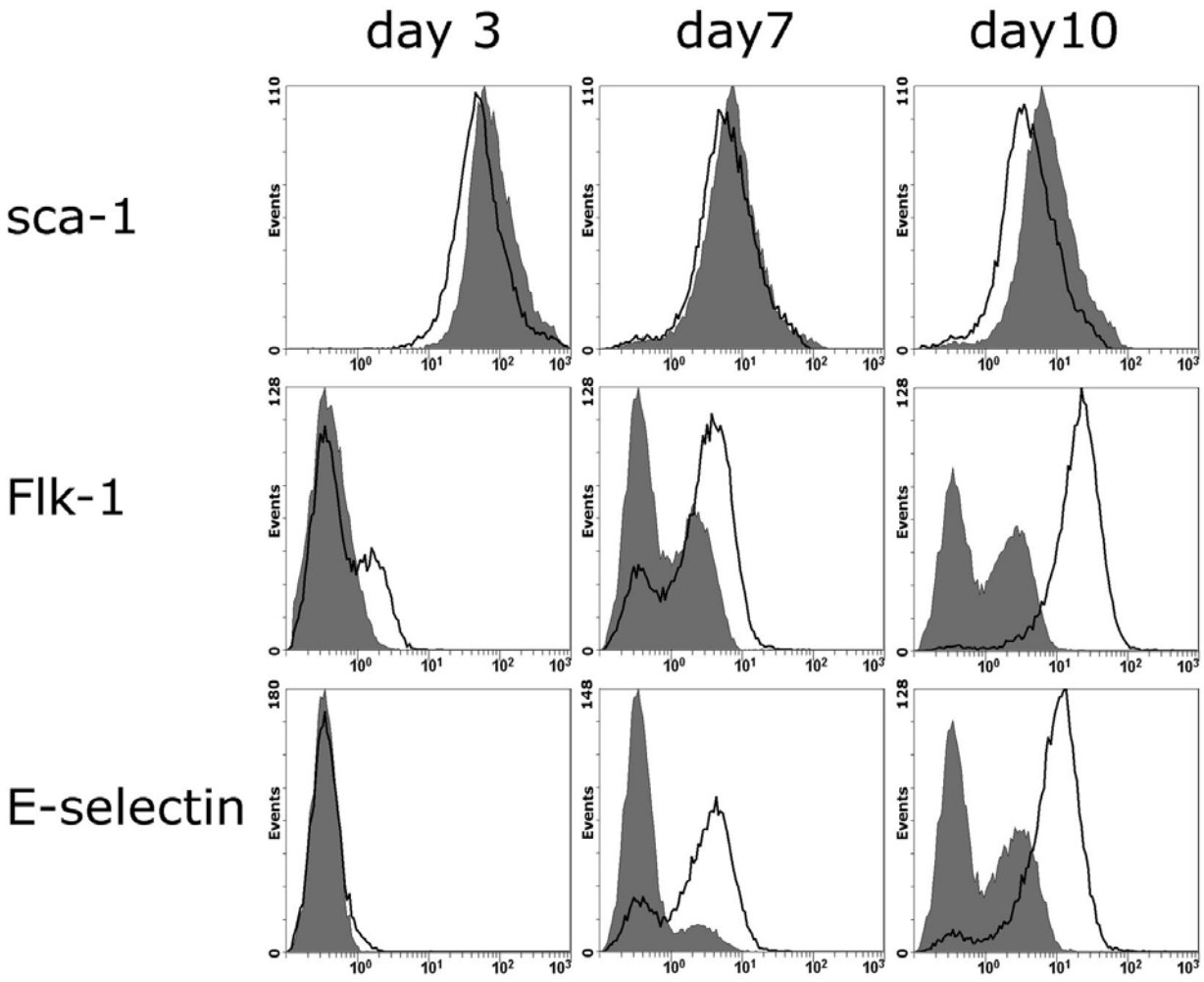
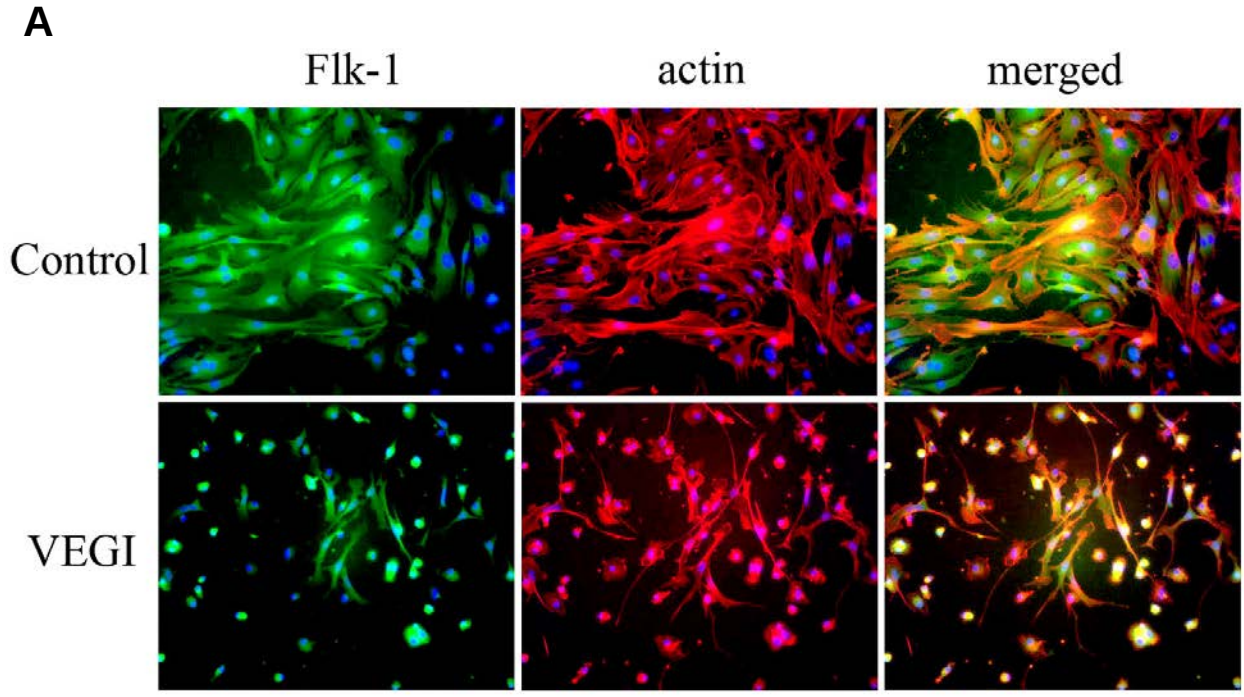


Figure 8. VEGI treatment decreases expression of EC markers.

Histograms from flow cytometric analysis of stem cell marker (Sca-1) and EC marker (Flk-1 and E-selectin) expression on BM-derived EPCs cultured in the absence or presence of VEGI. White areas, untreated; Shaded areas, VEGI-treated.

To confirm the change found in surface marker expression resulting from VEGI treatment, we immunostained day 3 and day 10 cultures with fluorescence-conjugated antibodies. We labeled multiple surface markers, including Flk-1, with their respective antibodies, as well as labeled F-actin to examine the cytoskeleton (Figure 9A). Analysis of the fluorescence intensity of stem cell markers Sca-1 and AC133 reveals that from day 3 to day 10, stem cell marker expression is decreased in both control and VEGI-treated cultures. However, while expression of EC markers Flk-1, Tie-2, E-selectin and VE-cadherin was found to increase in control cells, VEGI treatment significantly inhibited the expression of EC markers in day 10 cultures. Compared to untreated control cells, VEGI treatment resulted in a 50% decrease of EC marker expression, on average (Figure 9B). These results indicate that VEGI inhibits the differentiation of BM-derived EPC in culture by down-regulating EC gene expression.



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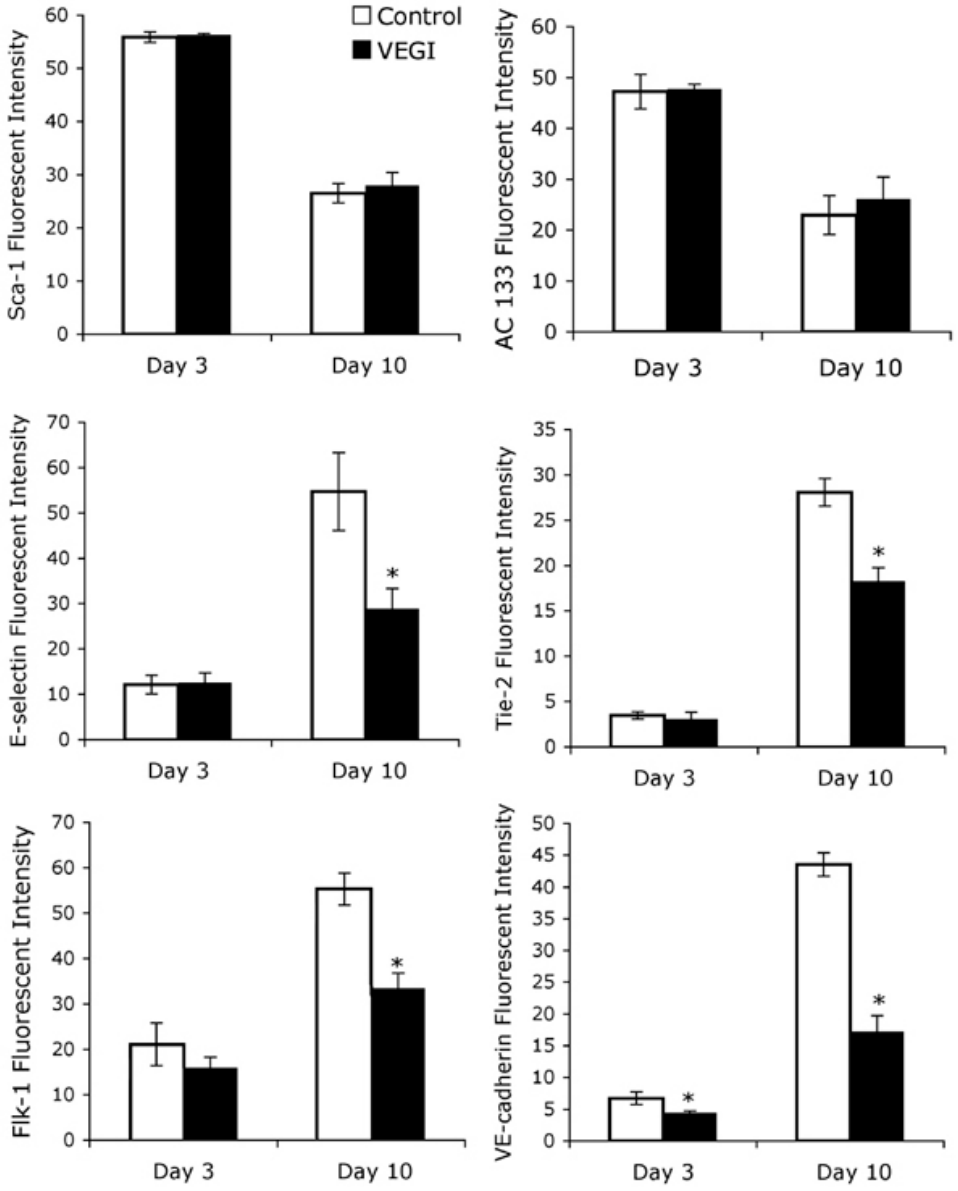
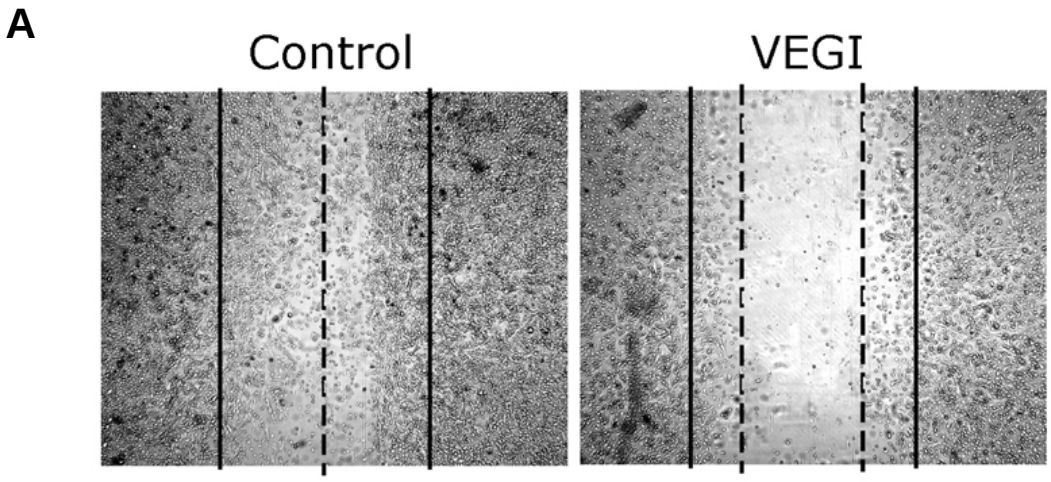


Figure 9. Analysis of fluorescence intensity of EPC markers.

A. Confocal microscopic images of immunofluorescence stained EC marker Flk-1 on EPCs cultured for 10 days in the absence or presence of VEGI (20x objective lens). Green, Flk-1; Red, actin; Blue, cell nuclei. B. A total of 3000 cells per well were analyzed for fluorescence intensity of various immunostained stem and EC markers.

2.4.3 VEGI inhibits EPC migration and formation of capillary-like structures

To determine whether VEGI has any impact on the function of BM-derived EPCs, we first examined the ability of the cultured cells to migrate. Sca-1^+ bone marrow cells were cultured in the presence or absence of VEGI for 10 days, then collected and reseeded. Next, utilizing the wound-healing assay, a scratch was made through the cell culture monolayer and cells were allowed to migrate for 48 hours. Control cells were capable of migrating into the wound and repopulating the exposed space. However, VEGI treatment inhibited the migration of the cultured cells, and as a result, VEGI-treated cells were unable to refill the open wound (Figures 10A, 10B). Additionally, the migration ability of EPCs on day 7 was determined using transwell chambers. After 8 hours, we observed that about 25% more cells migrated through the filters in control cultures than in VEGI-treated cultures (Figure 10C).



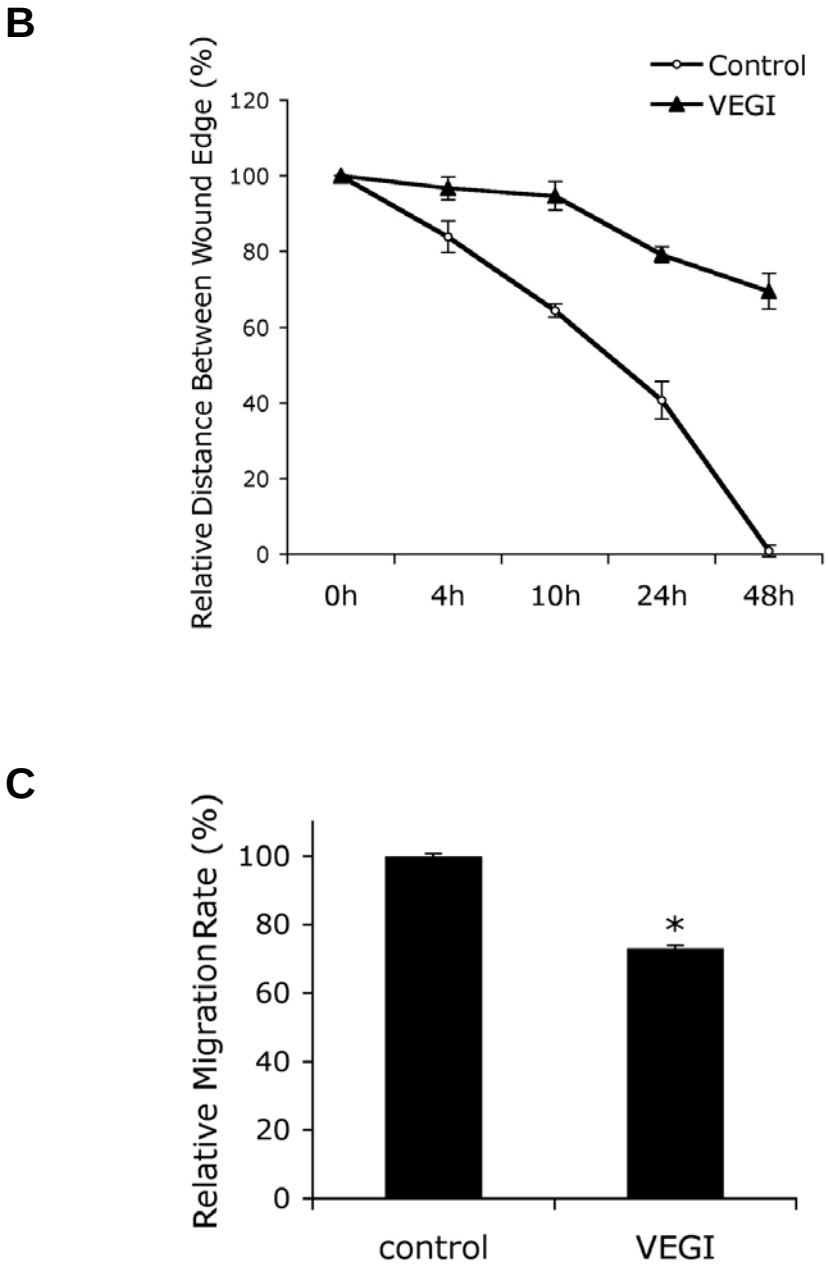


Figure 10. VEGI inhibits BM-derived EPC migration.

A. Phase-contrast images of cell migration into an open wound space. Solid line, wound edge; Dashed line, front edge of cell migration. B. Relative distance between the wound edge to the front edge of the migrating cells. C. Relative migration rate of BM-derived EPCs through a porous barrier in a transwell assay.

Next, we determined the ability of BM-derived EPCs to assemble into vascular networks on a reconstituted basement membrane. BM-derived EPCs in the control group were capable of forming capillary-like structures when placed on Matrigel (Figure 11A). Cells cultured in the presence of VEGI, however, exhibited an inability to form similar structures (Figures 11A, 11B). These results indicate that VEGI has an inhibitory effect on the migration abilities characteristic of differentiated ECs.

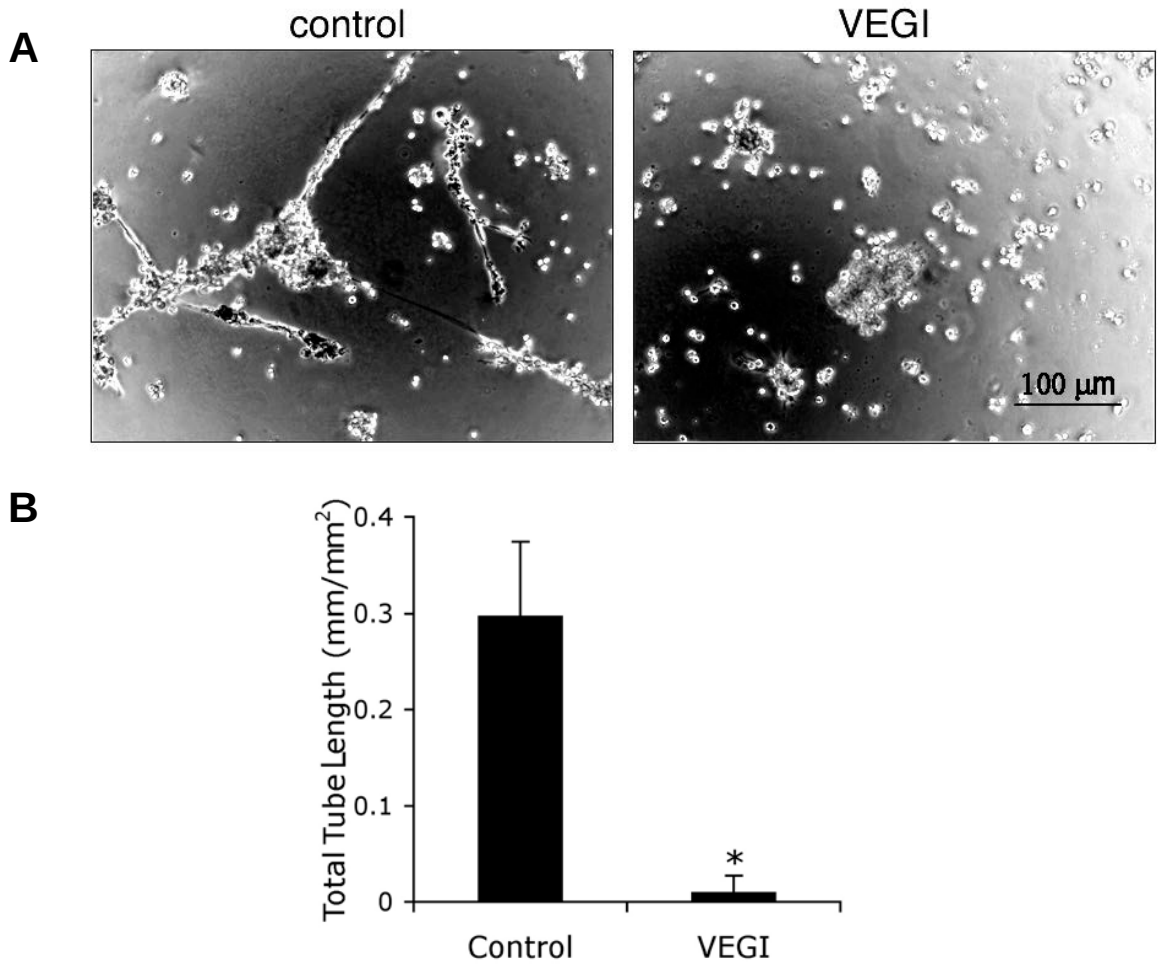


Figure 11. **VEGI inhibits capillary-like tube formation.**

A**.** Phase-contrast images of the formation of capillary-like structures by BM-derived EPCs. Cells cultured for 2 weeks in the absence or presence of VEGI were placed on Matrigel and allowed 24 hours for tube formation. B**.** Measurement of the total length of the capillary-like tubes after 24 hours.

2.4.4 VEGI inhibits EPC from adhering to fibronectin and vitronectin

To analyze the ability of BM-derived EPCs to adhere to a surface, freshly purified Sca-1^+ cells were cultured for one week in the presence or absence of VEGI, then collected and reseeded into culture plates coated with a battery of extracellular matrix proteins. Two hours after reseeding, we found that EPCs adhered best to fibronectin and vitronectin; however, adhesion to both these matrix proteins was reduced significantly in VEGI-treated cells (Figure 12). Additionally, when the cells adhered on fibronectin, the phosphorylation of cell adhesion molecules, FAK, paxillin, and Src, in the untreated control cells was 2-times that of VEGI-treated cells (Figure 13).

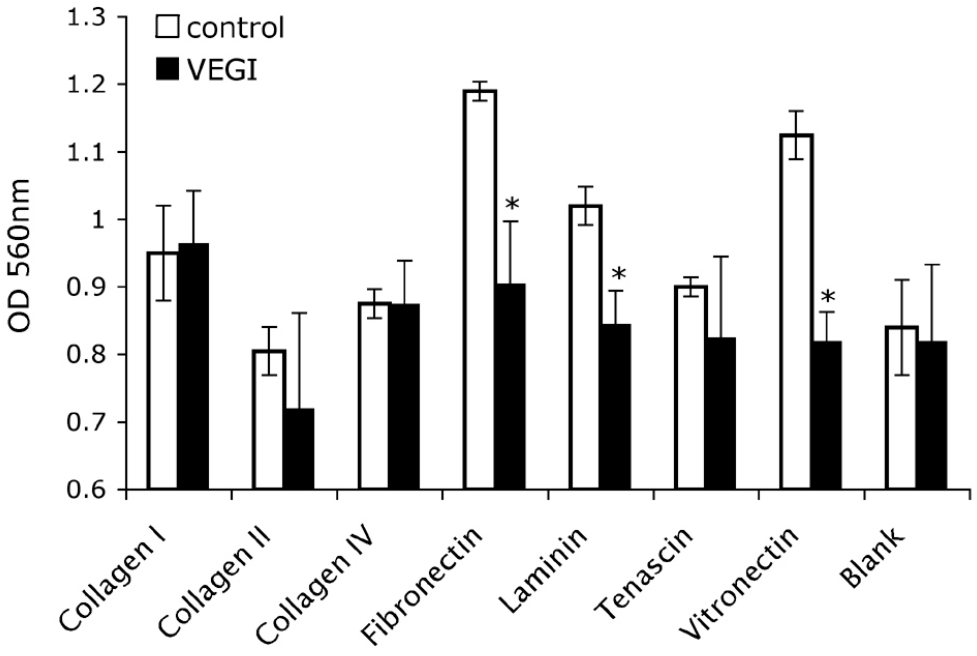


Figure 12. EPC adhesion to extracellular matrix proteins.
The ability of EPCs cultured for 7 days to adhere on surfaces coated with the indicated extra-cellular matrix proteins in the presence or absence of VEGI.

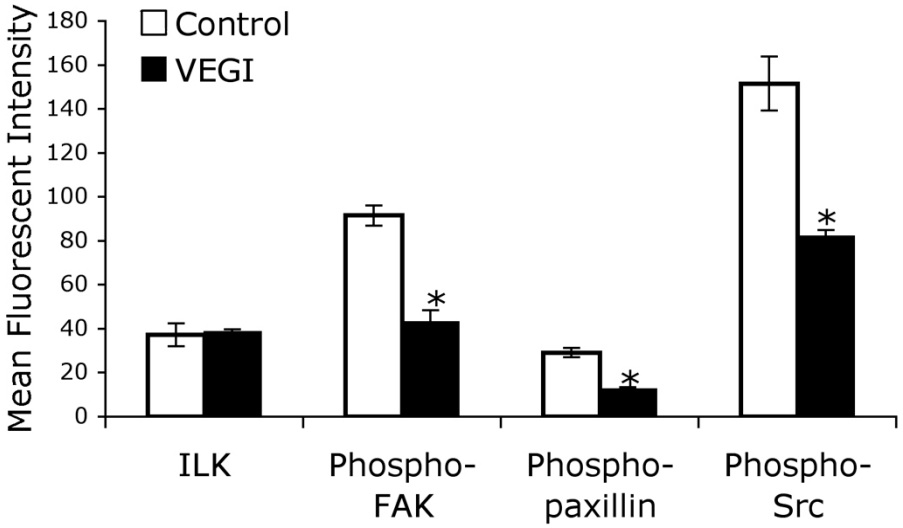


Figure 13. VEGI decreases cell adhesion signaling in EPCs.

EPC cultured for 10 days in the absence or presence of VEGI were immunostained for cell signaling molecules involved in adhesion and fluorescence intensity was analyzed.

We then examined the expression of integrin α5 and αv to see whether the decreased adhesion resulting from VEGI treatment was due to a decline in α5β1 and αvβ3, the receptors of fibronectin and vitronectin. Using immunofluorescence staining, we determined that morphologically, the integrin proteins formed clusters in focal adhesion structures only in the control group (Figure 14A). We analyzed the fluorescence intensity of the cells and found a significant decrease of integrin expression in VEGI-treated cultures. VEGI treatment yielded a 3-fold decrease in α5 expression and a 2-fold decrease in αv expression compared to controls (Figure 14B). These results suggest that VEGI inhibits BM-derived EPCs adhesion by inhibiting activation of signaling molecules and receptors involved in cell adhesion.

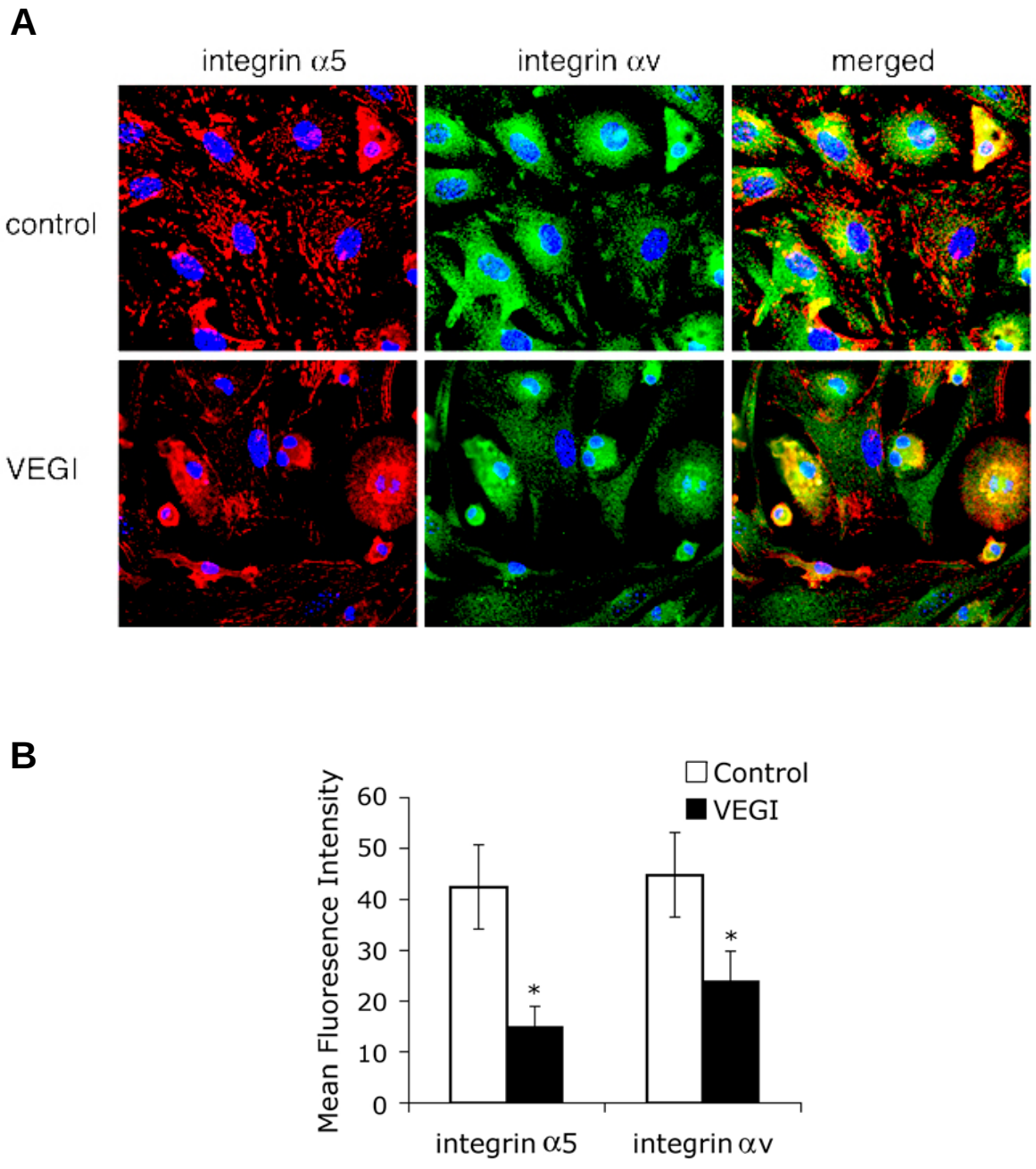


Figure 14. VEGI inhibits integrin expression on BM-derived EPCs.

A. Confocal microscopic images of immunofluorescence staining for integrins on EPCs cultured for 10 days in the absence or presence of VEGI. Green, integrin αv; Red, integrin α5; Blue, cell nuclei. B. Fluorescence intensity of integrins α5 and αv immunostaining.

2.4.5 Effect of VEGI on EPC viability and apoptosis

Given the diminished ability of BM-derived EPC to adhere when treated with VEGI, we examined the number of cells remaining suspended vs. becoming adhered in the presence of VEGI over a two-week culture. There is an increased number of suspended cells in VEGI-treated cultures during the first week (Figure 15A), but no difference was seen in the number of adhered cells (Figure 15B). During the second week of culture, while the number of suspended cells in both groups decline (Figure 15A), only the cells in the control group became adherent. In contrast, the number of adhered cells in the VEGI-treated group decreases substantially (Figure 15B).

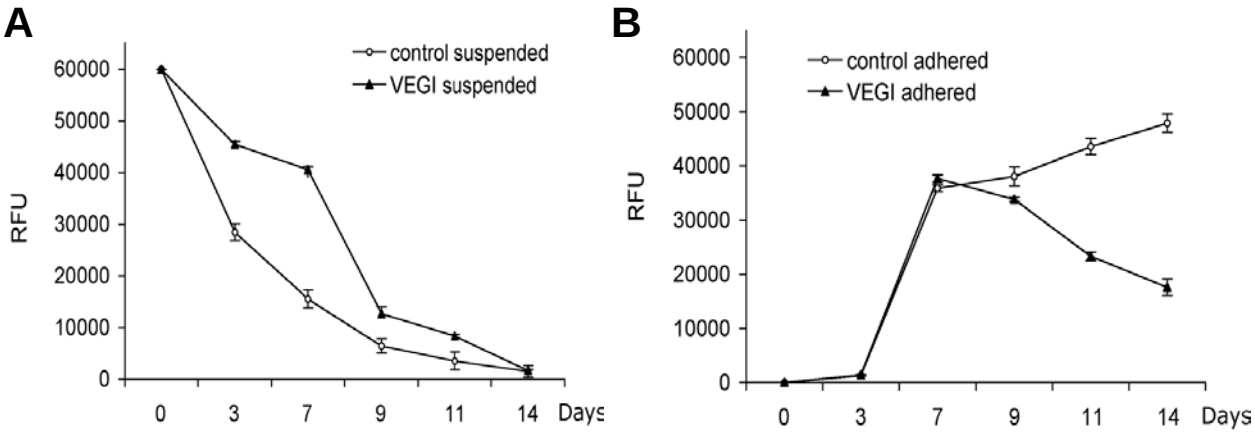


Figure 15. VEGI inhibits BM-derived EPC adhesion in culture.

A. The number of suspended cells and B. the number of adhered cells in EPC cultures measured by relative fluorescence units (RFU) of Calcein-AM staining.

To investigate the cause of the diminishing population of suspended and adhered cells in the VEGI-treated cultures, we analyzed the amount of apoptosis occurring in the cell cultures after one week. We stained the suspended and adhered cell population of control and VEGI-treated cultures for E-selectin to assess EPC differentiation and TUNEL to assess apoptosis (Figure 16).

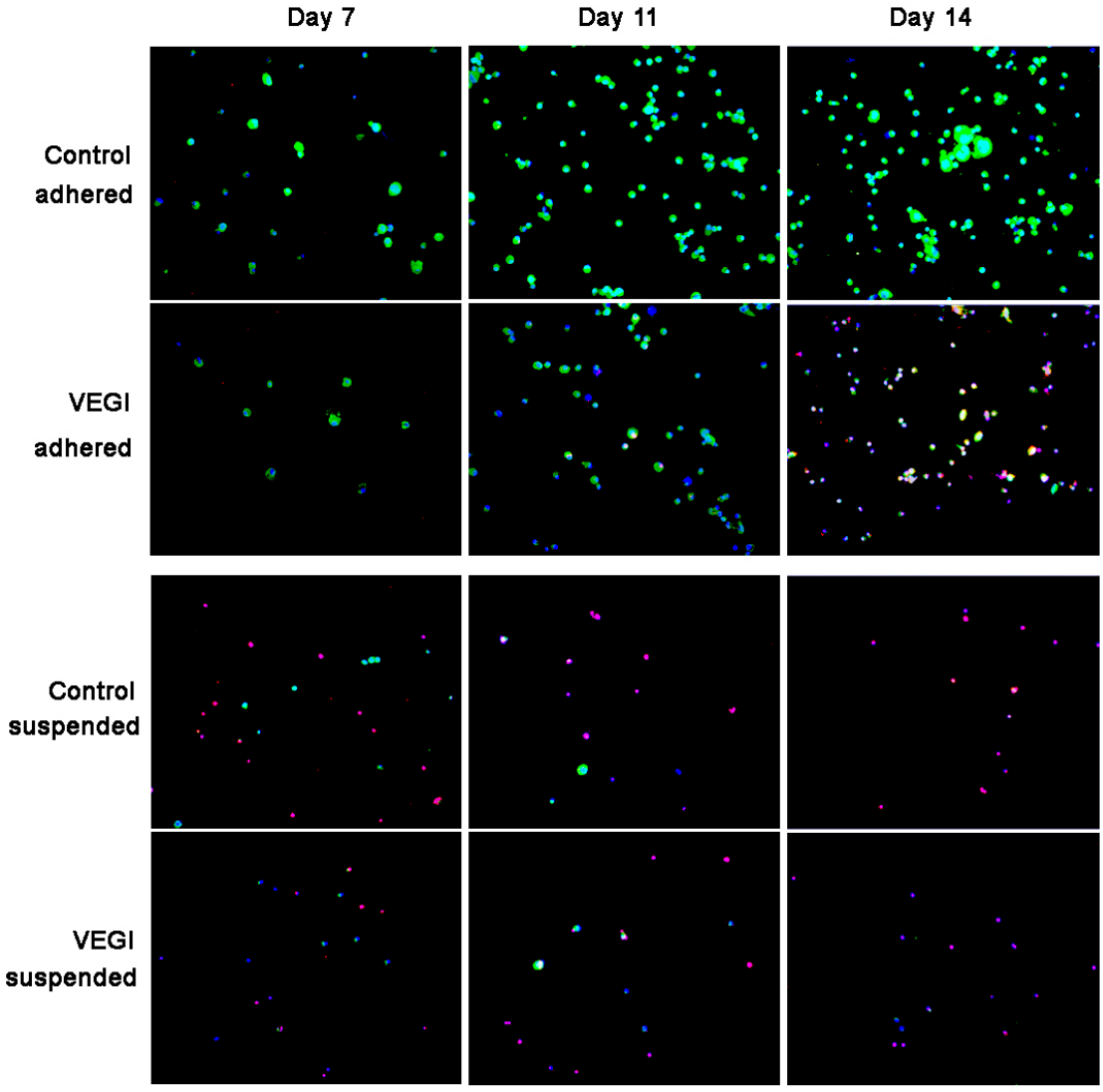


Figure 16. Relationship between EPC differentiation and apoptosis.
Fluorescence images of suspended and adherent cells undergoing differentiation or apoptosis at various time points as indicated. Red, TUNEL; Green, E-selectin; Blue, cell nuclei.

The total percentage of apoptosis in the adhered cell population of both the control group and VEGI-treated group was very low on day 7. However, while apoptosis rates in the control group did not increase, apoptosis rates in the VEGI-treated group increased 40% and 95% on days 11 and 14, respectively (Figure 17). In the suspended cell population, apoptotic cells were present regardless of VEGI-treatment. Interestingly, there was significantly less apoptosis in the VEGI-treated group only on day 7 (Figure 17).

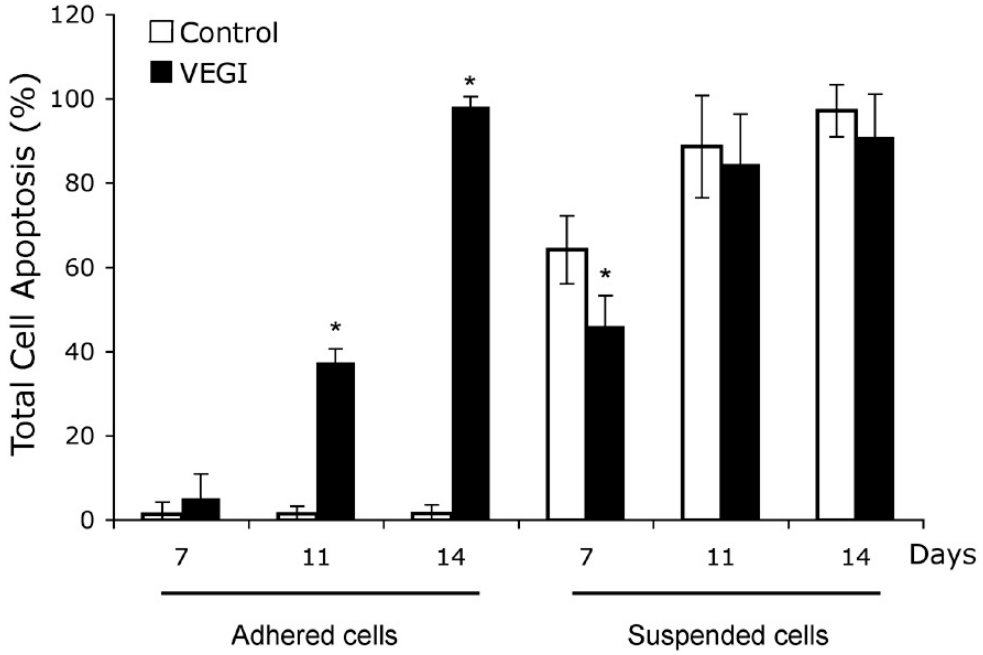


Figure 17. VEGI induces apoptosis of adhered cells.

Apoptosis rates of suspended cells and adhered cells in the presence or absence of VEGI, analyzed at various time intervals as indicated.

When we examined the fluorescence intensity of E-selectin, we found that in adhered cells, the expression level increased in control cultures from day 7 to 14. However, treatment with VEGI significantly prevented the expression of E-selectin compared to control cells (Figure 18). There was a low level of E-selectin in all suspended cells, suggesting this population of cells did not differentiate into EPC. Interestingly, the expression of E-selectin on day 7 suspended cells was significantly lower in the VEGI-treated group (Figure 18).

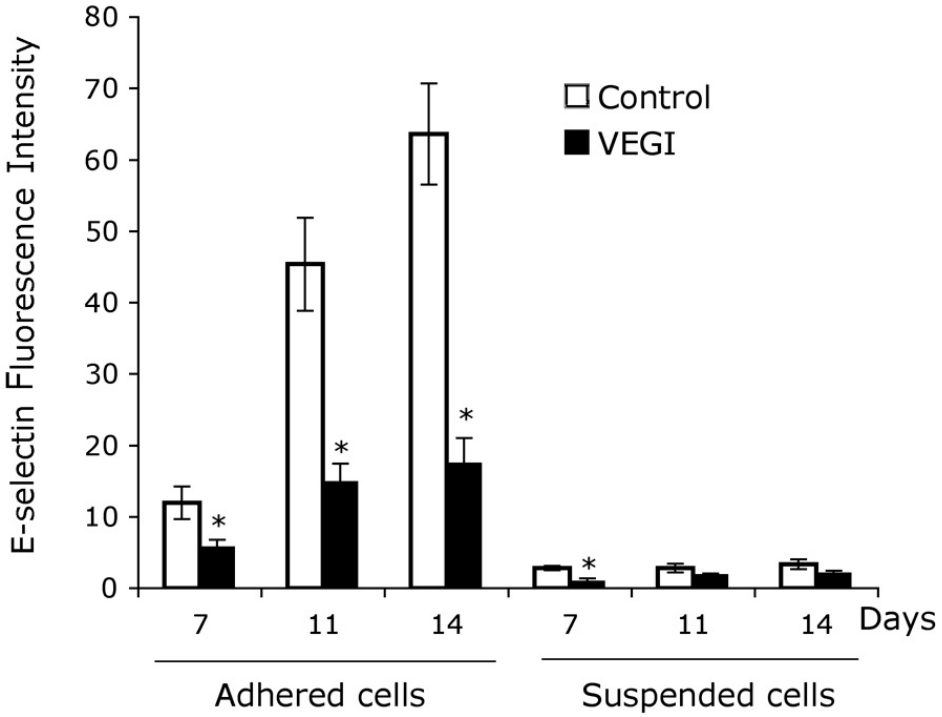


Figure 18. VEGI downregulates expression of E-selectin in adhered cells.
Fluorescence intensity of E-selectin positive EPCs analyzed at various time intervals as indicated.

Since we saw a marked decrease in apoptosis and E-selectin levels in day 7 suspended cells of the VEGI-treated group, we further analyzed the relationship between apoptosis and E-selectin expression on this suspended cell population (Figure 19). We found that in untreated controls, E-selectin positive cells did not undergo apoptosis, but E-selectin negative cells were almost completely apoptotic. Conversely, in VEGI-treated cells, E-selectin positive cells had a significant increase in apoptosis rates and E-selectin negative cells had a significant decrease of apoptosis rates (Figure 19). Together, these results demonstrate that VEGI has a slight protective effect on early-stage undifferentiated cells, but induces apoptosis of late-stage differentiated EPCs.

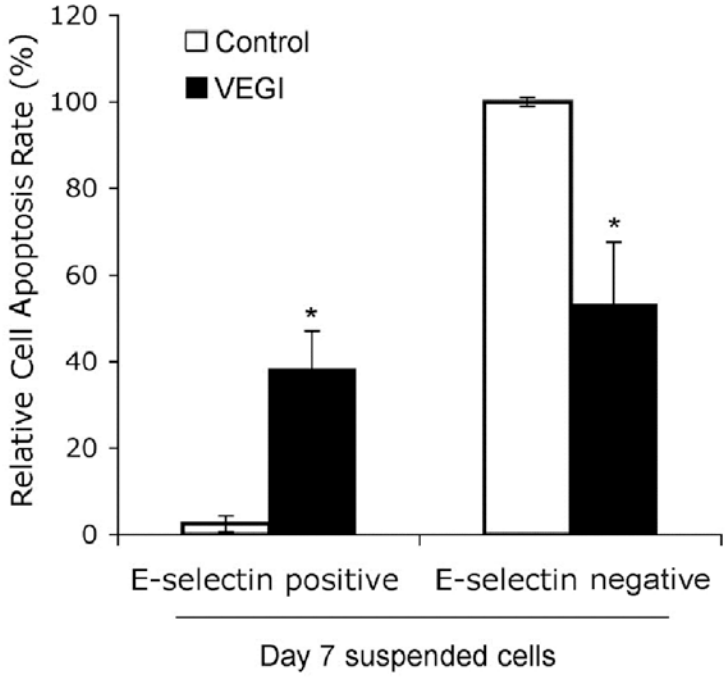


Figure 19. VEGI specifically induces apoptosis of E-selectin positive cells.
Apoptosis rates of E-selectin positive or negative cells in EPC cultures on day 7.

2.4.6 VEGI activates caspase-3 in late stage EPC

In order to determine the molecular mechanism of VEGI induced apoptosis on late stage BM-derived EPCs, we first examined expression levels of DR3 on differentiating EPC cultures. Western blot analysis revealed DR3 begins to be expressed around day 6 and increases markedly as BM-derived EPCs continue to differentiate toward ECs (Figure 20).

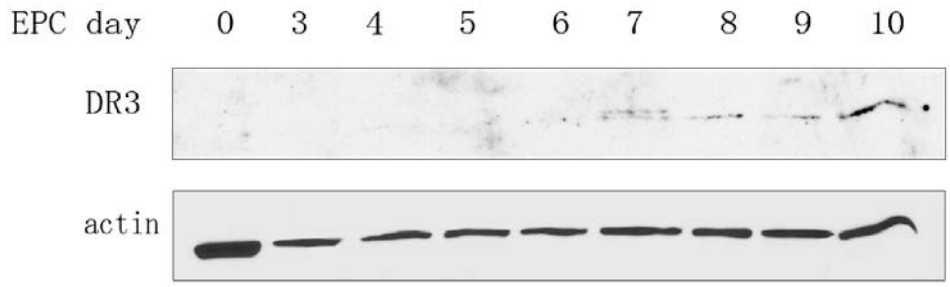


Figure 20. BM-derived EPCs express DR3.
Western blot showing increased DR3 expression in BM-derived EPC over 10 days of culture.

The increase in DR3 expression coincides with the increase of apoptosis seen in BM-derived cells in response to VEGI. To determine whether DR3 mediates the apoptotic effect in response to VEGI treatment, we examined caspase-3 activity to measure apoptosis of BM-derived EPCs cultured for 12 days. Caspase-3 activity was very low in EPC cultures on days 4 and 6 at all dosages of VEGI treatment. In contrast, there was a significant dose-dependent increase of caspase-3 activation on days 8 and 12 (Figure 21A). Using antibodies that neutralize DR3, caspase-3 activation in response to VEGI treatment was inhibited on days 8 and 12, while no effect was seen on days 4 and 6 (Figure 21B). These results indicate that DR3 likely mediates the apoptotic activity of VEGI on differentiated BM-derived EPCs.

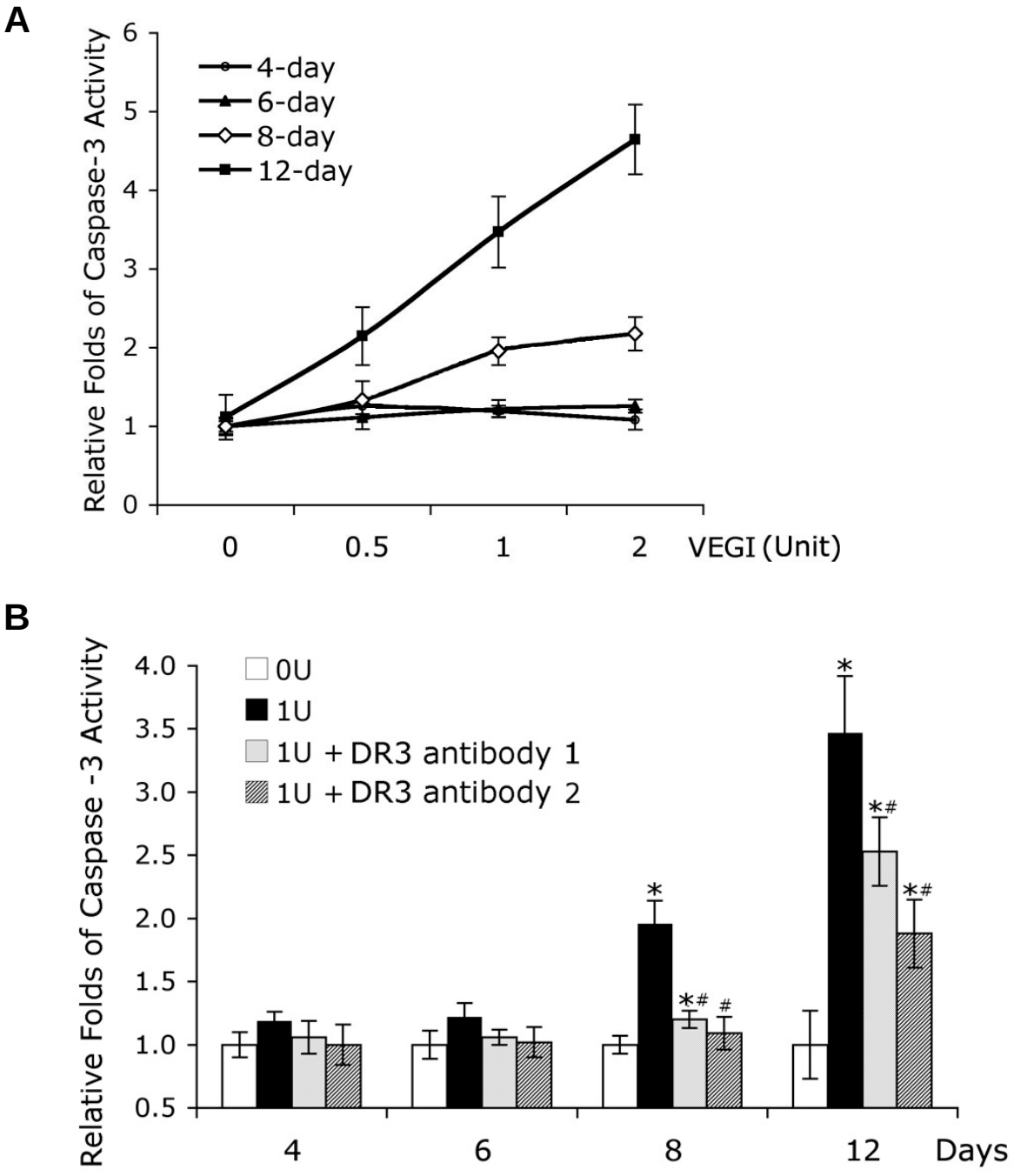


Figure 21. VEGI-induced apoptosis mediated by DR3.

A. Caspase-3 activation in EPC cultures induced with various concentrations of VEGI. B. Inhibition of VEGI-induced caspase-3 activation using DR3 neutralizing antibodies. Asterisk indicates $P < 0.05$ compared with untreated cells. Pound sign indicates $P < 0.05$ compared with VEGI-treated cells.

2.4.7 VEGI inhibits Akt, activates Erk and p38 in early stage EPC

To understand the molecular mechanism underlying the activity of VEGI on early stage cells, the activation of three important MAPKs (ErK, p38 and Akt) which modulate cell differentiation and growth was determined. We treated freshly purified Sca-1+ bone marrow cells with VEGI for 15 minutes to examine immediate early responses. We found a dose-dependent increase of phosphorylated Erk and p38 (Figure 22). In contrast, there was a dose-dependent inhibition of Akt phosphorylation (Figure 22). These findings indicate that VEGI activates cell growth signals and inhibits cell differentiation signals in early stage EPC cultures.

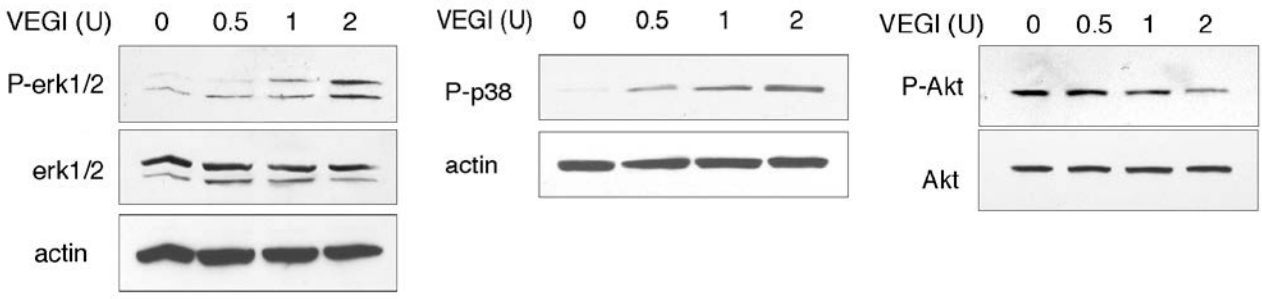


Figure 22. Cell signaling changes induced by VEGI treatment in early EPCs.
Western blots showing the phosphorylation of Erk, Akt or p38 in freshly purified Sca-1^{+} cells treated with various concentrations of VEGI for 15 minutes.

2.5 CONCLUSIONS

Neovascularization occurs via two pathways, angiogenesis and vasculogenesis. It has been shown that the process of vasculogenesis involves numerous growth factors that stimulate BM-derived EPC differentiation and migration to sites requiring vascularization [81, 89, 161, 162]. However, inhibitors of BM-derived EPC modulation are not well known. The discovery of an inhibitor of BM-derived EPCs is of value to understanding the regulation of post-natal vasculogenesis supported by EPCs. The data we show here indicate that VEGI, an endogenous cytokine, can inhibit differentiation of mouse BM-derived EPCs in culture, resulting in disrupted function of these cells and preventing formation of new vascular structures.

Bone marrow stem cells cultured under EC growth conditions can be divided into two distinct populations of cells, early and late stage EPCs. Early stage EPCs are also known as bone marrow EPCs, immature EPCs or vascular stem cells [31, 48, 163]. During the initial days of culture, we observe that most early stage EPCs are suspended in the culture medium and express a low level of EC surface markers. As cells differentiate toward late stage EPCs, they become adhered to the culture plates and express increased levels of EC markers Flk-1, Tie-1, E-selectin, and VE-cadherin, while their overall stemness decreases. Accordingly, the morphology of the cells elongate to resemble spindle shaped ECs. However, VEGI treatment inhibits the differentiation of early stage to late stage EPCs. We found that VEGI-treated cells do not express increased EC markers like their control counterparts, nor do they change morphologically. Compared to control cells, the level of EC marker expression in VEGI-treated cells was about 30% to 50%, indicating VEGI significantly inhibited BM-derived EPC differentiation.

Besides the observed inhibitory effect on morphology and EC marker expression, cells treated with VEGI also displayed decreased EPC functionality. VEGI-treated cells had a dramatic inhibition in migration across both a flat surface and through a porous membrane. In conjunction, VEGI-treatment resulted in almost a complete abolished ability of the cells to form capillary-like tubes on Matrigel. Moreover, the ability of VEGI-treated cells to adhere to extracellular matrix proteins, fibronectin and vitronectin, declined significantly as well. The stunted adhesion capability is likely due to the concurrent VEGI-induced inhibition of integrin $\alpha5\beta1$ and $\alpha v\beta3$ expression, which are the known receptors of fibronectin and vitronectin, and the inhibition of phosphorylated adhesion signaling molecules, FAK, paxillin, and Src. Inhibiting integrin expression may be one mechanism by which VEGI negatively modulates BM-derived EPC differentiation, as integrins have been shown to play an important role in EPC differentiation and homing [164-166].

By inhibiting adhesion in EPC cultures, VEGI-treatment maintains early stage EPCs in a suspended state. We found that many of the suspended cells found in day 7 cultures are also devoid of E-selectin expression, indicating the cells are undifferentiated. Additionally, these cells appear to undergo less apoptosis on day 7 compared to suspended control cells, indicating VEGI may have a transient protective effect on early stage undifferentiated EPCs. Conversely, VEGI treatment induced apoptosis in the adhered cell population. While the adhered cells in untreated controls remained viable and expressed high levels of E-selectin, indicating the cells had differentiated into EPCs, the adhered cells in VEGI-treated cultures had up to a 100% apoptosis rate by day 14, therefore inhibiting late stage EPC differentiation. These results indicate early stage and late stage EPCs have very different responses to VEGI.

The apoptosis activity in late stage EPCs as a result of VEGI treatment may be mediated by DR3, shown to be the receptor of VEGI in certain immune cells [133, 159]. We examined DR3 expression in EPCs and found that DR3 begins to emerge around one week of culture and continues to increase substantially by day 10. This timing of DR3 expression coincides with the onset of increased EPC apoptosis rates in VEGI-treated cultures. Furthermore, VEGI treatment induced caspase-3 activity only in EPCs cultured for more than 6 days. More importantly, antibodies that neutralize DR3 were able to inhibit caspase-3 activity in late stage EPCs treated with VEGI.

The mechanism behind the inhibitory activity observed in early stage EPCs appears to be DR3 independent, as early stage EPCs have not yet begun to express DR3. VEGI not only inhibits differentiation of early stage EPCs, but also protects them from undergoing apoptosis to a certain degree. As a result, we examined protein kinases involved in cell differentiation and proliferation. When we cultured freshly purified bone marrow stem cells for 15 minutes in the presence of VEGI, we were able to see an immediate dose-dependent decrease in the phosphorylation of Akt, which is known to be important for EPC homing and differentiation [99]. Therefore, the diminished Akt signaling may contribute to the inhibition of early EPC differentiation. When we examined Erk and p38 phosphorylation in the same conditions, we found a significant activation of both these MAPKs by VEGI. As Erk promotes cell proliferation and survival, activation of this kinase may provide the protective effect found on early stage undifferentiated EPCs. Meanwhile, p38 has been shown to inhibit EPC numbers [101]. Thus p38 activation may be an additional factor in inhibiting early stage EPC differentiation. These data suggest that the inhibitory effect of VEGI on the differentiation and apoptosis of early stage EPCs results from decreased Akt and increased Erk and p38 signaling.

In summary, this study demonstrates that VEGI has an inhibitory effect on the differentiation of BM-derived EPCs toward ECs. Our findings indicate that VEGI negatively modulates BM-derived EPCs by down-regulating EC marker expression in early stage EPCs and inducing apoptosis in late stage EPCs. In addition, VEGI inhibits the ability of BM-derived EPCs to adhere, migrate, and form capillary-like structures. Furthermore, we show that the inhibitory activity of VEGI is likely mediated by DR3 in late stage cultures and changes in cell signaling molecules in early stage cultures. These findings support the view that VEGI plays a role in regulating postnatal vasculogenesis by modulating BM-derived EPC differentiation.

3.0 VEGI INHIBITS BM-DERIVED EPC SUPPORTED TUMOR VASCULOGENESIS

3.1 ABSTRACT

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) have a critical role in tumor neovascularization. Vascular endothelial growth inhibitor (VEGI; TL1A) is a member of the TNF superfamily (TNFSF15). We have shown that recombinant VEGI suppresses tumor angiogenesis by specifically eliminating proliferating endothelial cells (ECs). We report here that treatment of tumor bearing mice with recombinant VEGI leads to a significantly decreased population of BM-derived EPCs in the tumors. We transplanted whole bone marrow from green fluorescent protein (GFP) transgenic mice into C57BL/6 recipient mice, which were then inoculated with Lewis lung carcinoma (LLC) cells. Intraperitoneal injection of recombinant VEGI led to significant inhibition of tumor growth and decrease of vasculature density compared to vehicle-treated mice. Tumor implantation yielded a decrease of BM-derived EPCs in the peripheral blood, while VEGI treatment resulted in an initial delay of such decrease. Analysis of the whole bone marrow showed a decrease of Lin^{-}-c-Kit^{+}-$Sca\text{-}1^{+}$ hematopoietic stem cell (HSC) population in tumor bearing mice; however, VEGI treatment caused a significant increase of this

cell population. In addition, the number of BM-derived EPC in VEGI-treated tumors was notably less than that in the vehicle-treated group, and most of the apoptotic cells in the VEGI-treated tumors were of bone marrow origin. These findings indicate that VEGI inhibits BM-derived EPC mobilization and prevents their incorporation into LLC tumors by inducing apoptosis specifically of BM-derived cells, resulting in the inhibition of EPC-supported tumor vasculogenesis and tumor growth.

3.2 INTRODUCTION

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) play a critical role in vasculogenesis, the *de novo* formation of new blood vessels which is essential for organ and tissue growth, and wound healing [11, 17, 18, 67, 82]. Abnormal neovascularization under disease conditions, such as that in cancer development, also involves postnatal vasculogenesis [4, 30]. Early theories of tumor neovascularization revolved solely around angiogenesis being the source for the tumor's vascular supply [22, 29]. New evidence shows that EPCs migrate from the bone marrow to the tumor site and differentiate into a new endothelium in the tumor bed, providing an alternative method of tumor neovascularization [19, 31, 68, 72, 81]. Contributions of EPCs to tumor neovasculature are evident from increased EPC markers in tumors of cancer patients [30, 32, 33]. It has also been shown that EPCs activate the "angiogenesis switch," a critical step in the transition of an avascular, dormant tumor to a vascularized, rapidly growing tumor [34].

Vascular endothelial growth inhibitor (VEGI; TNFSF15; TL1A) is a member of the tumor necrosis factor superfamily and an endogenous inhibitor of neovascularization [126].

VEGI is largely produced by vascular endothelial cells (ECs) of established blood vessels in a normal tissue, and is a specific inhibitor of EC proliferation, apparently playing a role in the modulation of vascular homeostasis. More specifically, VEGI has the ability to enforce growth arrest of ECs in G0 and G1 phases of the cell cycle, while inducing apoptosis of proliferating EC, leading to inhibition of angiogenesis [138, 155]. While VEGI is expressed in the vascular EC of normal human adult tissues, it is absent or expressed at low levels in tumor vasculatures in breast cancer [134], prostate cancer [147], urothelial cancer [148], as well as during wound healing [143], supporting the view that VEGI is a negative regulator of neovascularization whose expression needs to be turned down prior to the initiation of physiological or pathological blood vessel growth. Consistently, recombinant VEGI has been shown to be a highly potent inhibitor of EC proliferation in tumors, resulting in specific elimination of ECs in a tumor vasculature and inhibition of tumor growth [127, 131, 137].

In this study, we examine whether VEGI can inhibit BM-derived EPC-supported vasculogenesis in a Lewis lung carcinoma (LLC) tumor model. We found that systemically administered recombinant VEGI can inhibit the incorporation of BM-derived EPCs into LLC tumors and the differentiation of EPCs into ECs in these tumors. These findings provide important insights into the mechanism of modulation of EPC-supported vasculogenesis by this unique cytokine and endogenous inhibitor of angiogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Antibodies and reagents

Fluorochrome-conjugated anti-mouse Flk-1, CD133, c-Kit, and Sca-1 antibodies were from eBioscience (San Diego, CA). EasySep Mouse Hematopoietic Progenitor Enrichment Cocktail for lineage selection was from Stem Cell Technologies (Vancouver, BC, Canada). Anti-mouse CD31 antibody was from BD Biosciences (San Jose, CA). Cy3 and Cy5 conjugated secondary antibodies were from Jackson ImmunoLabs (West Grove, PA). Terminal Transferase, recombinant, kit for TUNEL staining was from Roche Applied Science (Indianapolis, IN). VEGI isoform VEGI-192 was prepared as described [131]. The endotoxin level in the VEGI preparation is 25 ng/mg.

3.3.2 Cells

Lewis lung carcinoma (LLC) cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA).

3.3.3 Mice

Eight week old female C57BL/6 mice (wild-type mice) and C57BL/6 EGFP10sb/J mice (transgenic mice that express GFP in all cell types) were purchased from the Jackson Laboratory (Bar Harbor, ME). All procedures involving experimental animals were performed in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.3.4 Bone marrow transplantation and engraftment analysis

Whole bone marrow from C57BL/6 EGFP10sb/J donor mice was harvested by flushing the femurs and tibias of adult animals. C57BL/6J recipient mice were lethally irradiated with 9 Gray of a cobalt source. After lethal irradiation, the recipient mice received 5×10^6 whole bone marrow cells via lateral tail vein injection. One month after bone marrow transplant (BMT), blood was obtained from the tail veins and engraftment was verified by flow cytometric analysis to confirm stable and complete chimerism, as indicated by >85% of GFP^+ cells in peripheral blood.

3.3.5 Tumor inoculation and VEGI administration

One month after BMT, the chimeric mice were injected subcutaneously with 5×10^6 LLC suspended in phosphate-buffered saline (PBS, Lonza). Tumors were allowed to grow for 4 days and measured in a blinded manner with a dial caliper. The tumor volumes were determined using the formula, volume = width x width x length x 0.52. The experimental animals were then

randomized and divided into two groups on day 4. The treatment group received one daily i.p. injection of VEGI while the control group received comparable injections of the vehicle (on days 4, 5 and 6 for a total of three treatments). The experimental animals were sacrificed on day 7. Tumor tissues, peripheral blood and bone marrow were collected for pathologic analysis.

3.3.6 Flow cytometry

Cells were harvested (for peripheral blood analysis samples, 100 µl of blood was collected from the tail vein of each experimental mouse, and for bone marrow samples, whole bone marrow was collected from femurs and tibia via bone marrow flush) and lysed with ACK cell lysis buffer (Lonza). Cells were then washed with 2 mL of fluorescence-activated cell sorter (FACS) buffer (1% bovine serum albumin and 0.05% sodium azide in PBS). The cells were collected by centrifugation, resuspended in 100 µl of FACS buffer containing 1 µg of the indicated antibody, dispensed in a minimum of 1×10^5 cells/sample, gently mixed, and incubated at room temperature for 15 minutes. The cells were washed with FACS buffer, centrifuged, resuspended in 0.5 mL PBS and analyzed within 1 hour. Coulter FACS equipment and EXPO analysis software (Beckman Coulter, Fullerton, CA) were used.

3.3.7 Immunohistochemistry

Tumors were fixed with 2% paraformaldehyde in PBS at room temperature for 2 hours, then cyroprotected by transferring to a 30% sucrose solution overnight at 4°C. Samples were flash frozen in liquid nitrogen cooled 2-Methylbutane for 30 seconds, then immersed in liquid nitrogen for an additional 10 second and stored at -80°C until cryostat sectioning. Tumor sections (8 µm

thickness) were immunostained by first being blocked for non-specific antibody binding with 2% BSA, followed by incubation with primary antibodies for 1 hour at room temperature. After 5 washes with PBS, sections were incubated with secondary antibodies for 1 hour at room temperature and then washed again with PBS. After nuclei staining with DAPI, sections were mounted. Immunostained sections from peripheral and central regions of each tumor were imaged using a 60x oil objective on a Fluo-view 1000 confocal microscope (Olympus, Center Valley, PA). A minimum of 10 fields/section and 10 sections/tumor sample were analyzed.

3.3.8 Detection of apoptotic cells

Cryosections were washed 3x with PBS and incubated with TUNEL mixture at 37°C for 30 minutes, washed with PBS, then streptavidin secondary antibody was added for 30 minutes at room temperature. DAPI was used to stain for the cell nucleus and slides were mounted before imaging.

3.3.9 Statistical analysis

At least three repeat experiments (n=3-5 per group) were performed yielding similar results. Results shown are of 1 experiment. Student's t-test for independent samples was used to compare data between experimental groups. All data presented as the mean ± SE; $P<0.05$ was considered statistically significant and is indicated with an asterisk.

3.4 RESULTS

3.4.1 VEGI inhibits tumor growth and vascularization

Female mice (C57BL/6) with transplanted bone marrow from green fluorescent protein (GFP) transgenic mice were inoculated on day 0 with LLC cells subcutaneously ($5x10^6$ cells per injection). On day 4 following the initial inoculation of LLC cells, the experimental animals were treated with recombinant VEGI by intraperitoneal injection (I.P.) (5mg/kg), with repeats on day 5 and day 6. The control group was treated with vehicle. The size of the tumors was measured daily. We found a significant inhibition of tumor growth rate in the VEGI-treated group compared to that in the control group (Figure 22). On day 7, the average tumor volume of the VEGI-treated group was 62% of that of the control group.

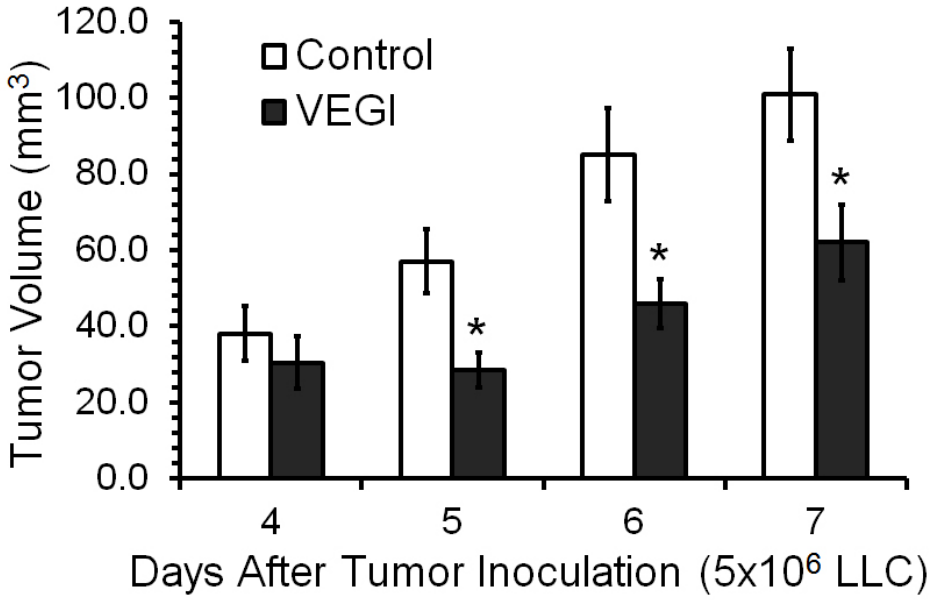
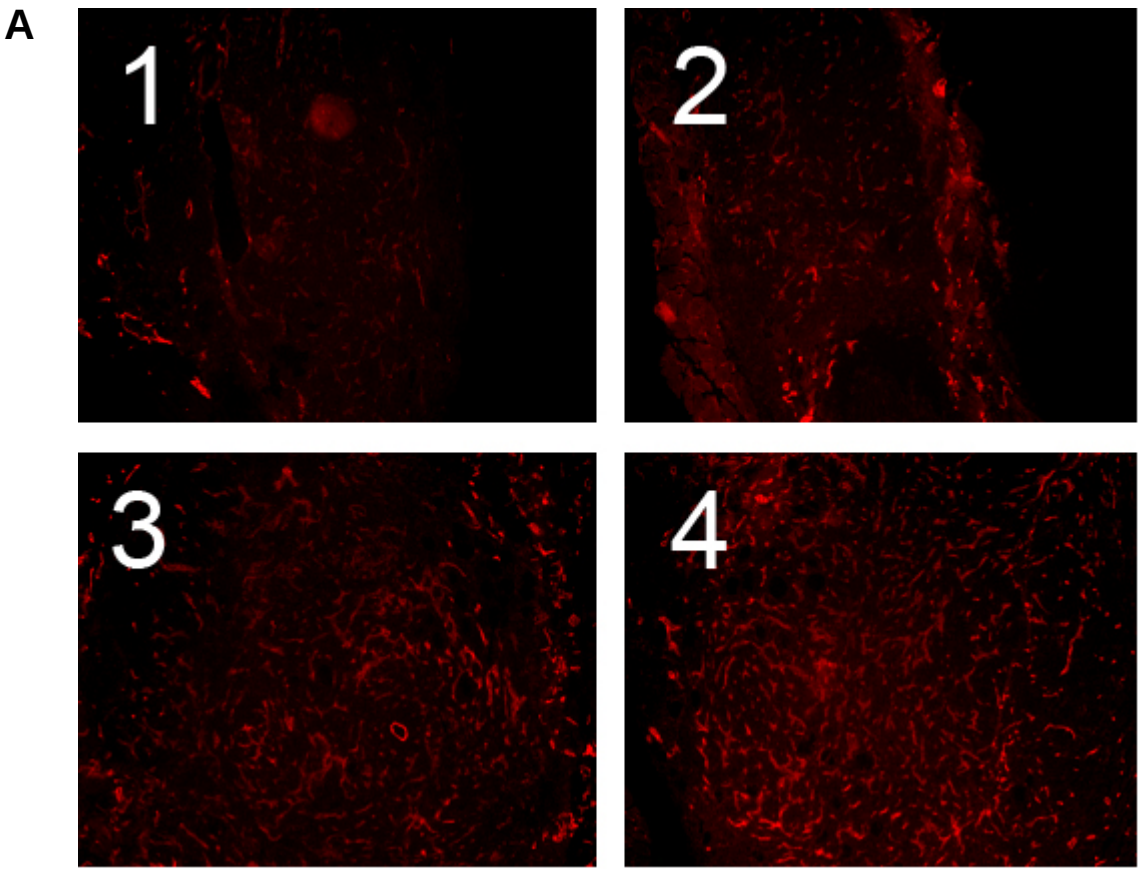


Figure 23. VEGI inhibits the growth of LLC tumors.

Average tumor size in mice treated with either vehicle (Control, n=6) or VEGI (n=6) given by intraperitoneal injection.

The tumors were harvested on day 7. Cryostat sections of the tumor samples were analyzed for vascular density using immunohistochemical staining for CD31, a marker of ECs. The intensity of CD31 immunostaining was scored on a scale of 1-4 (Figure 23A). We found that the mean intensity score was significantly lower in the VEGI-treated tumors than in vehicle-treated tumors (2.06 ± 0.07 vs 3.05 ± 0.06, respectively), indicating less vascular formation (Figure 23B). These results demonstrate that VEGI treatment leads to retarded growth of the LLC tumors and decreased vascularization in the tumors.



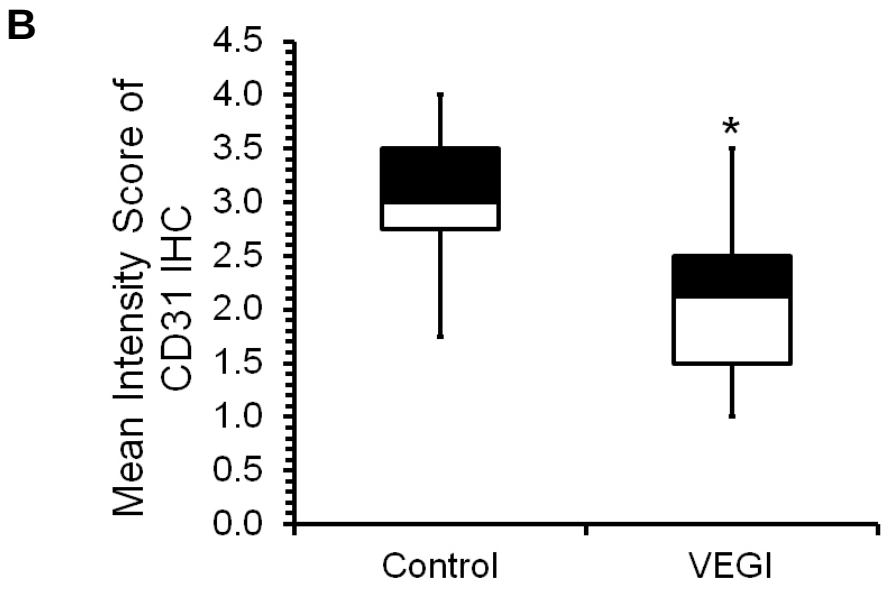


Figure 24. VEGI treatment inhibits tumor vascularization.

A. Representative images of tumor samples immunostained for CD31 and scored on a scale of 1-4, with 4 being the most. Red, CD31. B. Box plot showing average intensities of CD31 scored on Day 7 LLC tumor samples.

3.4.2 VEGI impact on peripheral blood EPC and bone marrow hematopoiesis

To determine whether VEGI has any impact on BM-derived EPCs in the periphery blood as well as in the bone marrow, we first analyzed the number of circulating EPC in the peripheral blood of tumor bearing mice treated with VEGI or vehicle. Blood was collected immediately prior to cancer cell inoculation and analyzed by using flow cytometry to identify BM-derived GFP^+ cells that were also positive for both Flk-1 (vascular endothelial growth factor receptor 2), an EC marker, and CD133 (Prominin-1), an indicator of stem cells. Blood was collected again four days after tumor inoculation, prior to VEGI treatments, in order to determine any change in the

number of BM-derived EPCs because of the tumor burden. Comparing to the baseline of day 0, we found an about five-fold decrease in the number of GFP^+-$Flk\text{-}1^+$-$CD133^+$ cells four days after tumor inoculation (Figure 24), suggesting active recruitment of periphery blood EPC into the tumor. We also analyzed blood collected on day 5, 6, and 7 for changes in BM-derived EPC resulting from the daily VEGI treatments. Interestingly, 24 hours after the initial VEGI treatment, we found a 64% increase in the number of GFP^+-$Flk\text{-}1^+$-$CD133^+$ cells compared to the vehicle-treated group. However, this difference between the VEGI-treated group and vehicle-treated group disappeared on the following days (Figure 24). These findings suggest that VEGI-treatment results in an inhibition of the recruitment of the blood-borne EPC by the tumor, albeit transient under the experimental conditions.

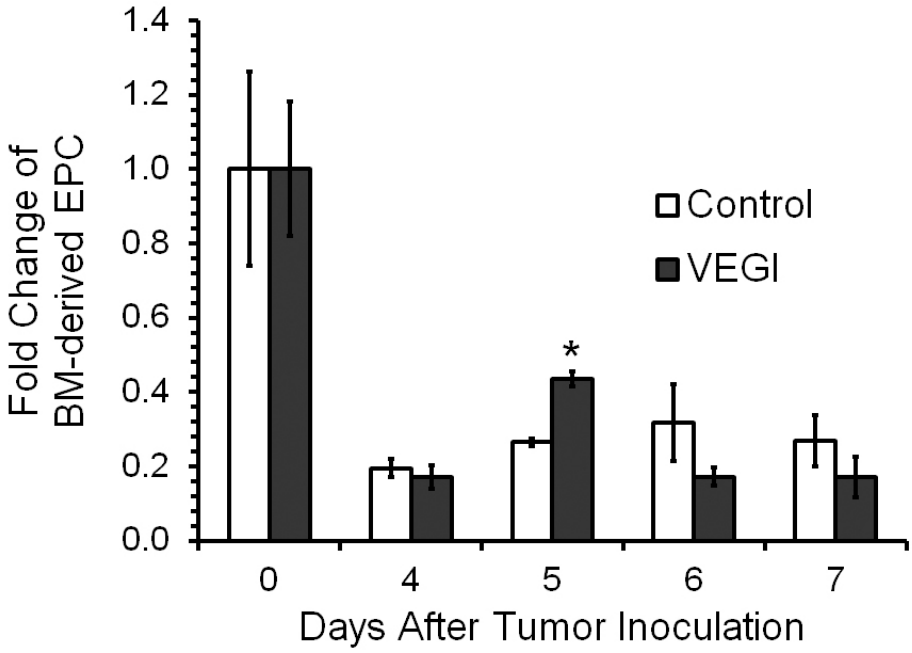


Figure 25. Effect of VEGI treatment on peripheral blood EPCs.

Fold change of circulating EPC (GFP^+-$Flk\text{-}1^+$-$CD133^+$). Blood was collected via tail vein on day 0 prior to tumor inoculation and on days 4, 5, and 6 prior to VEGI treatment (n=3 per group). Day 7 blood samples collected 24 hours after the last treatment.

We also looked for changes in the hematopoietic stem cell (HSC) population (Lin^--c-Kit^+-Sca-1^+) in the bone marrow of the VEGI- or vehicle-treated groups by flow cytometry, and compared the results with those of the bone marrow of normal non-tumor bearing mice. We found that there was an about 80% increase in the population of HSC in the bone marrow without tumor burdens because of VEGI treatment (Figure 25). By solely having a tumor burden, the population of purified HSC in the bone marrow exhibited a 53% decrease (Figure 25), possibly because of their mobilization induced by vascular trauma such that occurs in tumor neovascularization. VEGI treatment of the tumor bearing group caused the percentage of Lin^--c-Kit^+-Sca-1^+ HSC in the bone marrow to increase to a level similar to those found with VEGI-treated, non-tumor bearing mice (Figure 25). These findings suggest that VEGI treatment has an inhibitory effect on the mobilization of BM-derived EPCs into the circulation.

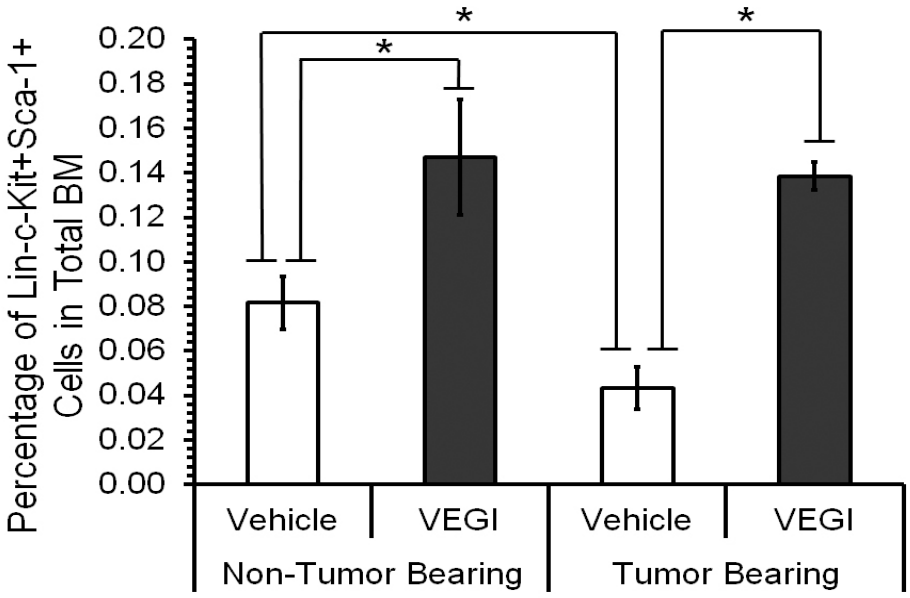


Figure 26. Effect of VEGI treatment on bone marrow HSCs.

Percentages of HSCs (Lin^--c-Kit^+-Sca-1^+) in whole bone marrow on Day 7. Samples analyzed by using flow cytometry.

3.4.3 VEGI inhibits BM-derived EPC incorporation into LLC tumors

In order to investigate the impact of VEGI treatment on the incorporation of BM derived-EPCs into the LLC tumors, tumor sections were immunostained for EC marker CD31. GFP and CD31 double positive cells were identified by confocal microscopy. In the tumor samples of the vehicle-treated animals, we were able to detect areas highly populated with GFP^+-$CD31^+$ cells (Figure 26A). In contrast, the tumor samples from the VEGI-treated group consisted of significantly fewer GFP^+-$CD31^+$ cells (Figure 26A). By counting the number of GFP^+-$CD31^+$ cells in the tumor sections (Figure 26B), we found that the number of double positive cells in the tumors of VEGI-treated animals was 56% of that in the control group (Figure 26C). This finding indicates that VEGI treatment resulted in a lowered number of EC originating from BM-derived EPCs in the tumors compared with that of vehicle-treated tumors.

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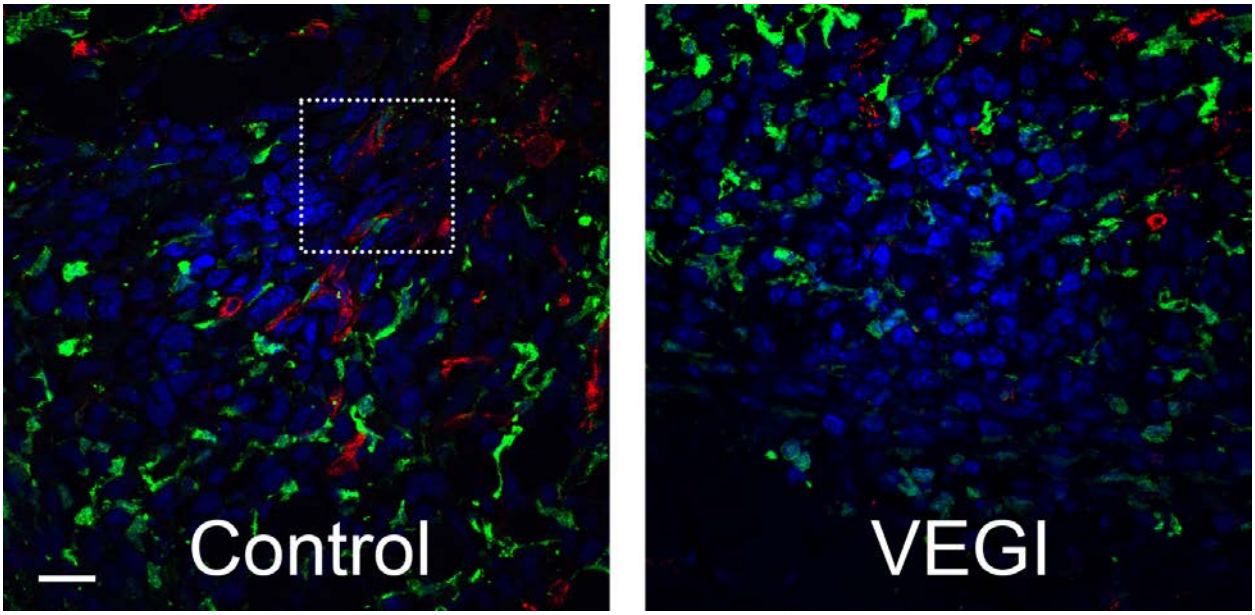

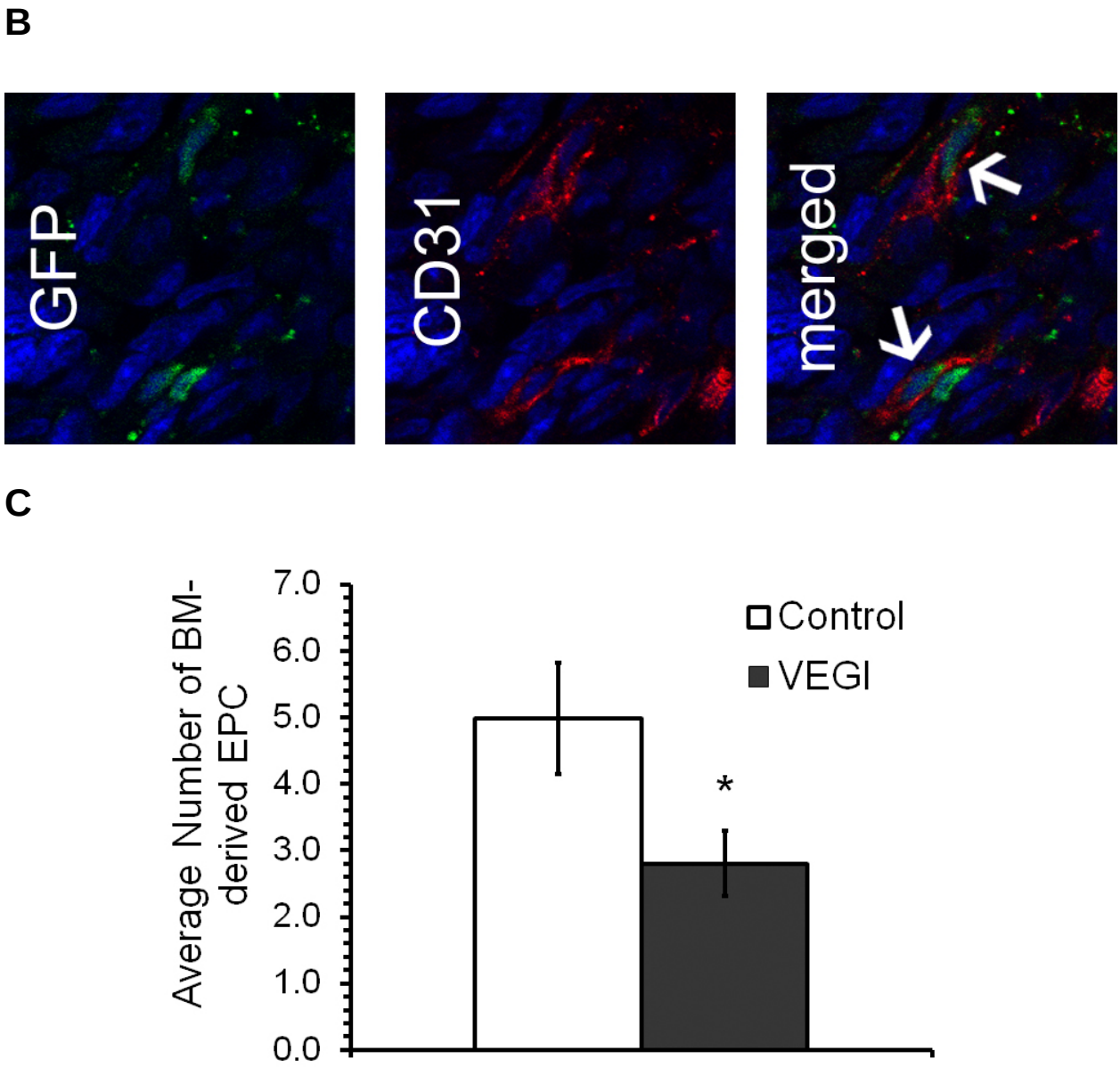

Figure 27. VEGI inhibits EPC incorporation into LLC tumors.

A. Representative confocal images of immunostaining for BM-derived EPCs in LLC tumors collected on Day 7 following cancer cell inoculation and 24-hours after the last of three daily VEGI treatments. Green, GFP; Red, CD31; Blue, cell nuclei; Scale bar, 20 μm. B. High magnification of the boxed area shown in Panel A. Arrows indicate GFP^+-$CD31^+$ cells. C. Quantification of the number of GFP^+-$CD31^+$ cells from randomly selected fields on each tumor section; a minimum of 10 fields per tumor sample were counted from each animal (n=4 per group).

3.4.4 VEGI induces apoptosis of BM-derived cells

To investigate the cause of the diminishing population of BM-derived ECs in the tumors of VEGI-treated animals, we used TUNEL staining to determine the extent of the apoptosis of BM-derived cells (Figure 27A). More specifically, we determined overall apoptosis in the tumors as well as apoptosis of GFP^+ cells derived from the bone marrow (Figure 27B). In the VEGI-treated tumors, the total number of apoptotic cells was more than two-times of that in the controls. Remarkably, the number of apoptotic GFP^+ cells in the VEGI-treated group was more than three-times of that in the controls (Figure 27C). Additionally, while about 40% of all apoptotic cells in the control group were GFP^+, nearly 70% of the apoptotic cells in the VEGI-treated group were GFP^+ (Figure 27C). These findings suggest that VEGI treatment gives rise to apoptosis of BM-derived cells that have been recruited into the tumors.

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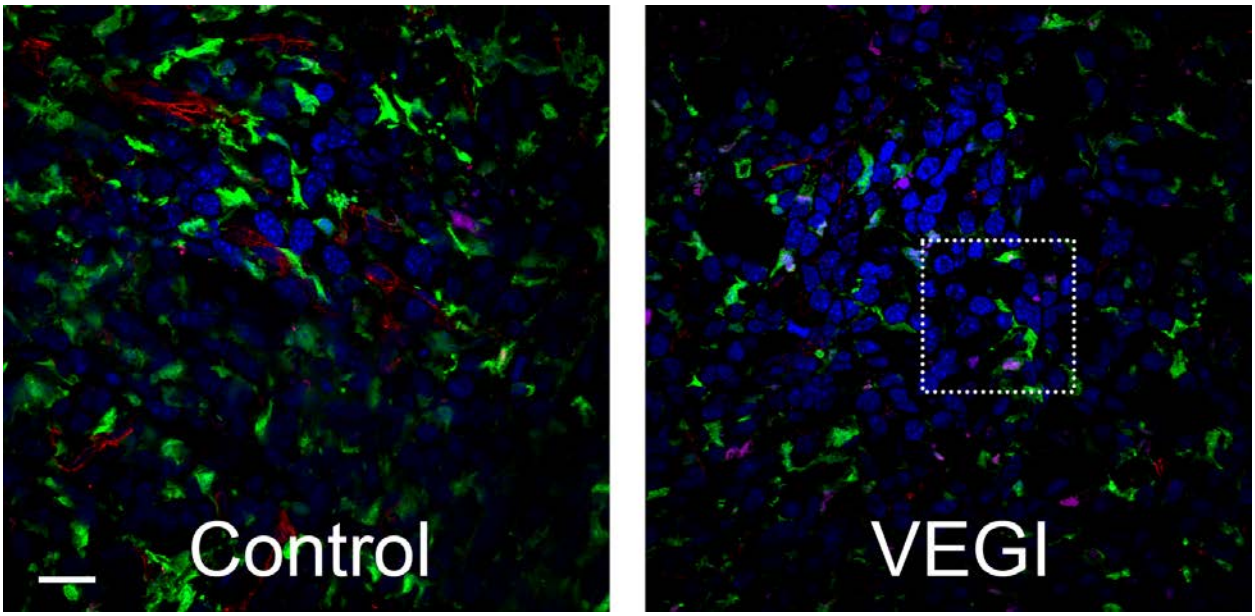

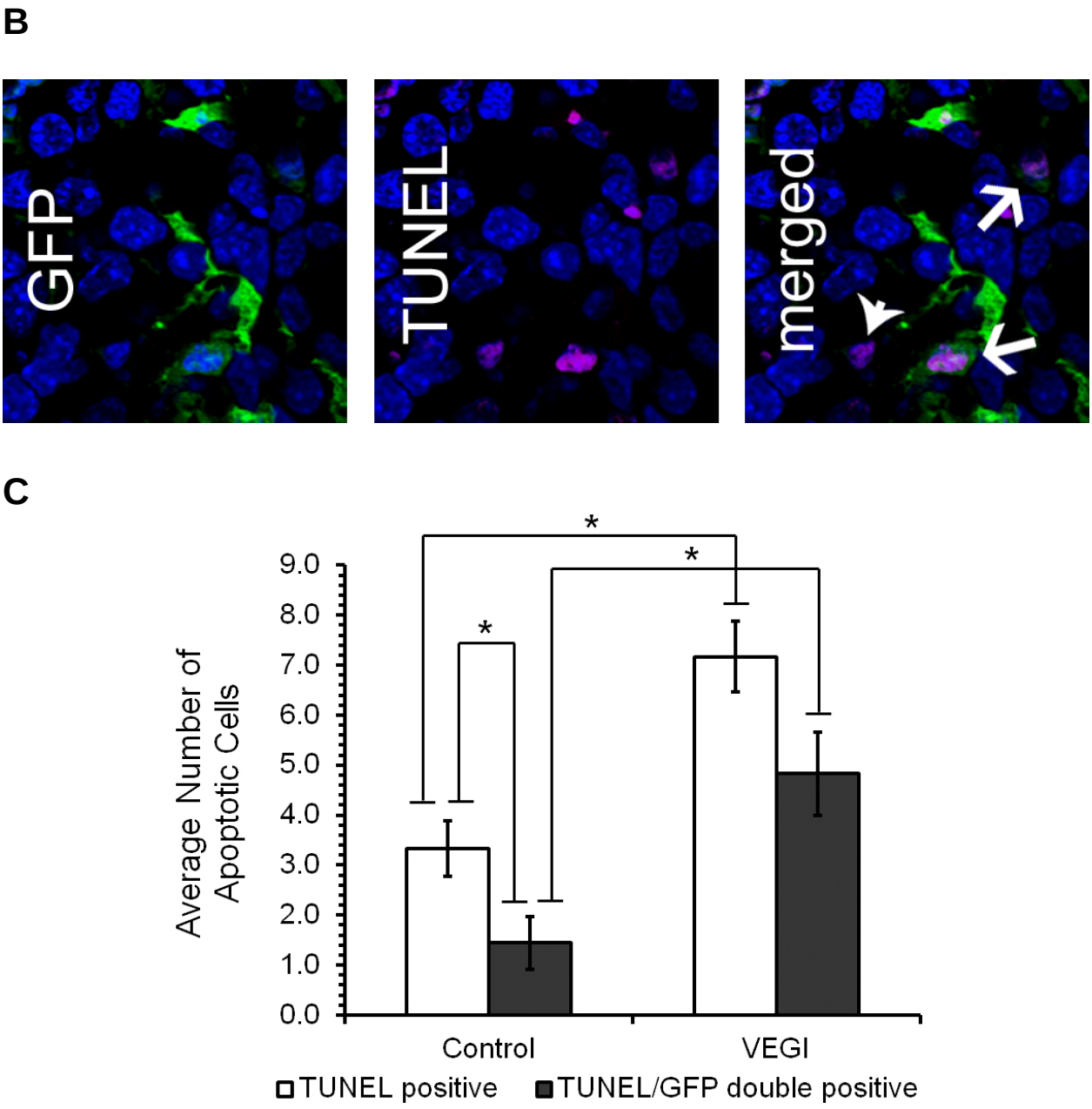

Figure 28. Induction of apoptosis of BM-derived cells by VEGI-treatment.

A. Representative confocal images of Day 7 LLC tumor samples analyzed for apoptosis. Green, GFP; Red, CD31; Magenta, TUNEL; Blue, cell nuclei; Scale bar, 20 μm. B. High magnification of the boxed area shown in Panel A. TUNEL positive cells (arrow heads) and GFP^{+}-$TUNEL^{+}$ cells (arrows) are indicated. C. Average number of TUNEL positive cells per field in vehicle-treated control or VEGI-treated tumors. A minimum of 10 fields per tumor sample were counted from each animal (n=4 per group).

3.5 CONCLUSIONS

Tumor vasculature arises through two processes: angiogenesis supported by ECs from existing blood vessels [4], and vasculogenesis supported by an alternative source of ECs, known as BM-derived EPCs [18, 82]. VEGI inhibits angiogenesis by specifically inhibiting EC proliferation and inducing apoptosis of proliferating ECs, resulting in suppression of tumor growth [131, 155]. To demonstrate in animal tumor models whether VEGI, an endogenous cytokine, can inhibit BM-derived EPC-supported vasculogenesis and thereby suppress this alternate method of neovascularization is important to the understanding of the mechanism of tumor neovascularization. The study may also yield pivotal insights into potential anticancer therapeutic approaches. The data we show here indicate that VEGI is able to inhibit EPC-supported vasculogenesis in tumors.

The function of EPCs in supporting tumor vasculogenesis has been debated extensively in previous studies. In some reports, the role of EPCs in tumor vascularization has been shown to be highly significant, not only contributing between 50-100% of the tumor vasculature, but also inducing neovasculogenesis [33, 34, 107]. BM-derived EPCs are shown to be critical for activating the angiogenic switch, which induces the vascularization of a tumor, as blocking EPCs not only inhibits angiogenesis and impairs tumor metastases, but also leads to prolonged survival of tumor bearing mice [34]. Other studies have shown, however, that EPC contribution is negligible, compromising no more than 5% of the ECs in the tumor [119, 120, 167, 168]. Apparently an important factor attributing to the discrepancies is the timeframe of when EPCs incorporation into the tumor vasculature was analyzed. It has been reported that BM-derived EPCs participate in early tumor vascularization, forming BM-derived vessels, before their incorporation is diluted by peripheral vessels resulting from angiogenesis [123]. Therefore, we

examined the contribution of EPCs to tumor vasculogenesis within the timeframe when subcutaneously implanted LLC form palpable tumors, approximately within a few days of cancer inoculation. Treatment of the tumor bearing mice with VEGI resulted in a marked retardation of tumor growth, accompanied by a decrease in vascular density in the tumors. These data are consistent with the view that VEGI is able to negatively regulate EPC-supported nascent neovascularization during the early formation of LLC tumors.

EPCs are thought to migrate out of the bone marrow into the periphery and home to a site of vascular trauma, such as occurs during tumor vasculogenesis [31, 68, 81]. We examined the peripheral blood of the experimental mice before and after tumor cell inoculation as well as after VEGI treatment to determine if there is any difference in the circulating BM-derived EPC population. Previous studies have indicated that EPCs are mobilized from the BM into the circulation, then incorporated into the vascular bed within 48 hours after tumor inoculation in animal models [18, 33, 81]. We determined the baseline amount of circulating BM-derived EPCs prior to tumor inoculation, and found that four days of possessing a tumor burden significantly deprived the amount of BM-derived EPCs in the blood. It is likely that the BM-derived EPC population in the blood of the experimental animals diminishes because these cells are incorporated into the tumor. We then treated the tumor bearing mice with VEGI. While no further change was seen in the amount of circulating BM-derived EPCs in the vehicle-treated control group, there was an increase of circulating BM-derived EPCs in the blood of VEGI-treated mice compared to the control group one day after initial VEGI treatment. Interestingly, however, the levels of circulating BM-derived EPCs in the VEGI treated group declined to the same level seen for the vehicle-treated control group within the next 24 hours. It is plausible that the temporary increase in BM-derived EPC arises from VEGI inhibiting the ability of EPCs to

adhere to a site of vascular trauma, causing a transient accumulation of BM-derived EPCs in the blood.

The subsequent decline of circulating EPCs in VEGI-treated, tumor bearing animals may be explained in terms of the inhibition of cancer cell-induced HSC mobilization from the bone marrow. We analyzed the whole bone marrow of the experimental mice for the percentage of Lin^--c-Kit^+-$Sca\text{-}1^+$ HSC, which share a common lineage with EPCs [60, 169], after three days of VEGI treatment. We compared the percentage of the HSCs in the tumor bearing mice to non-tumor bearing mice and found that the tumor burden leads to a marked decrease of the percentage of these cells, while VEGI treatment caused the number of Lin^--c-Kit^+-$Sca\text{-}1^+$ cells in the bone marrow to increase by about three-fold. A relatively larger percentage of HSCs within the bone marrow, but a lower percentage of BM-derived EPCs in the circulation, suggests that VEGI inhibits tumor-induced HSC mobilization in the bone marrow. Alternatively, the increase of the percentage of Lin^--c-Kit^+-$Sca\text{-}1^+$ cells in the bone marrow by VEGI treatment suggests that VEGI promotes HSC production. This possibility will be investigated in future studies.

The significant decrease in the number of EPCs incorporated into the tumor site of VEGI-treated mice, compared to that in vehicle-treated mice, may be because of not only VEGI inhibition of the mobilization of HSCs in the bone marrow and inhibition of EPC adherence, but also the possibility of VEGI inducing apoptosis of BM-derived EPCs. Given the unique ability of VEGI to induce apoptosis of only proliferating EC, we utilized CD31, a widely accepted marker of EC lineage, to visualize BM-derived EPCs and their response to VEGI. We found that VEGI treatment not only caused an increase in the number of overall apoptotic cells in the tumor, but specifically induced the apoptosis of cells deriving from the bone marrow, giving rise to a diminished BM-derived EPC population in the tumor.

In summary, this study illustrates the role of VEGI as an inhibitor of BM-derived EPC incorporation and differentiation in LLC tumors. Our findings indicate that VEGI inhibits BM-derived EPC-supported tumor vasculogenesis, ultimately inhibiting tumor growth. In addition, we show that tumor burdens mobilize EPCs from the bone marrow and recruit circulating EPCs, and that VEGI suppresses this process. Further, we show that VEGI directly inhibits BM-derived EPC-supported tumor vasculogenesis by specifically inducing apoptosis in BM-derived cells in the tumors. These findings are consistent with the view that VEGI plays a critical role in the modulation of EPC-supported postnatal vasculogenesis.

4.0 DISCUSSION

The emphasis of these studies was to investigate the effect of VEGI activity on BM-derived EPCs to gain a better understanding of the potential therapeutic value of VEGI for inhibiting tumor vasculogenesis. In our effort, we utilized recombinant VEGI to treat cultures of bone marrow stem cells stimulated to differentiate into ECs in an attempt to deduce whether VEGI is able to inhibit their proliferation *in vitro*. We also determined whether VEGI could specifically inhbit BM-derived EPC supported vasculogenesis in a tumor model of LLC. Furthermore, we collected preliminary evidence on the mechanism of action by VEGI in these experiments. Our results support the hypothesis that VEGI is a biological inhibitor capable of inhibiting tumor vasculogenesis.

4.1 MODULATION OF BM-DERIVED EPC *IN VITRO*

4.1.1 VEGI inhibits differentiation of BM-derived EPC

The discovery of progenitor cells of endothelial lineage, EPCs, which can differentiate into mature ECs has led to many studies on the role of these cells in vascularization [11]. It has been widely reported that EPCs derive from the bone marrow and contribute to neovascularization by homing to a site of vascular injury where it aggregates, proliferates, and differentiates to form an

entirely new vessel. This process of vasculogenesis has been determined to be important to normal physiological vascular repair as well as in pathological uncontrolled blood vessel formation [18]. It has been shown that there are multiple factors that contribute to the upregulation of BM-derived EPCs, such as VEGF and FGF-2 [81, 162]. However, there are few reports dedicated to the understanding of negative regulators of BM-derived EPCs.

One of the more prominent roles of VEGI is its regulation of EC proliferation by targeting those cells for apoptosis [155]. Because VEGI has a specific role in attenuating angiogenesis, we wanted to determine if VEGI was also able to exhibit anti-proliferative and pro-apoptotic actions on EPCs. To delineate VEGI's role in regulating BM-derived EPCs, we initially had to isolate and characterize EPCs. To date, there have been multiple methods described for EPC isolation, but no distinct consensus on one method [48, 151]. The characterization of EPCs have been further hindered based on the fact that EPCs and HSCs, both derived from hemangioblasts [66], share many of the same cell surface markers, the primary method of determining EPCs from more primitive HSCs. In our lab, we utilized Sca-1^+ cell selection to isolate early stage EPCs directly from mouse bone marrow, because Sca-1 is a widely recognized marker for adult murine HSCs and typically utilized by other investigators to isolate EPCs from mouse bone marrow. We found that a high density of Sca-1^+ cells and low serum concentration in the culture media is essential for BM-derived EPC survival and differentiation. This observation is in accordance with previous reports [170, 171].

Isolated cells were stimulated in culture media containing essential EC growth factors. We found that during the initial days of culture, freshly isolated cells were unable to adhere to the culture plates. This may be due to the fact that these cells express only stem cell markers and are considered to be the early population of EPC. These early stage EPCs slowly differentiate

toward ECs, which was immediately visible in their change in morphology. Concurrently, it was seen that these later stage EPCs begin to express surface markers indicative of committed EC lineage while expression of their stemness was decreased. In our determination of BM-derived EPCs, we examined a battery of markers that have been repeatedly distinguished by many groups as identifying markers for EPC.

Overall, we determined that a mixture of both stem cell markers (Sca-1 and CD133) and EC markers (Flk-1, Tie-2, and E-selectin) allowed for characterization of BM-derived EPCs over a course of a two-week culture. We found that VEGI inhibits early stage EPC progress into differentiated EPCs by examining these same markers. Unlike EPCs in the control group, cells cultured in the presence of VEGI have up to a 50% lowered expression of only endothelial markers (Flk-1, Tie-1, E-selectin, and VE-cadherin). However, there was no difference in the expression level of Sca-1 and CD133, which in both control and VEGI treated groups, was lowered after 10 days of culture. These data suggest that VEGI could largely inhibit, but not completely prevent, EPC differentiation from bone marrow cells.

4.1.2 VEGI inhibits BM-derived EPC functionality

We state that VEGI is able to inhibit BM-derived EPC differentiation based on decreased levels of surface markers, but we also tested the functionality of the cells to confirm this finding. EPCs are known to have migratory and adhesive functionality required for their transport to sites requiring neovascularization. In our culture system, VEGI treatment significantly diminished the ability of EPC to adhere to extracellular matrix proteins fibronectin and vitronectin. Besides limiting EPCs adhesion to culture coated plates, VEGI also downregulated EPC ability to migrate across both an open space and a porous barrier. Utilizing the wound healing assay to

determine the ability of cells to move into an open space on a flat surface in a unit of time, control cells exhibited a mobility that is nearly two times faster than VEGI-treated cells. However, when measured in a transwell assay to determine the ability of the cells to pass through a porous barrier, the speed of the untreated cells is only about 25% faster than VEGI-treated cells. This discrepancy likely resulted from the larger size of the untreated cells, which were able to differentiate and elongate, ultimately hindering their mobility through the porous filters in the latter assay. The different migration time (48 hours vs. 8 hours) may also cause the difference of the inhibition rate. Moreover, the ability of EPCs to form capillary-like tubes on extracellular matrix proteins decreases substantially as a result of VEGI treatment. These findings strongly suggest that VEGI inhibits early stage EPC differentiation toward ECs and preventing their functional properties.

4.2 MECHANISMS OF VEGI ACTION IN BM-DERIVED EPC INHIBITION

The mechanism involved in VEGI inhibition of BM-derived EPC differentiation and function is unknown. However it is presumed that VEGI is able to regulate factors involved in EPC growth signaling. Additionally, it is thought that VEGI may downregulate proteins necessary for EPC mobilization. The importance of integrin receptors α5β1 and αvβ3 are known for EC migration, in which ECs interact with the extracellular matrix via these proteins [164, 165]. Since VEGI treated cells are unable to adhere to fibronectin and vitronectin in culture conditions, we looked for the expression of integrins on these cells and found a decreased expression. It appears as though VEGI, by inhibiting the production of these integrins on BM-derived EPC, leads to the

decreased migration and adhesion seen *in vivo*. This could be one part of the mechanism by which VEGI negatively modulates EPC differentiation.

VEGI treatment inhibits early stage EPCs adhesion and causes the cells to remain largely undifferentiated and suspended in culture. In control cultures without VEGI, many of the cells were able to adhere, and those cells which did not adhere undergo apoptosis. We were able to determine that VEGI decreased the rate of apoptosis in the suspended cells, giving rise to the idea that VEGI may have a protective effect on early stage EPC. On the other hand, VEGI treatment led to increased apoptosis of adherent cells while no apoptosis was detected in untreated cells. These results suggest that differentiated and undifferentiated EPCs respond to VEGI activity in a very different manner that is yet to be determined.

One possible variable in the different apoptotic response by differentiated vs. undifferentiated EPCs may be due to the expression of DR3 on these cells. DR3 has been described as the receptor for VEGI and mediates the apoptotic pathway response [133]. We found that DR3 is not detected in BM-derived EPCs until approximately 1 week in culture, consistent with when cells begin to undergo apoptosis in response to VEGI treatment. DR3 expression increases markedly in differentiated EPCs after 10 days in culture and accordingly, apoptosis rates increase profoundly after 11 days of culture. An activated caspase cascade in VEGI-treated EPCs is observed only after the expression of DR3 and can be reversed using DR3-neutralizing antibodies. These data indicate that DR3 is likely the mechanism by which VEGI can exert its inhibitory effect on BM-derived EPCs.

Activation of protein kinases have been reported to be essential for VEGI induced apoptosis and inhibition of ECs. It appears that these proteins are also involved in the inhibition of EPCs by VEGI, but not via pro-apoptosis. As the early EPCs have yet to express DR3, it is

unknown how VEGI is signaling to these cells. However, it is apparent the VEGI does interact with early EPC to inhibit differentiation. In BM-derived EPC cultures, treatment with VEGI leads to an immediate decline of Akt phosphorylation. Akt signaling pathway plays an important regulatory role in the differentiation of EPCs [99], therefore the inhibition of Akt by VEGI may contribute to the reason early EPCs are retained in an undifferentiated state. Not only does VEGI inhibit differentiation of cells in culture, but it has been shown that VEGI actually supports undifferentiated cell survival, protecting it from undergoing apoptosis, which was unexpected. This mechanism of VEGI may relate to Erk and p38, which are both activated by VEGI treatment in early EPCs. Activation of the Erk signaling pathway is essential for cell proliferation and survival, presenting a plausible mechanism underlying the protection of early-stage EPCs and increased cell numbers compared to untreated controls. Activation of p38 has been shown to inhibit EPC proliferation [101], perhaps contributing to the inhibitory effect of VEGI on EPC differentiation.

4.3 MODULATION OF BM-DERIVED EPC *IN VIVO*

4.3.1 BM-derived EPC-supported vasculogenesis

It has been well established that VEGI plays a role in inhibiting the proliferation of mature ECs [131, 155], therefore it is highly likely that it can exert a similar effect on the precursors of ECs. These precursors derived from the bone marrow are termed BM-derived EPCs. EPCs have been postulated to play a main role in triggering the onset of vascularization in tumors, which results in uncontrolled growth of the tumor [34]. The tumor vasculature has been shown to be a vital for

the survival and growth of a tumor, providing necessary nutrients and oxygen to hypoxic regions and forming a critical vascular network allowing for the metastasis of tumor cells to secondary sites [21, 27]. As metastasis will lead to the eventual death of the tumor host, it is imperative to prevent or eliminate this vascular pathway, which can not only halt the progression of the disease, but perhaps abolish the primary tumor as well.

The contribution of EPCs to tumor vasculogenesis has been debated extensively in previous studies. While some groups have reported that the role of EPCs in tumor vascularization is highly significant [18, 30, 33, 34], other have stated that EPC contribution is negligible [119, 120, 167, 168]. It has been reported that the different methods of analyzing EPC incorporation into a tumor and the tumor model itself may be an important factor attributing to these discrepancies. One model that has been used repeatedly and has been shown to involve EPC-supported tumor vasculogenesis is the LLC model [34]. In our hand, we found that EPC do incorporate into a tumor bed, albeit at a low percentage that is consistent with other groups utilizing a LLC tumor model [34]. It was seen that EPC constitute a small percentage of the overall circulating blood and low numbers were found at the tumor site on day 7. Our decision to utilize such an early tumor mass was based on recent reports that BM-derived EPC participate in early tumor vascularization before their incorporation is diluted by peripheral vessels [123].

4.3.2 VEGI inhibits BM-derived EPC in circulation

BM-derived EPCs are shown to be the critical factor inducing the vascularization of a tumor. It has been shown that blocking EPCs not only inhibits angiogenesis and impairs tumor metastases, but also leads to prolonged survival of tumor bearing mice [34]. Endogenous inhibitors of angiogenesis play a pivotal role in controlling the angiogenic balance but they become

downregulated during tumor progression, allowing for uncontrolled vascular growth [172]. Re-introduction of endogenous inhibitors in the tumor microenvironment may help to restore the angiogenic balance. With this in mind, we tested the ability of VEGI to block EPC-supported vasculogenesis. First, it was shown that treatment of the tumor bearing mice with VEGI resulted in a marked retardation of tumor growth that was accompanied by a decrease in vascular density in the tumors, suggesting VEGI negatively regulates neovascularization during the early formation of LLC tumors.

We examined the peripheral blood of experimental mice for circulating levels of BM-derived EPCs in order to determine whether the tumor growth inhibition and decreased vasculature caused by VEGI treatment was due to EPC-supported vasculogenesis and not solely VEGI's inhibitory activity on ECs and angiogenesis. It was determined that circulating BM-derived EPC populations drastically decreased 4 days after tumor inoculation, consistent with previous studies that have indicated EPCs incorporate into tumor vasculature within the first 2 days of tumor burden in mice [18, 33, 81]. It is likely that the decreased population of BM-derived EPCs found in the blood of the experimental animals diminished because these cells had already incorporated into the tumor. Interestingly, there was an increase of circulating BM-derived EPCs in the blood of VEGI-treated mice compared to the control group after initial VEGI treatment. However, the increase was temporary and BM-derived EPC populations in the VEGI-treated group quickly declined to similar amounts seen for the vehicle-treated control group. The temporary increase in BM-derived EPCs is hypothesized to be due to the inhibitory activity of VEGI on the ability of EPCs to adhere to a site of vascular trauma, causing a transient accumulation of BM-derived EPCs in the blood. This theory is supported our *in vitro* findings that EPCs in the presence of VEGI are unable to attach to culture plates coated with extracellular

matrix proteins and that the expression of integrin-α5 or -αV was substantially down-regulated by VEGI. Additionally, it is thought that after inhibiting EPC adhesion, VEGI then induces apoptosis of these cells, leading the ensuing population decline of BM-derived EPCs in the circulation. A future study of the apoptosis rates of EPCs in blood after VEGI treatment can be of value in further exploring this possibility.

Another possibility for the decline of EPCs in blood after treatment by VEGI may be due to the inhibition of HSC mobilization from the bone marrow. It has been shown that EPCs and HSCs mobilize from the blood together [173]. We found an accumulation of HSCs in the bone marrow of VEGI-treated mice, regardless of tumor bearance, and it was seen that the percentage of HSCs actually increased to levels almost double that of normal controls. This view is consistent with our *in vitro* data indicating that the ability of EPCs to migrate markedly declines after VEGI treatment. Furthermore, the increase of the percentage of Lin$^-$c-Kit$^+$-Sca-1$^+$ cells in the bone marrow by VEGI treatment suggests that VEGI not only inhibits the mobilization of HSCs from bone marrow into the periphery, but that VEGI may promote HSC production. This thought is in line with our data showing VEGI activates cell growth signals in early EPCs derived from Sca-1$^+$ cells. However, future studies on the whole bone marrow will be performed to further evaluate this possibility.

4.3.3 VEGI inhibits BM-derived EPC in tumors

BM-derived EPCs are low in their frequency, which makes it difficult to stain for their multiple markers while allowing for visualization of their co-localization. Given the unique ability of VEGI to induce apoptosis of only proliferating EC [155], we therefore utilized CD31, a

widely accepted marker of EC lineage, to visualize BM-derived EPCs and their response to VEGI. It is important to note that CD31 is not solely found on ECs, but on other cells such as myeloid cells. However, in conjunction with GFP, we were able to determine which CD31 cells derived from the bone marrow, a good indicator of BM-derived EPC. A significant decrease in the number of EPCs incorporated into the LLC tumor site of VEGI-treated mice was observed. This phenomenon may occur in part to the inhibition of HSC mobilization from the bone marrow and the inhibition of circulating EPC adherence, as previously stated.

The decrease in EPC incorporation may also be due to an apoptotic effect. We determined the possibility of VEGI induced apoptosis of BM-derived EPCs, which is likely when taking into account the apoptosis of BM-derived cells seen in our VEGI-treated cultures. We found that VEGI treatment not only caused an increase in the number of overall apoptotic cells in the tumor, but specifically induced the apoptosis of cells deriving from the bone marrow, giving rise to a diminished BM-derived EPC population in the tumor. Subsequent studies on tumor cell suspensions analyzed by flow cytometry could help confirm the decline of BM-derived EPC as well as the increase in apoptosis.

4.4 CONCLUDING REMARKS

We focused on characterizing the inhibitory action of VEGI on BM-derived EPCs. We have shown that VEGI treatment of BM-derived cells in culture prevents their differentiation towards an EC lineage by suppressing EC-specific gene expression in early stage EPCs and inducing apoptosis of late stage EPCs. Moreover, we have demonstrated that VEGI treatment in a LLC

tumor model diminished BM-derived EPC incorporation into the tumors, hindering BM-derived EPC-supported tumor vasculogenesis and ultimately inhibiting tumor growth. Together, these studies support our hypothesis that VEGI is a biologically significant inhibitor of tumor vasculogenesis and have resulted in a better understanding of VEGI and its potential impact for cancer therapy.

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