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Vascular Precursor Cells in Tissue Injury Repair

XIN SHI^a, WEIHONG ZHANG^b, LIYA YIN^a, WILLIAM M. CHILIAN^a, JESSICA KRIEGER^a, and PING ZHANG^a

^aDepartment of Integrative Medical Sciences, College of Medicine, Northeast Ohio Medical University, Rootstown, OH 44272, USA

^bDepartment of Basic Medicine, School of Nursing, Zhengzhou University, Zhengzhou, Henan Province 450052, PR China

Abstract

Vascular precursor cells include stem and progenitor cells giving rise to all mature cell types in the wall of blood vessels. Upon tissue injury, local hypoxia and inflammation result in generation of vasculogenic mediators which orchestrate migration of vascular precursor cells from their niche environment to the site of tissue injury. The intricate crosstalk among signaling pathways coordinates vascular precursor cell proliferation and differentiation during neovascularization. Establishment of normal blood perfusion plays an essential role in effective repair of the injured tissue. In recent years, studies on molecular mechanisms underlying the regulation of vascular precursor cell function have achieved substantial progress, which promotes exploration of vascular precursor cell-based approaches to treat chronic wounds and ischemic diseases in vital organ systems. Verification of safety and establishment of specific guidelines for the clinical application of vascular precursor cell-based therapy remain major challenges in the field.

Keywords

vascular precursor cells; stem cells; progenitor cells; tissue injury; repair; vasculogenesis; angiogenesis

Introduction

Reestablishment of blood circulation is essential for repair of tissue injury. Tissue vascularization commonly involves vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis is the formation of new blood vessels from vascular precursor cells. Angiogenesis is the process of outgrowing vessels from the existing vasculature.

Address for correspondence: Ping Zhang, MD, PhD, Department of Integrative Medical Sciences, College of Medicine, Northeast Ohio Medical University, 4209 State Route 44, Room F339, Rootstown, OH 44272, Tel: 330-325-6342, Fax: 330-325-6841, pzhang@neomed.edu.

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Arteriogenesis involves remodeling of arteries where collateral arterial anastomoses undergo abluminal expansion. Cell composition in blood vessels is highly heterogeneous and dynamic. Endothelial cells assemble in a monolayer lining the inner surface of all blood vessels. Pericytes are cell components of the microvasculature, including capillaries, arterioles, and venules. In addition to these cells, large blood vessels have many other cell types, such as smooth muscle cells (SMCs), fibroblasts, mast cells, dendritic cells, and macrophages.

Since the discovery of unique cell subpopulations with the functional similarity to embryonic angioblasts in the peripheral blood of adults^{1,2}, vascular precursor cells have drawn a broad attention for their role in repair of the blood vasculature. At the site of tissue injury, hypoxia and inflammation result in an increase in local production of bioactive mediators which function as chemoattractants and/or activators. These mediators orchestrate migration of vascular precursor cells from their niche environment to the site of tissue injury. Proliferation and differentiation of vascular precursor cells, particularly endothelial progenitor cells (EPCs), contribute to formation of new blood vessel islets. The initially formed vascular cores via vasculogenesis are pruned and extended by angiogenesis. Maturation of the blood vasculature can be subsequently achieved through remodeling processes with the participation of different vascular precursor cell types, such as mesenchymal stem cells (MSCs) and smooth muscle progenitor cells (SMPCs) (Figure 1 sketches the involvement of vascular precursor cells in neovascularization during the process of tissue injury repair). This review discusses the recent progress in characterization of vascular precursor cells and highlights signaling mechanisms underlying the regulation of their function in promoting neovascularization during the process of tissue injury repair. Efforts in exploring the application of vascular precursor cells for the treatment of tissue injury are also addressed.

Vascular Precursor Cells

Vascular precursor cells denote stem and progenitor cells that give rise to mature cell types in the wall of blood vessels, including endothelial cells, SMCs, and fibroblasts. The existence of circulating EPCs in adults was initially reported by Asahara and colleagues in 1997¹. In their study, CD34 and Flk1/KDR (Flk1 or fms-like tyrosine kinase-1 is also called vascular endothelial growth factor receptor-2 or VEGFR-2 in mice; KDR or kinase insert domain receptor is the human homolog of VEGFR-2) positive mononuclear cells isolated from human peripheral blood were able to differentiate into endothelial cells *in vitro* and to participate in neovascularization *in vivo*. Since then, numerous efforts have been devoted to characterization of adult EPCs. Blood EPCs observed by Asahara and colleagues were in a spindle shape with a limited potential of proliferation for up to 4 weeks in culture¹. In 1998, Shi and colleagues isolated an EPC population from human bone marrow and demonstrated that these precursor cells could proliferate to form cobblestone-shaped cell monolayers in the presence of vascular endothelial growth factor (VEGF) *in vitro*³. These marrow-derived EPCs exhibited a strong potential of proliferation as evidenced by their lifespan of over 30 passages in cell culture. Shortly thereafter, Hur and colleagues reported the presence of two EPC types termed “early” and “late” EPCs, respectively⁴. Early and late EPCs appeared sequentially in the culture of peripheral blood mononuclear cells (PBMCs). Early EPCs in

the spindle shape showed peak growth at 2 to 3 weeks and died at 4 weeks, whereas late EPCs with the cobblestone shape appeared late at 2 to 3 weeks, exhibited exponential growth from 4 to 8 weeks, and lived up to 12 weeks. Early EPCs were proved as a heterogeneous cell population including clones that generated late EPCs. Further investigations on the origin, phenotype, and vasculogenic properties of these cell types have improved understanding about them. Early EPCs are now preferably called “circulating angiogenic cells” (CAC) and late EPC are termed “endothelial colony forming cells” (ECFC)⁵. The CAC gene signature is highly enriched for markers of M2 macrophages⁶. These cells do not possess the ability of becoming endothelial cells or directly incorporating into the microvascular network. Instead, they can substantially induce endothelial tube formation *in vitro* and vascular repair *in vivo* through generation of vasculogenic mediators. ECFCs are most likely the true EPCs responsible for giving rise to endothelial cells and participating in neovasculization *in vivo*⁷.

Ample evidence indicates that EPCs have a close relationship with hematopoietic precursor cells⁸. In early embryogenesis, endothelial and hematopoietic cells appear to share a common ancestor, i.e. hemangioblasts, in the yolk sac^{9,10}. Shortly later in the embryonic development, hematopoietic stem cells (HSCs) are derived from the hemogenic endothelium in the dorsal aorta^{9,11}. In adults, some surface markers identifying putative EPCs are co-expressed by hematopoietic precursor cells, such as CD34 in humans^{1,12}, and CD117 (stem cell growth factor receptor or c-kit) as well as stem cell antigen-1 (Sca1) in mice^{13,14}. The lineage (lin)⁻CD34⁺ cell population or panleukocyte marker CD45⁻CD34⁺ cell population in human bone marrow and peripheral blood contains enriched HSCs, while EPCs or ECFCs exhibit a phenotype of CD45⁻CD34⁺KDR⁺¹⁵. These facts indicate that human CD45⁻CD34⁺ cells are a heterogeneous population of cells containing precursors for both hematopoiesis and vasculogenesis⁸. Since mature endothelial cells may also express CD34¹⁶, CD133 has been introduced for further verification of EPC identity. CD133 antigen (also known as prominin-1) is a marker for hematopoietic stem cells. Mature endothelial cells do not express CD133. CD34⁺ cells with co-expression of KDR and CD133 has been identified as a unique population of circulating endothelial precursors (CEPs) in humans². In murine models, transplantation of marrow lin⁻c-kit⁺Sca1⁺ cells (a primitive precursor cell population enriched with HSCs) can not only repopulate blood cells, but also give rise to endothelial cells that are integrated into blood vessels in various organ tissues in the recipients¹⁷. A subset of mouse lin⁻c-kit⁺Sca1⁺ cells expressing VEGFR-2/Flk1 is believed to contain enriched EPCs¹⁸. In rats, a subset of blood cells bearing lin⁻Hoechst⁺CD36⁺ markers have been characterized to contain putative EPCs¹⁹. Blood-derived EPCs in rabbits express CD34⁺VEGFR-2⁺Ulex europaeus agglutinin-1(UEA1)⁺ markers²⁰. These progenitors are also positive for uptake of acetylated low-density lipoprotein.

Numerous efforts have been made for characterizing the vasculogenic/angiogenic activity of cells from the myeloid lineage, particularly of monocytes and macrophages. It is now accepted that monocytes and macrophages do not possess the capacity to further differentiate into fully functional endothelial cells^{8,21,22}. Instead, they promote blood vessel formation through generation of chemoattractants and vasculogenic factors for local vascular precursor cell homing, proliferation, and differentiation. Monocyte/macrophage activation through different pathways can reach two extreme phenotypes: M1 classically activated

macrophages that exhibit a pro-inflammatory phenotype, and M2 alternatively activated macrophages that are pro-angiogenic and anti-inflammatory²³. Macrophages have a full-range of plasticity allowing them to switch from one phenotype to another under the influence of local environment. Accordingly, either *in vitro* or *in vivo*, the distinct milieu is considered to be critical for developing pro-angiogenic activity of monocytes and macrophages²¹.

Bone marrow houses and produces SMPCs. The existence of SMPCs in the circulation was documented in 2001²⁴. In the following year, confirmation for the capacity of human bone marrow-derived mononuclear cells for differentiation into SMCs was reported²⁵. Bone marrow-derived mononuclear cells represent a highly heterogeneous population containing cells from the myeloid origin, such as CD14 positive monocytes and macrophages. Similar to the angiogenic activity described above, accumulated observations suggest that differentiated monocytes/macrophages may primarily generate paracrine effect on remodeling of artery and other large vessels rather than function as the true SMPCs^{26,27}. A CD14 negative SMPC phenotype has been identified recently²⁸. Cells with this phenotype can differentiate into alpha smooth muscle actin (α SMA) expressing cells in the culture system with stimulation of platelet-derived growth factor (PDGF)-BB. Studies using the promoter-sorting method have shown that adhered bone marrow cells transfected with a human smooth muscle (SM)-22 α promoter driven green-fluorescent protein (GFP) gene construct express GFP at 5 days after the transfection²⁹. These cells express platelet-derived growth factor receptor (PDGFR)- β but neither mature (calponin) nor immature (embryonic form smooth muscle myosin heavy chain or SMemb) SMC-specific proteins at that time. The cells can eventually grow into individual clones expressing SMC-specific proteins (α SMA, calponin, and SM-1). Culture of murine bone marrow cells with PDGF-BB and monomeric collagen has also been reported to produce SMPCs³⁰. These SMPCs exhibit high proliferative rates prior to their expression of SMC marker α SMA, SM22- α , and smooth muscle-myosin heavy chain (SM-MHC). *In vivo* studies have revealed that transplantation of bone marrow labelled with enhanced green-fluorescent protein (eGFP) or β -galactosidase (LacZ) in experimental animals and by sex-mismatching in humans result in neointimal SMCs with the respective labeling or gene marker^{31,32}. Furthermore, co-transplantation of adult human peripheral blood-derived EPCs and SMPCs to a nude/SCID mouse model of hindlimb ischemia has been reported to induce a robust neovascularization, improvement of blood perfusion, and enhancement of tissue injury repair³³.

At the present time, no marker or makers have been confirmed specific for SMPCs^{26,27}. SMPCs, by definition, can only be specified for their potential of a smooth muscle fate but not expressing differentiated SMC marker proteins²⁷. It has been documented that five surface markers regulating various SMPC functions, including PDGFR- β , carboxypeptidase M (CPM), carbonic anhydrase 12 (CA12), receptor activity-modifying protein 1 (RAMP1), and low-density lipoprotein receptor-related protein (LRP1) can be used for detecting circulating SMPCs in humans³⁴. The reliability of these markers remains to be further validated.

Stem/progenitor cell (SPC) types other than those from hematopoietic tissue may possess the potential to develop into vascular cells. The blood vessel wall is a reservoir for resident

precursor cell^{35,36}. The wall of a mature blood vessel typically consists of three layers: the tunica intima, tunica media, and tunica adventitia. All of these three layers contain resident progenitor cells, including EPCs³⁷, SMPCs, and multipotent vascular precursor cells^{38,39}. The initial evidence for the existence of vascular precursors in the vascular wall was obtained from an *ex vivo* study in which embedding rings of human embryonic aorta in collagen gels led to outgrowth of capillary-like structures with cells expressing markers of endothelial differentiation, such as CD31, CD34, von Willebrand factor (vWF), and Flk1/VEGFR-2⁴⁰. Literatures suggest that precursor cells residing in the vessel wall may divided into two categories, one of which is a typical vascular progenitor cell type giving rise to endothelial cells, SMCs, or both, while the other resembles MSCs^{37,41}.

The intima encompasses a layer of endothelial cells lining the luminal surface of the blood vessel and an elastic lamina of subendothelial connective tissue called the basement membrane. Evidence suggests that the vessel wall-associated endothelial cell pool contains a complete hierarchy of endothelial progenitors⁴². EPCs isolated from human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) exhibit similar clonogenic potential and endopoietic activity compared to EPCs derived from human umbilical cord blood. EPCs from the blood vessel wall express a profile of endothelial cell-specific antigens including CD31, CD141, CD105, CD146, CD144, vWF, and Flk1, but not the hematopoietic cell surface markers CD45 and CD14. In addition to EPCs associated with the vascular endothelial pool, the intima of the vessel wall contains MSCs that can differentiate into different types of mesenchymal cells^{43,44}. Gene expression by MSCs from the intima shows a strong similarity to that by MSCs from other sources except for two genes related to angiogenesis, interleukin-8 (IL-8) and matrixmetalloproteinase-2 (MMP-2, or gelatinase A), that are expressed more in intima-associated MSCs than in MSCs from other sources, such as bone marrow and umbilical vein⁴⁴. Furthermore, the expression of both genes is shared with endothelial cells from the umbilical cord vein, suggesting that they may be of particular importance in vascular development and physiology.

Pericytes, also known as Rouget cells or mural cells, predominately reside in the subendothelial space surrounding smaller blood vessels or microvasculature, such as capillaries, precapillary arterioles, and postcapillary venules^{45,46}. These cells are continuous with SMCs of larger-sized arteries and veins⁴⁶. In addition, pericyte-like cells have been reported to exist in the inner intimal layer, primarily in the subendothelial layer, in large, medium, and small arteries in humans⁴⁷. Since pericytes are contractile, they apparently play a role in the regulation of vessel tension, vessel permeability, and blood pressure⁴⁸. Different precursor cell types, including embryonic stem cells (ESCs)^{49,50}, vascular MSCs^{45,51}, bone marrow-derived MSCs⁵², SMCs⁵³, fibroblasts⁵⁴, and hematopoietic precursor cells⁵⁵, have been reported to be able to differentiate into pericytes. Pericytes themselves are multipotent in producing different mature cell types, including SMCs, adipocytes, osteoblasts, chondrocytes, and neurons^{56–60}, which suggests their high degree of plasticity. Pericytes appear to be in a unique status with the capacity to switch phenotypic characteristics in a large range. As capillaries are remodeled into larger vessels to meet increased functional demand, pericytes can further differentiate into true SMCs in order to accommodate the requirement for strengthening the vessel wall⁵⁶. In addition, the multipotency of pericytes may play a role in the pathogenesis of developing lesions in the vasculature. Pericytes can

contribute to plaque formation through differentiation into adipocytes in the lipid core, chondrocytes in the fibrous cap, or osteoblasts in the typically late-stage calcified atherosclerotic plaques³⁷.

Pericytes actively participate in angiogenesis and vasculogenesis. They can stabilize vascular sprouts by migrating along angiogenic sprouts of endothelial cells⁴⁶. Pericytes can also invade tissues in the absence of endothelial cells to form tubes enabling the subsequent penetration of endothelial cells^{61,62}. These precursors produce a number of mediators, including VEGF^{63,64}, angiopoietin 1 and 2 (Ang-1 and 2)⁶⁵, and inflammatory cytokines^{66,67}, to regulate endothelial cell migration, survival, and proliferation.

Most of surface markers expressed by pericytes are not specific. Further, the expression of cell markers by pericytes is dynamic depending on the location and functional status of these cells. The currently accepted identity of human pericytes is the combination of CD146⁺ PDGFR- β ⁺ phenotype along with negative for hematopoietic, endothelial, and myogenic cell markers (CD34⁻CD31⁻CD45⁻CD56⁻)^{45,68-70}. Cultured mouse brain vascular pericytes are CD146⁺PDGFR- β ⁺neural/glial antigen 2 (NG2, also known as chondroitin sulfate proteoglycan 4, or CSPG4)⁺CD31⁻⁷¹. “Activated” pericytes associated with vascular remodeling and neovascularization express an elevated level of regulator G-protein signaling 5 (RGS5)⁷². Pericytes in normal capillaries typically express desmin, but not α SMA. However, pericytes in venules are immunoreactive for both⁴⁶. The expressional pattern of NG2 and α SMA helps to distinguish between three subsets of human pericytes associated with capillaries (NG2⁺ α SMA⁻), venules (NG2⁻ α SMA⁺), and arterioles (NG2⁺ α SMA⁺)⁶⁹. Particularly, NG2 is considered as an angiogenic marker of pericytes⁷³, which appears to be involved in capillary sprouting⁷⁴. The function of NG2 during angiogenesis is assumed to facilitate the activity of several angiopoietic mediators, including PDGF-AA, basic fibroblast growth factor (bFGF), and transforming growth factor- β (TGF- β)⁷³.

The media layer of blood vessel primarily consists of concentrically arranged SMCs along with collagen fibers, elastic fibers, elastic lamellae, and proteoglycans. SMCs in media of artery walls can switch between two typical phenotypes: the “contractile/quiescent phenotype” and “synthetic/proliferative phenotype.” The former is for contraction and the later for synthesis of extracellular matrix proteins. In the process of vascular injury repair, the fully differentiated SMCs switch from their contractile phenotype to synthetic phenotype and proliferate at sites of vascular injury. This activation of SMC proliferation is associated with an increase in production of extracellular matrix components and reduction in expression of SMC marker gene products including α SMA, SM-22 α , and SM-MHC^{26,27}.

The media layer houses vascular precursor cell types. The majority of these vascular precursor cells are multipotent for differentiation. In the media of adult mouse aorta, a side population (SP) of cells displaying the lin⁻c-kit^{-low}Sca1⁺CD34^{-low} surface characteristics have been isolated³⁸. These SP cells can differentiate into CD31, VE-cadherin, and vWF expressing endothelial cells when in culture with VEGF, or into α SMA, calponin, and SM-MHC expressing SMCs when in culture with TGF- β 1/PDGF-BB. In addition, they can generate vascular-like branching structures, composed of both VE-cadherin⁺ cells and smooth muscle actin⁺ cells in Matrigel. SP cells also form myeloid or lymphoid

hematopoietic colonies in methylcellulose-based medium. In human thoracic aorta, two distinct cell populations have been observed between the media and adventitia⁷⁵. One is composed of CD34⁺ cells and the other is made up with c-kit⁺ cells. Cells in both of these two populations actively proliferate. In culture systems, these cells co-express mesenchymal stromal cell markers, including CD44, CD90, and CD105, and stem cell gene products, such as octamer-binding transcription factor 4 (OCT4), c-kit, and breakpoint cluster region pseudogene 1 (BCRP1). They can acquire an endothelial cell phenotype in the presence of VEGF, characterized by increase in KDR and vWF expression. These precursor cells are also able to form capillary-like structures in *in vitro* assessments of angiogenesis.

The adventitia is the outer coating of blood vessels consisting of connective tissue. This layer of the vessel wall has a complex structure containing perivascular nerves, nourishing microvessels, and diverse cell types (including resident vascular progenitor cells) embedded in a collagen-rich extracellular matrix⁴¹. Similar to those residing in the media layer, precursor cells in the adventitia are multipotent for differentiation. In adult mice, clusters of cells expressing stem cell markers, such as Sca1⁺ (21%), c-kit⁺ (9%), CD34⁺ (15%), and Flk1⁺ cells (4%), have been observed in the adventitia of aortic roots⁷⁶. Sca1⁺ cells from this region are able to differentiate into SMCs in response to PDGF-BB stimulation *in vitro* and develop into SMC-like cells in atherosclerotic lesions of the intima following transplantation to the adventitial side of vein grafts in recipient mice. This observation also demonstrates that precursor cells in the adventitia possess the potential to migrate across the vessel wall and differentiate into SMCs. In adult human vascular wall, a “vasculogenic zone” has been described, which is located between the smooth muscle and adventitial layer⁷⁷. It predominantly contains CD34⁺CD31⁻ cells that also express VEGFR-2 and Ang 1 receptor (TIE2). *In vitro*, CD34⁺ cells from human arterial wall form capillary sprouts. New vessels formed by these precursor cells express markers for angiogenically activated endothelial cells, such as carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), and for mature endothelial cells, such as VE-cadherin or occludin. Vascular precursor cells are found in large and middle-sized arteries and veins from various organs. Studies have shown that Notch homolog 1 (Notch1)⁺, Stro1 (a MSC marker)⁺, Sca1⁺, and Oct4⁺ cells are distributed along the vasculogenic niche in the arterial wall³⁹. These cells homogeneously express markers of stemness (Stro1⁺Notch1⁺Oct4⁺) and the MSC lineage (CD44⁺CD90⁺CD105⁺CD73⁺CD29⁺CD166⁺), but are negative for hematopoietic and endothelial markers (CD34⁻CD45⁻CD31⁻vWF⁻). Vascular wall MSCs exhibit characteristics of stem cells, such as a high efflux capability for Hoechst 33342 dye, the ability to form spheroids when growing in suspension, and to generate colonies when seeded at low density. Furthermore, their multipotency of differentiation along the adipogenic, chondrogenic, and leiomyogenic pathways has been identified by culturing them in the respective induction media. They are also able to differentiate into mature SMCs and pericytes⁷⁸.

It is now accepted that bone marrow and blood vessel walls of almost all organ tissues in the body provide niche for MSCs^{70,79–84}. In addition to being locally recruited through direct cell migration, tissue resident MSCs may enter the blood stream to travel to tissue of distant organ systems⁸⁵. MSCs in the peripheral circulation share similarly biological characters with those of bone marrow derived MSCs^{86,87}. Circulating MSCs can also exit the blood

stream to home to the blood vessel wall niche environment through transendothelial migration⁸⁸. It appears that MSCs residing in different organ systems within the body constitute a MSC network, through which they dynamically organize their functional properties, including quiescence, self-renewal, and differentiation. Although sharing similar characteristics, MSCs in different niche environments may vary in phenotype, morphology, capacity for proliferation, and potential for differentiation⁸⁵. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has suggested the following fundamental standards to better define characteristics of MSCs⁸⁹: 1) MSCs must be plastic-adherent when maintained in standard culture conditions; 2) MSCs must express CD105, CD73, and CD90 without expression of CD45, CD34, CD14, CD11b, CD79α, CD19, and human leukocyte antigen-D related (HLA-DR) surface molecules; and 3) MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. Further investigation on the signaling regulation of MSC functional states and travel patterns will provide a deeper insight into MSC biology as well as their role in repair of vascular injury. Table 1 lists phenotypic markers and key properties of vascular precursor cell types.

Mediators Regulating the Activity of Vascular Precursor Cells

Vascular precursor cells should appropriately respond to injurious stimuli in order for them to participate in injury repair. Tissue injury caused by various factors, such as trauma, hemorrhage, vascular disease, infection, and inflammation, is commonly accompanied by destruction of nourishing blood vessels and disruption of local blood perfusion. The resulting hypoxic state evokes the adaptive response of local cells. Hypoxia-inducible transcription factors (HIFs) play a critical role in the regulation of cell responses to hypoxic stimulation^{90,91}. In mammals, HIFs are inactivated through ubiquitination-associated degradation of their α subunits (HIF-1α, HIF-2α, and HIF-3α)⁹². Hypoxia inhibits degradation of HIF-α subunits in the proteasome, allowing translocation of stabilized α subunits to the nucleus to form a complex with constitutively expressed HIF-β and co-activators, which together activate transcription of hypoxia-response element (HRE)-bearing genes^{90,93}. Besides the hypoxia-dependent pathway, activation of HIFs can be induced by various cytokines, growth factors, reactive oxygen/nitrogen species, and microbe-derived components in normoxic conditions⁹⁴. Mechanisms underlying normoxic activation of HIFs remain to be elucidated, which apparently involve regulations at both transcriptional and post-transcriptional levels. HIFs are master transcription factors that activate expression of over 60 genes crucial for cell survival and metabolism under hostile conditions⁹⁵.

The promoter region of VEGF gene contains binding sites for HIFs⁹⁶. Binding of HIFs to the VEGF promoter activates VEGF gene expression^{97,98}. In a rat model of gastric mucosal injury, co-localization of HIF-1α protein with VEGF protein was observed in endothelial cells lining regenerating capillaries⁹⁹. Along with endothelial cells, many other cell types like macrophages, master cells, and fibroblasts express VEGF under the regulation of the HIF activity^{90,100}. In humans, the VEGF gene contains eight exons occupying a coding region of approximately 14kb in chromosome 6p12¹⁰¹. Due to alternative mRNA slicing, VEGF protein molecules may contain different numbers of amino acid residues. At least six VEGF protein isoforms have been identified, including VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206. Most cell types express multiple variants of VEGF

with the predominant expression of VEGF₁₂₁ and VEGF₁₆₅^{102,103}. VEGF (or its recent reclassification as VEGF-A) and other members of the VEGF family [VEGF-B, C, D, E and placenta growth factor (PGF)] have been well characterized. The biological features of each VEGF family member and their correspondent receptors have been described in detail in several recently published review articles^{101,103,104}.

VEGF is a secreted protein crucial for vasculogenesis and angiogenesis¹⁰². VEGF produced by local cells following tissue injury can diffuse into the blood stream, resulting in an increase in VEGF concentration in the systemic circulation^{105–107}. The active forms of VEGF are mostly homodimers with a molecular weight of 45kDa¹⁰⁸. VEGF initiates cell signaling through binding to its receptors, VEGF receptor-1 (VEGFR-1) and VEGFR-2. VEGFR-1 has approximately 10-fold higher affinity for VEGF than that of VEGFR-2, but the kinase activity of VEGFR-1 is about one-tenth that of VEGFR-2¹⁰⁴. Furthermore, the VEGFR1 gene produces two products, membrane-bound VEGFR-1 and soluble VEGFR-1. Membrane-bound VEGFR-1 is the full-length, fully functional VEGFR, while soluble VEGFR-1 consists of only the extracellular domain of VEGFR1 without ability to transmit signal across cell membrane. Therefore, VEGFRs may have different roles in vasculogenesis and angiogenesis. VEGFR-2 essentially serves as a positive signal transducer, while VEGFR-1, particularly soluble VEGFR-1, functions as an attenuator¹⁰⁹. Both EPCs and differentiated endothelial cells in the blood vasculature express VEGFR-2^{18,110}. Engagement of a VEGF homodimer with its receptor causes dimerization of two receptors, triggering their autophosphorylation through the receptor-associated tyrosine kinases. This is followed by activation of several downstream signaling components, including the phospholipase C-gamma (PLC- γ)/protein kinase C (PKC), protein kinase D (PKD), phosphatidylinositol-3 kinase (PI3K), Ras pathway members, and mitogen-activated protein kinase (MAPK), to mediate cell proliferation, differentiation, migration, and contraction^{103,106,111–117}.

The stromal cell-derived factor-1 (SDF-1, also known as CXC motif chemokine 12 or CXCL12)/CXC receptor 4 (CXCR4) axis provides a major driving force for stem/progenitor cell mobilization and homing. EPCs express CXCR4 and respond to SDF-1. The promoter region of SDF-1 contains HIF binding sites¹¹⁸. Engagement of HIF with the SDF-1 promoter activates SDF-1 gene transcription. Cells, particularly endothelial cells, at the site of tissue injury increase their expression and release of SDF-1. In normal circumstance, SDF-1 expression is higher in the bone marrow than in peripheral tissues, but the marrow-periphery gradient of SDF-1 is reversed with the increased release of SDF-1 from sites of injury. Marrow EPCs are then mobilized to the peripheral circulation and recruited to tissue sites with active SDF-1 expression. In addition, SDF-1 causes increases in EPC proliferation and expression of genes for endothelial differentiation, including vWF, TIE2, and VE-cadherin¹¹⁹. SDF-1/CXCR4 signaling has been observed to promote angiogenic activity of EPCs in both *in vitro* and *in vivo* experimental models¹²⁰. SDF-1/CXCR4 signaling is also involved in mediating homing of MSCs and SMPCs^{121,122}.

Macrophage migration inhibitory factor (MIF) is a member of the lately defined 'chemokine-like function' (CLF) chemokine family that promotes EPC migration and angiogenesis¹²³. Hypoxia-induced production of MIF by different cell types, including

endothelial cells and vascular SMCs, involves the HIF-1 α dependent pathway^{124,125}. CD74 is a plasma membrane receptor which binds MIF with a high affinity¹²⁶. CD74 forms heteromeric complexes with either CXCR2 or CXCR4^{127,128}. These activated complexes may initiate cell signaling through the MAPK, PI3K, and protein kinase B (AKT) cascades^{126,128,129}. MIF potently stimulates EPC chemotaxis^{124,130}. Anti-MIF or anti-CXCR4 antibody is able to block EPC migration in response to supernatants of hypoxia-conditioned HUVECs. Clinical studies have shown that both the serum level of MIF and the number of circulating EPCs in patients receiving flap operations increase markedly following the surgery¹³⁰. A significant correlation exists between increases in the serum level of MIF and the number of circulating EPCs. Additionally, serum samples from flap patients promotes EPC migration, which can be partially blocked by anti-MIF antibody. MIF also mediates endothelial cell migration and tube formation *in vitro* as well as to induce angiogenesis *in vivo*¹²³.

Ang-1 and Ang-2 play important roles in facilitating angiogenesis, especially in EPC adhesion to and migration across established endothelium. HIF regulates the expression of Ang-2 by endothelial cells¹³¹. Macrophages can produce Ang-2 in response to different stimuli, including lipopolysaccharide (LPS), interferon-gamma (INF γ), prostaglandin E2 (PGE2), and VEGF¹³². In human adults, Ang-2 is expressed only at sites of vascular remodeling¹³³. Ang-2 is a ligand for the receptor tyrosine kinase TIE2. An important function of Ang-2 is to destabilize established vasculature allowing formation of new vessels. EPCs express Ang-2 receptor. Ang-2 binding to TIE2 causes a marked stimulation of EPC migration¹¹⁷. Ang-2 and VEGF have an additive effect on EPC migration. Ang-2 also promotes adhesion of EPCs to the endothelial cell monolayer. Such adhesion localizes these progenitor cells to angiogenic endothelial cells in order for them to participate in formation of new blood vessels. Besides Ang-2, Ang-1 has been shown to enhance EPC migration and adhesion to endothelial cells¹¹⁷. Hypoxia and VEGF up-regulate Ang-1 gene transcription by pericytes¹³⁴. Therefore, Ang-1 appears to participate in the regulation of EPC migration and homing to injured tissue sites during repair and/or the formation of blood vessels.

Several members of the interleukin family regulate vascular precursor cell function during the process of new vessel formation. Interleukin-1 β (IL-1 β) is a cytokine that promotes vasculogenesis and angiogenesis either through directly exerting its effect on EPCs or indirectly via the HIF-VEGF pathway¹³⁵. Cultures of murine EPCs with IL-1 β display increased numbers of cells and colonies. IL-1 β also significantly increases the number of vessel-like structures in a Matrigel assay of EPCs, demonstrating its ability to augment EPC vasculogenic function. IL-1 β significantly stimulates human EPC proliferation, migration, and adhesion through activation of the PI3K-AKT signal pathway and extracellular-signal-regulated kinases 1/2 (ERK1/2) signaling¹³⁶. EPCs express IL-1 receptor-I (IL-1R). In *in vivo* Matrigel plug experiments, it has been observed that IL-1 produced from infiltrated marrow myeloid cells stimulates VEGF production by recruited endothelial cells from the neighboring tissues, which in turn promotes angiogenesis¹³⁷. Subcutaneous administration of interleukin-1 α (IL-1 α) to mice can cause a strong angiogenic response locally, which is accompanied by infiltration of VEGF-expressing cells. Treatment with VEGFR-2 neutralizing antibodies blocks this IL-1 α -induced angiogenic response¹³⁸. Interleukin-6

(IL-6) has also been found to stimulate EPC proliferation, migration, and Matrigel tube formation. EPCs express IL-6 receptor (IL-6R)¹³⁹. Binding of IL-6 to its receptor activates the gp80/gp130 signaling pathway, leading to activation of the downstream ERK1/2 and signal transducer and activator of transcription 3 (STAT3) cascades.

Genes encoding PDGFs are targeted by hypoxia and/or HIF^{140–143}. Certain inflammatory cytokines and growth factors can also stimulate PDGF expression by different cell types^{144,145}. Mammalian PDGFs are divided into two classes (class I and II), depending on the presence of basic retention motifs (PDGF-A and B) or CUB domains (PDGF-C and D)¹⁴⁵. Ligand PDGFs predominantly form homodimers except for one heterodimer (PDGF-AB) that has been identified in the culture of human platelets¹⁴⁶. PDGFs bind to two similar protein tyrosine-kinase receptors: PDGFR- α and β . PDGF-AA and PDGF-CC function via binding to PDGFR- α , while PDGF-BB acts through engagement with PDGFR- β . Cell types from mesenchymal, hematopoietic, endothelial, and epithelial origins, including MSCs, pericytes, vascular SMCs, blood mononuclear cells, and EPCs, express PDGFRs. The expression of PDGFRs by cells can be substantially upregulated by inflammatory cytokines, growth factors, and microbe-derived substances^{145,147}. Binding of PDGFs to their receptors initiates dimerization of receptors, allowing for receptor autophosphorylation on tyrosine residues in the intracellular domain. Downstream signaling involves activation of several major pathways, including the Ras/MAPK, PI3K, PLC- γ , and signal transducer and activator of transcription 5 (STAT5) cascades, during the process of modulating cell proliferation, differentiation, migration, and secretion¹⁴⁴. Endothelial cells in the developing vasculature, particularly at the tip of angiogenic sprouts and in the growing arteries, strongly express PDGF-BB¹⁴⁵. Locally produced PDGF-BB by endothelial cells plays a crucial role in recruiting pericytes in order for both cell types to function coordinately during the development of new vessels and maturation of existing vessels. PDGF signaling regulates EPC survival, proliferation, and migration^{148,149}. In addition, PDGFs are major mitogens for a number of vascular cell types, including pericytes, fibroblasts, SMPCs, and SMCs¹⁵⁰.

TGF- β is another important mediator for formation and remodeling of blood vessels. In mammals, three isomeric forms (TGF- β 1-3) have been identified in the TGF- β family¹⁵¹. In humans, TGF- β 1 is the predominant isoform that commonly generates homodimers with a mass around 25kDa. Almost all types of cells produce TGF- β 1. Members of the TGF- β family exert their effects by binding to specific receptors¹⁵². In mammals, seven type I [T β RI, also known as activin receptor-like kinase (ALK) 1 to 7] and five type II (T β RII) TGF- β receptors have been identified. TGF- β binds to the type II receptor with a high affinity. Upon binding, a type I receptor is recruited to form a ligand-induced heteromeric receptor complex within which the constitutively active type II receptor phosphorylates the type I receptor on specific serine and threonine residues to activate the type I receptor¹⁵³. ALK5 and ALK1 are major type I receptors for TGF- β signaling in vasculogenic cells¹⁵⁴. Interaction of ligands with TGF- β receptors can be affected by soluble ligand binding proteins and accessory type III receptors, such as endoglin and betaglycan^{155,156}. Signaling molecules positioned immediately downstream of the type I receptor activation are receptor Smads (R-Smads). With the activation of the receptor complex, R-Smads are recruited to and phosphorylated by type I receptors. Typically, activation of ALK5 will induce activation of the Smad 2/3 pathway, whereas activation of ALK1 will lead to activation of the Smad

1/5/8 pathway¹⁵⁷. A complex interplay exists between TGF- β /ALK5 and TGF- β /ALK1 signals, which adjust the overall effect of TGF- β on the downstream signaling activities¹⁵⁸. Activated R-Smads will interact with the common Smad4 (C-Smad) to form heteromeric complexes that will then translocate to the nucleus to regulate the transcription of target genes with the assistance of other partner proteins.

Studies on genetic TGF- β deficiencies have demonstrated that TGF- β 1 and T β RII are critical for both vasculogenesis and angiogenesis^{152,153,158}. TGF- β 1 promotes EPC vasculogenic activity¹⁵⁹. TGF- β signaling also regulates differentiation of EPCs from human pluripotent stem cells¹⁶⁰. TGF- β 1 alone or with PDGF-BB promotes proliferation and SMC differentiation of bone marrow-derived multipotent progenitor cells¹⁶¹. TGF- β 1 also promotes SMC differentiation from MSCs¹⁶² and enhances contractile function of vascular constructs generated from hair follicle mesenchymal stem cells¹⁶³. Except for the direct effect on vascular precursor cells, TGF- β acts on HIF-1 vasculogenic activity through enhancing HIF-1 α gene expression, increasing HIF-1 α protein stability, and inducing HIF-1 DNA binding activity^{164–166}.

In contrast to mediators stimulating the activity of vascular precursor cells as discussed above, certain bioactive molecules exert inhibitory effects on vascular precursor cell function in various physiological and pathological conditions. Vascular endothelial growth inhibitor (VEGI), also termed as tumor necrosis factor superfamily 15 (TNFSF15), is a cytokine produced predominantly by endothelial cells in established blood vessels. Three isoforms of VEGI including VEGI-174, VEGI-192, and VEGI-251 have been identified¹⁶⁷. Since VEGI inhibits endothelial cell proliferation and induces apoptosis in these cells, VEGI appears functioning as an autocrine cytokine to inhibit angiogenesis and stabilize the established vasculature¹⁶⁸. VEGI also exerts a strong inhibitory effect on EPC activities, including their differentiation into endothelial cells, adherence, migration, and vasculogenesis^{169,170}. In addition, VEGI induces apoptosis of differentiated EPCs, but not early-stage EPCs. VEGI exerts its effect on EPCs at least partially through engagement with the death domain-containing receptor 3 (DR3), a member of the TNF receptor superfamily, that is expressed by differentiated EPCs, endothelial cells, and other mature cell types^{169,171}. One possible mechanism underlying VEGI-mediated inhibition of EPC function is to stimulate soluble VEGFR-1 expression by activating the PKC, Src, and Erk1/2 signaling pathway¹⁷⁰. VEGI promotes alternative splicing of the VEGFR-1 gene in favor of soluble VEGFR-1 production by down-regulating nuclear protein Jumonji domain-containing protein 6 (Jmjd6), thus alleviating Jmjd6-inhibited soluble VEGFR-1 expression. Since soluble VEGFR-1 essentially functions as a VEGF trapper/inhibitor¹⁰⁴, the biological activity of VEGF is subsequently inhibited by VEGI. Immunohistochemical staining has shown that VEGI expression in tissues of acute wounds is down-regulated¹⁷². The underlying mechanisms remain to be elucidated. It is apparent that this reduction of VEGI expression is in favor of revascularization during the process of tissue injury repair.

Pigment epithelium-derived factor (PEDF) is a 50-kDa secreted glycoprotein in the serine proteinase inhibitor (Serpins) family¹⁷³. PEDF is a potent anti-angiogenic factor¹⁷⁴. The anti-angiogenic activity of PEDF is selective in that it is effective against newly forming vessels but spares existing ones. PEDF functions on the target cells likely through interaction

with its receptor(s). A putative PEDF receptor of 80–85-kDa has been isolated¹⁷⁵. There is a reciprocal interaction between PEDF and VEGF^{176,177}. *In vitro* studies have observed that PEDF decrease VEGF expression by cultured cells¹⁷⁶. Silencing of the PEDF gene upregulates VEGF expression at both the RNA and protein levels. PEDF inhibits VEGF expression likely through inhibiting hypoxia-induced increase in VEGF promoter activity, HIF-1 nuclear translocation, and mitogen activated protein kinase phosphorylation. In diabetic patients with foot ulcer, the plasma level of PEDF is elevated¹⁷⁸. In animal experiments, it has been observed that the increase in plasma level of PEDF is correlated with the reduction of EPC counts in the peripheral circulation. PEDF also inhibits EPC functional activities, including adhesion, migration, and tube formation, in *in vitro* culture systems. In addition to function on EPCs, PEDF stimulates the surface expression of Fas ligand by endothelial cells¹⁷⁴. Activated endothelial cells that are migrating out from the established vasculature to form new vessels in response to inducers of angiogenesis (such as VEGF) display Fas receptor. The interaction of Fas ligand with Fas receptor induces apoptosis in these activated endothelial cells. Since regular endothelial cells in existing vessels do not express Fas receptor, they appear to be protected from PEDF-induced apoptosis.

Interleukin-10 (IL-10) is generally considered as an anti-inflammatory cytokine. *In vitro* culture of human EPCs with IL-10 has shown that IL-10 causes a dose-dependent inhibition of EPC differentiation into mature endothelial cells¹⁷⁹. The presence of IL-10 in serum is inversely correlated with EPC function in patients with chronic inflammatory diseases, such as systemic lupus erythematosus (SLE). Observations have also shown that activation of the inflammasome pathway in patients with SLE leads to elevation of serum interleukin-18 (IL-18) level, which is correlated with EPC dysfunction¹⁸⁰. IL-18 can cause a dose-dependent inhibition of EPC differentiation into mature endothelial cells in the *in vitro* culture system. In addition, culturing EPCs with angiotensin II (Ang II) has been shown to accelerate the rate of senescence as well as reduction of proliferation in EPCs^{181,182}. Inhibition of telomerase activity appears playing an important role in Ang II-induced impairment of EPC function. Table 2 lists major activators and inhibitors for vascular precursor cell activities.

Vascular Precursor Cells in Vascularization during Tissue Injury Repair

A typical process of wound healing encompasses distinct, yet overlapping, phases of hemostasis, inflammation, proliferation, and maturation. Effective vascularization is essential for delivering oxygen and nutrients to cells in organ tissues. Upon traumatic injury, the initial effort of the host defense is to establish hemostasis, where damaged blood vessels constrict along with local formation of platelet plugs and blood clots. Tissue injury caused by pathological changes of the vascular wall is also accompanied by either bleeding from the rupture of blood vessel or ischemia due to occlusion of blood vessel. Activation of the HIF pathway caused by interruption of tissue oxygen supply ignites chain reactions of cytokine production⁹⁰. Lipid and peptide mediators derived from aggregated platelets participate in initiation of the inflammatory reaction¹⁸³. In the meanwhile, activation of pattern recognition receptors (PRRs) in cells through engagement with damage-associated molecular pattern ligands (DAMPs) and pathogen-associated molecular pattern ligands

(PAMPs, in the presence of infection) evokes the local and/or systemic inflammatory response¹⁸⁴. Recruitment of leukocytes to the site of tissue injury reinforces generation of cytokines, chemokines, and growth factors during the inflammatory phase. These soluble mediators work in concert to recruit vascular precursor cells from their niche environment, such as the bone marrow and vascular wall, to the site of tissue injury. Clinical investigations have shown that EPCs in the peripheral circulation can increase by 50-fold within the first 6 to 12 hours in patients with burn injuries or coronary artery bypass grafting¹⁸⁵.

The bone marrow hematopoietic environment contains an endosteal niche for retention of quiescent stem cells and a permissive sinusoidal site for precursor cell proliferation and their release into the systemic circulation^{186–188}. NG2⁺ pericytes around small arterioles in the endosteum appear to be the major player for maintaining quiescence in marrow stem cells, whereas leptin receptor⁺ cells in the perisinusoidal niche facilitate stem/progenitor cell cycling and proliferation¹⁸⁹. Mobilization of stem and progenitor cells from bone marrow involves cell detachment from the stromal niche and egress into the circulation. During this process, cells are activated for proliferation to expend the pool of these precursors. Programming of precursor cells to improve their potential for differentiation into vascular cells also takes place.

Bone marrow contains diverse types of stromal cells¹⁸⁷. The retention of stem and progenitor cells in their niche relies on ligand binding to the cell membrane. Several ligand/receptor pairs, including kit ligand (kitL)/c-kit, SDF-1/CXCR4, and vascular cell adhesion molecule-1 (VCAM-1)/integrins ($\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$), play important roles in the retention of these cells^{187,188,190}. Many cytokines, such as SDF-1, VEGF, stem cell growth factor (SCF), granulocyte colony-stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF- α , interleukin-3 (IL-3), IL-6, IL-8, and pattern recognition receptor ligands generated from the site of tissue injury stimulate marrow stromal cells and hematopoietic cells to secrete matrix metalloproteinase-9 (MMP-9)^{188,191,193}. MMP-9 converts kitL from a membrane-bound adhesion- and survival-promoting molecule to a soluble survival/mitogenic factor through proteolytic cleavage. Increase in soluble kitL drives transfer of stem cells from the quiescent niche to the proliferative niche¹⁸⁸. In addition, MMP-9 can cleave c-kit from cell surfaces to facilitate their mobilization¹⁹⁴.

Stromal cells in perivascular regions express high levels of SDF-1 that binds to CXCR4 expressed by stem cells to retain them in the marrow niche^{187,195,196}. These perivascular stromal cells, including SDF-1-abundant reticular (CAR) cells, nestin⁺ stromal cells, and leptin receptor⁺ stromal cells, are essentially mesenchymal precursor cells in a considerable overlap with each other. Uncoupling of SDF-1 from CXCR4 in the marrow facilitates the release of these stem cells¹⁹⁷. Certain soluble mediators, such as G-CSF, Flt3 ligand (Flt3L), and SCF induce down-regulation of SDF-1 expression by marrow niche cells^{198,199}. Membrane associated SDF-1 is a substrate for MMP-9 and neutrophil elastase. Degradation of SDF-1 by MMP-9 and elastase in the marrow niche promotes stem cell mobilization^{200,201}. *In vitro* studies have shown that soluble SDF-1 induces transendothelial migration of hematopoietic stem and progenitor cells across marrow endothelium²⁰². Marrow endothelium constitutively expresses endothelial VCAM-1. Engagement of

VCAM-1 with its receptors (including integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$) expressed by stem/progenitor cells helps retain these precursors in the bone marrow²⁰³. G-CSF stimulates marrow neutrophil release of elastase and cathepsin G, which cleave VCAM-1 expressed by marrow stromal cells to further facilitate stem cell mobilization²⁰⁴. In addition to the regulation of stem/progenitor cell mobilization mediated by ligand/receptor pairs described above, actions of other cellular and molecular factors contribute as well. These include changes in cell membrane lipid rafts, activation of the complement cascade and fibrinolytic system, interaction of heat-resistant bioactive lipids [such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P)] with their receptors, and regulation through the sympathetic nervous system^{205,206}.

Due to the close relationship between EPCs and HSCs, these two types of precursors may share similar mechanisms during their mobilization from the bone marrow. Comparable pathways appear to be used for mobilization of other vascular precursor cells. HIF signaling through VEGF and SDF-1 has been shown to promote mobilization of MSCs from the bone marrow²⁰⁷, and SDF-1 induces marrow mobilization of vascular SMPCs²⁰⁸. Nevertheless, evidence suggests that mechanisms do exist for differentially mobilizing subsets of stem/progenitor cells from the bone marrow²⁰⁹. Administration of VEGF, followed by treatment with CXCR4 antagonist AMD3100 in mice has been reported to mobilize both EPCs and stromal progenitor cells, while suppressing hematopoietic stem and progenitor cell activation. Further investigation on mechanisms for optimal mobilization of vascular precursor cells appears to be helpful for developing an effective approach to enhance vascularization during the process of injury repair.

Mobilized marrow precursors gain access to tissue primarily through the systemic circulation. Homing of vascular precursors, requires a series of coordinated efforts, including chemotaxis, adhesion, migration, and vascular integration. Chemoattractants generated at the site of tissue injury build up a gradient of chemoattractive force to guide the migration of vascular precursor cells. Locally produced HIF stimulates surface membrane expression of SDF-1 by endothelial cells, injured SMCs, and activated platelets²¹⁰, which mediates the homing of CXCR4⁺ progenitor cells²¹¹. Additionally, IL-8, human growth-regulated oncogene (GRO), keratinocyte chemoattractant (KC), and neutrophil-activating peptide 2 (NAP-2) expressed by cells in the injured tissue facilitate homing of EPCs through interaction with CXCR2²¹². The coupling of CC chemokines and their receptors, including monocyte chemoattractant protein-1 (MCP-1)/CC receptor 2 (CCR2) and RANTES/CCR5, play a significant role in promoting EPC homing to sites of new vessel formation^{213–215}. Interactions between selectins (E-, L-, and P-selectins) with their ligands helps EPC tethering and recruitment to the site of tissue injury^{216–218}. Functional activities of integrin adhesion molecules are critical in various steps of EPC homing²¹⁹. Specifically, integrins $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha v\beta 3$ and $\alpha v\beta 5$ are major contributors for EPC homing, invasion, differentiation, and paracrine factor production. $\beta 2$ integrins mediate EPC attachment and transendothelial migration. Both VEGF and SDF-1 stimulate expression of intercellular adhesion molecule-1 (ICAM-1, a ligand of $\beta 2$ integrins) by vascular endothelial cells²²⁰. The upregulated expression of ICAM-1 mediates EPC homing by interacting with their surface $\beta 2$ integrins. In addition to EPCs, other marrow-derived vascular precursors, such as MSCs and SMPCs, are able to home to sites of new vessel formation through diverse

mechanisms including the interactions between their expressed integrins and endothelial adhesion molecules, the SDF-1/CXCR4 axis, and PDGF-BB/PDGFR- β ligand-receptor signaling^{46,121,122,221–223}. Currently, mechanisms for recruitment of vascular precursor cells from the blood vessel wall niche are less well defined. Recruitment of cells from remote locations requires transportation through the blood circulation, while cell mobilization from nearby sites might be achieved via direct migration of cells.

Vascular precursor cells contribute to neovascularization during tissue injury repair through two major functions: structural incorporation by converting to functional vascular wall cells and signaling regulation via generation of paracrine and autocrine mediators. Recruited EPCs actively proliferate and differentiate into endothelial cells during vascularization. One estimate suggests that EPCs contribute anywhere between 2–25% of endothelial cells in newly formed vessels²²⁴. In a murine model of new corneal vessel formation, 53% of pericytes were derived from the bone marrow²²⁵. Other *in vivo* studies of injury repair reported that 11–50% of regenerated vascular SMCs were from the bone marrow origin²²⁶. Besides differentiation into mature vascular cells along the same lineage, translineage commitment of vascular precursor cells may also contribute to new vessel formation. With appropriate stimulation, EPCs can give rise to vascular SMCs through endothelial-to-mesenchymal transdifferentiation (EnMT)^{227,228}. MSCs can differentiate into multiple types of vascular cells, including SMCs, pericytes, and endothelial cells^{229–231}. Figure 2 illustrates differentiation, transdifferentiation, and interchange of vascular precursor cells and vascular cells. Currently, the relative contributions of recruited and resident cells to new vessel formation during the process of injury repair remains uncertain^{225,232}. It may vary depending on the type and extent of injury as well as the tissue type involved in injury.

Vascular precursor cells are potent producers for bioactive mediators²³³. Mediators expressed by EPCs include HIF-1 α , VEGF, PDGF, TGF- β 1, SDF-1, insulin-like growth factor-1 (IGF-1), and IL-8^{234–238}. Attachment of EPCs to extracellular matrices upregulates expression of vasculogenic mediators by these precursors²³⁵. EPCs also release extracellular vesicles, including microvesicles (50–1000 nm) and exosomes (30–120 nm), containing cell-associated protein, RNA or microRNA, and DNA components^{239,240}. These extracellular vesicles activate the angiogenic activity of vascular cells, particularly mature endothelial cells, through horizontal transfer of signaling molecules. Exposure to EPC-derived exosomes enhances proliferation, migration, and tube formation of endothelial cells in *in vitro* culture models^{241,242}. Endothelial cells stimulated with these exosomes have been shown to increase the expression of angiogenesis-related molecules, including FGF, VEGF, VEGFR-2, Ang, E-selectin, CXC motif chemokine 16 (CXCL16), nitric oxide synthase (eNOS) and IL-8. Encountering extracellular vesicles, pericytes increase expression of VEGF²⁴³, which further promotes the survival and stabilization of endothelial cells. *In vivo* treatment with EPC-derived exosomes has been reported to accelerate re-endothelialization in the early phase post endothelial damage in the carotid artery in rats²⁴¹ and to enhance cutaneous wound healing in diabetic rats²⁴². MSCs are known to produce many paracrine factors for neovascularization, including VEGF, IGF-1, TGF- β 1, MCP-1, and IL-8^{244–247}. Activation of MSCs following exposure to inflammatory cytokines (such as TNF- α , IL-6, and TGF- α) and PAMPs (such as LPS) enhances their production of vasculogenic factors^{248–251}. Like EPCs, MSCs produce extracellular vesicles to promote migration and

angiogenic activity of endothelial cells and pericytes^{252,253}. Activities of MSC-derived extracellular vesicles in promoting neovascularization as well as the potential of their application for clinical treatment have been comprehensively discussed in a recent review article²⁵⁴.

At the present time, recognition about the relative contribution of direct cell integration and generating paracrine/autocrine mediators by vascular precursors to new vessel formation during tissue injury repair remains incomplete. Discrepancy also exists in reports regarding the significance of vascular precursor cells in revascularization during injury repair²⁵⁵. Improvement of techniques for tracing vascular precursor cells and further investigation on activities of vasculogenic mediators produced by vascular precursor cells will be helpful for understanding the integrated efforts in the regulation of new vessel formation.

Application of Vascular Precursor Cells in the treatment of Tissue Injury

Recently, efforts in exploring the potential of using vascular precursor cells in the treatment of chronic wounds (such as diabetic ulcers and delayed healing bone fractures), traumatic injury (such as bone fracture repair), and ischemic damage in vital organ systems (such as myocardial infarction and stroke) has been substantially expanded. Non-healing ulcers in lower extremities are frequent complications of diabetes. Insufficiency in the number and function of vascular precursor cells is one of the common causes for the development of these wounds^{256–258}. Accordingly, stimulating release of vascular precursor cells from their niche environment has been investigated as a therapeutic approach for the treatment of these chronic ulcers. G-CSF stimulates bone marrow release of granulocytes, HSCs, and EPCs. In a randomized placebo-controlled study of 40 patients with diabetic foot infection, administration of G-CSF as adjunctive therapy was beneficial for earlier eradication of pathogens from the infected ulcer, quicker resolution of cellulitis, and avoiding surgical interventions²⁵⁹. An analysis of five clinical trials including a total of 167 patients with diabetic foot infection has also revealed that G-CSF administration significantly reduces likelihood of lower extremity surgical interventions, such as amputation, and shortens the duration of hospital stay²⁶⁰. In these cases, the beneficial effect of G-CSF appears to be linked to the improvement of neutrophil-mediated immune defense. It remains unclear if mobilization of EPCs plays a role. Ang-1 is another potent factor stimulating EPC mobilization and activation. Overexpression of Ang-1 at the site of injury through adenoviral vector mediated Ang-1 gene transduction has been reported to result in improvement of EPC recruitment, neovascularization, and re-epithelialization in diabetic mice²⁶¹. Other strategies have also been found to improve diabetic wound closure and neovascularization in mice, such as systemic administration of agent AMD3100 promoting stem and progenitor cell mobilization along with local application of PDGF-BB²⁶².

Cell based-therapies aiming at increasing local vascular precursor cells and/or enhancing functional activities of these cells have been shown to improve healing outcomes of chronic wounds in diabetes. One prospective clinical trial phase I/IIa study reported that patients with non-healing diabetic foot ulcers achieved complete wound closure with increased vascular perfusion at an average of 18 weeks following local transplantation of autologous blood CD34⁺ cells²⁶³. Local implantation of EPCs or topical application of vascular

progenitors (EPCs and CD133⁺ progenitor cells) has been documented to promote neovascularization and accelerated wound healing in diabetic murine models^{236,237,264}. These precursors actively produce vasculogenic mediators (such as VEGF, IL-8, and PDGF) and other growth factors [such as bFGF, keratinocyte growth factor (KGF)] to exert paracrine stimulation of vascularization and injury repair. Local proangiogenic priming by injecting a mixture of VEGF, bFGF, and PDGF prior to implantation of EPCs has been observed to further improve the effectiveness of neovascularization and wound healing in diabetic animals²⁶⁵. Enhancing growth of marrow-derived EPCs in PolyCaprolactone-Gelatin (PCG) nano-fiber matrix in culture and then applying this PCG-EPCs matrix to the wound of diabetic mice can achieve sustained delivery of EPCs onto the diabetic wounds and enhance fibrosis-free wound healing²⁶⁶. In addition to EPCs, topical application of MSCs can also achieve inducing early granulation tissue formation and stabilizing neovasculture at wound sites²⁶⁷. Implantation of collagen scaffolds containing MSCs in murine skin wounds has been reported to enhance vascularization during the dermal regeneration process²⁶⁸. Since paracrine interactions among vascular precursor cells, inflammatory cells, and tissue cells at the site of tissue injury play a pivotal role in the regulation of wound repair, developing scaffold matrices containing optimal components of EPCs, MSCs, and vasculogenic mediators highlights an area of current interest for improving the treatment of chronic diabetic wounds.

Vascularization is also critical for healing of bone injury. Strategies for utilizing vascular precursor cells to enhance vascularization during bone injury repair include stimulation of native vascular precursor cell homing to the site of injury, local delivery of vascular precursor cells, and the combination of both techniques. The advantages of stimulating vascular precursor cell homing are the simplicity in application, inexpensive cost, and utilization of self-cell resources. VEGF, PDGF, SDF-1, and FGF have been tested in this application²⁶⁹. The direct absorption of vasculogenic mediators can achieve an immediate burst of stimulation, while continuing release from carrier materials entrapping vasculogenic factors, such as hydrogels, microspheres, and nanoparticles, allows generation of sustained and/or dynamic stimulations. Success has also been reported in enhancing local production of vasculogenic factors by tissue cells with genetic-engineering techniques^{270,271}. Local delivery of marrow-derived EPCs to the site of bone fractures has been reported to enhance VEGF expression and to promote bone healing in experimental animals^{272,273}. Development of bioengineered scaffolds modified with vasculogenic mediators and containing stem and progenitor cells may enhance vascularization in large bone grafts²⁴². Along this path, a variety of precursor cells, including EPCs, MSCs, HSCs, pericytes, and ESCs, have been tested. Since interplays among cells in the bone and marrow regulate bone regeneration and its vascularization, including elements of bone marrow in bioengineered scaffolds deserves more attention in the future investigations.

Ischemia-induced myocardial infarction is a life threatening injury. Myocardial ischemia causes a rapid mobilization of EPCs into the systemic circulation²⁷⁵ and increase in homing of these vascular precursor cells to the injured heart^{276,277}. These observations identify that EPCs actively participate in the host response to ischemic injury in the heart. EPCs infused intravenously can actively incorporate into foci of myocardial neovascularization²⁷⁶. Administration of G-CSF to promote mobilization of stem and progenitor cells from the

bone marrow has been shown to improve vascular precursor cell homing, infarcted tissue repair, and left ventricular function in animal models of myocardial ischemia^{278–280}. However, results of G-CSF treatment in patients with ischemic heart diseases remain controversial^{281–285}. In experimental studies, implantation of EPCs and other vascular precursor cells around the ischemic areas appears having promising effects. Direct transplantation of human EPCs or MSCs into the border regions of ischemic heart tissue is able to improve cardiac function and enhance vascularization in rats^{233,286}. Infusion of autologous EPCs in the circumflex artery following myocardial infarction can substantially reduce the size of infarction with an enhancement of vascularization in pigs²⁸⁷. Sequential transplantation of EPCs followed by implantation of fetal cardiomyocytes or EPCs again to marginal zones of infarction has been reported to additively improve myocardial function and vascularization in rats²⁸⁸. In addition, local implantation of genetically engineered EPCs and MSCs overexpressing vasculogenic mediators (IGF-1, FGF-1, and SDF-1) has been demonstrated to further enhance the beneficial effects of these precursor cells on neovascularization and myocardial function in animal models of myocardial ischemia^{289–291}. Treatment of mouse $\text{lin}^{-}\text{Sca1}^{+}\text{CD31}^{+}$ EPCs and human CD34^{+} cells with inhibitors of DNA methyltransferases (5-Azacytidine), histone deacetylases (valproic acid), and G9a histone dimethyltransferase (BIX-01294) can globally increase the activity of the transcriptome, including reactivation of pluripotency-associated genes and up-regulation of cardiomyocyte- and endothelial cell-specific gene expression²⁹². Intramyocardial transplantation of these reprogrammed mouse and human EPCs into mice with acute myocardial infarction has been shown to significantly improve ventricular function, enhance de novo cardiomyocyte differentiation of transplanted cells, and increase capillary density. Several clinical trials have reported that intracoronary transfer of autologous bone marrow cells or bone marrow-derived stem cells result in a better recovery of regional systolic function and reduction in infarct size in patients with myocardial infarction^{293,294}. Recent meta-analyses of several clinical studies have confirmed that stem/progenitor cell therapy improves left ventricular contractility in patients with myocardial infarction^{294–298}. Moreover, intracoronary cell therapy has been reported to be accompanied with a decrease in incidence of death, recurrent of acute myocardial infarction, and readmission for heart failure^{294,297}. At the present time, however, clinical reports regarding the effect of intracoronary stem/progenitor cell treatment on the infarct volume and remodeling remain inconsistent^{294,295,297,299}. A phase 3, randomized, double-blinded, active-controlled, unblinded standard of care study (the RENEW study) is underway to assess the efficacy and safety of intramyocardial administration of autologous CD34^{+} cells in patients with refractory angina³⁰⁰.

Stroke is a devastating insult to the brain. Optimal repair of brain injury relies on effective revascularization to support neurogenesis and synaptic plasticity. Like what has been observed in ischemic-induced myocardial infarction, stroke causes mobilization of vascular precursor cells into the systemic circulation^{301–304}. In a rat model of stroke, endogenously mobilized EPCs were observed to participate in neovascularization in the boundary zone of ischemia, which was beneficial for maintaining neurobehavioral functions of animals³⁰⁵. Active homing of nanoprobe-labeled EPCs to the peri-infarct area in the brain of mice has been detected by non-invasive imaging after the ischemic attack. Transplanted EPCs are able

to incorporate into vessels around areas of infarction through neovascularization³⁰⁶. Studies on patients suffering from strokes have revealed that an increase in EPC concentration in the circulation is a positive sign for better outcomes of recovery^{302,303,307}, while failure to elevate blood EPCs often implies a poorer prognosis^{301,308}. In animal models of brain ischemia, systemic or intra-carotid arterial administration of EPCs has been shown to enhance neovascularization and neurogenesis, reduce the severity of brain injuries, and improve long-term neurobehavioral outcomes^{309–311}. EPC-derived paracrine mediators also play an important role in promoting neovascularization and neurogenesis following the ischemic attack^{312,313}. Treatment with G-CSF in combination with transplantation of human umbilical cord blood cells in animal models of stroke has been reported to be beneficial in coordinating efforts of both transplanted precursor cells and endogenously mobilized stem/progenitors in integration of graft-host cells, production of growth factors, and promotion of neurogenesis³¹⁴. In patients with stroke, treatment with G-CSF has been shown to substantially increase CD34⁺ cells in the circulation^{315,316}, which is associated with a tendency toward reduction in ischemic lesion in the brain³¹⁶. Several phase I clinical trials have been completed involving intra-arterial or intravenous transplantation of autologous marrow mononuclear cells, including CD34⁺ precursor cells, in patients with stroke^{317,318}. The results suggest that this therapy is feasible and seems to be safe. A phase II, randomized, dose-finding, controlled multicenter trial on intra-arterial bone marrow cells transplantation in patients with acute ischemic stroke (IBIS trial) has been launched recently³¹⁹.

Perspective Remarks

Vascular precursor cells in adults represent a highly heterogeneous population of tissue stem/progenitor cells residing in different niche environments. A complex signaling network regulates their self-renewal, proliferation, differentiation, transdifferentiation, mobilization, and homing. These cells actively participate in tissue injury repair through structural integration into the vasculature and generation of soluble mediators that promote the coordinated efforts among various types of cells in new vessel formation and tissue regeneration. Improved understanding of underlying mechanisms will enhance the development of vascular precursor cell-based therapies for effective treatment of wounds and ischemic attacks in vital organ systems. Current attention is primarily focused on exploring the maximal efficacy of treatment using optimized combinations of vascular precursor cells and vasculogenic agents. Genetic engineering of vascular precursor cells to boost their function in promoting vascularization represents another new direction of research. However, identifying the risk and safety of vascular cell-based therapies are vital for their clinical application in the future.

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Definition of abbreviations

7AAD	7-Aminoactinomycin D
αSMA	alpha smooth muscle actin
AKT	protein kinase B
ALK	activin receptor-like kinase
Ang	angiopoietin
Ang II	angiotensin II
BCRP1	breakpoint cluster region pseudogene
bFGF	basic fibroblast growth factor
C1P	ceramide-1-phosphate
CA12	carbonic anhydrase 12
CAC	circulating angiogenic cell
CAR	SDF-1-abundant reticular
CCR	CC receptor
CD	clusters of differentiation
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CEP	circulating endothelial precursor
c-kit	stem cell growth factor receptor
CLF	chemokine-like function
CPM	carboxypeptidase M
CSPG4	chondroitin sulfate proteoglycan 4
CXCL12	CXC motif chemokine 12
CXCL16	CXC motif chemokine 16
CXCR	CXC receptor
DAMP	damage-associated molecular pattern ligand
DR3	death domaincontaining receptor 3
ECFC	endothelial colony forming cell
eGFP	enhanced green-fluorescent protein
EnMT	endothelial-to-mesenchymal transdifferentiation

eNOS	nitric oxide synthase
EPC	endothelial progenitor cell
ERK1/2	extracellular-signal-regulated kinases 1/2
ESC	embryonic stem cell
FGF	fibroblast growth factor
Flk1	fms-like tyrosine kinase-1
Flt3L	Flt3 ligand
G-CSF	granulocyte colony-stimulating factor
GFP	green-fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO	human growth-regulated oncogene
HAEC	human aortic endothelial cell
HIF	hypoxia-inducible transcription factor
HLA-DR	human leukocyte antigen - antigen D related
HRE	hypoxia-response element
HSC	hematopoietic stem cell
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-gamma
IGF-1	insulin-like growth factor-1
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-1R	interleukin-1 receptor
IL-3	interleukin-3
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IL-8	interleukin-8
IL-10	interleukin-10
IL-18	interleukin-18

Jmjd6	Jumonji domain-containing protein 6
KC	keratinocyte chemoattractant
KDR	kinase insert domain receptor
KGF	keratinocyte growth factor
kitL	kit ligand
LacZ	β -galactosidase
Lin	lineage
LRP1	low-density lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MIF	macrophage migration inhibitory factor
MMP-2	matrixmetalloproteinase-2
MMP-9	matrixmetalloproteinase-9
MSC	mesenchymal stem cell
NAP-2	neutrophil-activating peptide 2
NG2	neural/glial antigen 2
Notch1	Notch homolog 1
OCT4	octamer-binding transcription factor 4
PAMP	pathogen-associated molecular pattern ligand
PBMC	peripheral blood mononuclear cell
PCG	PolyCaprolactone-Gelatin
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PEDF	pigment epithelium-derived factor
PGE2	prostaglandin E2
PGF	placenta growth factor
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C

PKD	protein kinase D
PLC-γ	phospholipase C-gamma
PRR	pattern recognition receptors
RAMP1	receptor activity-modifying protein 1
RGS5	regulator G-protein signaling 5
S1P	sphingosine-1-phosphate
Sca1	stem cell antigen-1
SCF	stem cell growth factor
SDF-1	stromal cell-derived factor-1
SLE	systemic lupus erythematosus
SM-22a	smooth muscle-22a
SMC	smooth muscle cell
SMem	embryonic form smooth muscle myosin heavy chain
SMPC	smooth muscle progenitor cell
SP	side population
SPC	stem/progenitor cell
STAT	signal transducer and activator of transcription
TβR	transforming growth factor beta receptor
TGF-β	transforming growth factor beta
TIE2	receptor for angiopoietin
TNF	tumor necrosis factor
TNFSF15	Tumor necrosis factor superfamily 15
UEA1	Ulex europaeus agglutinin-1
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor receptor-2
VEGI	vascular endothelial growth inhibitor
vWF	von Willebrand factor

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Background

Tissue injury is commonly associated with damage to blood vasculature. Restoring blood supply is essential for wound healing. Crosstalk among signaling pathways coordinates recruitment of vascular precursor cells to the site of tissue injury and regulates their activity during neovascularization. In recent years, substantial progress has been achieved in basic studies on molecular mechanisms underlying the regulation of vascular precursor cell function during their participation in tissue injury repair.

Translational Significance

Novel information obtained from these basic investigations drives exploration of vascular precursor cell-based approaches to treat chronic wounds and ischemic diseases in vital organ systems.

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Figure 1.
Involvement of vascular precursor cells in neovascularization during tissue injury repair.

Figure 2. Differentiation, transdifferentiation, and interchange of vascular precursor cells and vascular cells.

Table 1

Phenotypic markers and key properties of vascular precursor cells

Precursor cell	Origin	Phenotypic markers
EPC	Human	CD45 ⁻ CD34 ⁺ CD133 ⁺ VEGFR-2(KDR) ⁺
	Mouse	Lin ⁻ c-kit ⁺ Sca1 ⁺ VEGFR-2(Flk1) ⁺
	Rat	Lin ⁻ Hoechst ⁺ CD36 ⁺
	Rabbit	CD34 ⁺ VEGFR-2 ⁺ UEA1 ⁺ with positive for uptake of acetylated low-density lipoprotein
MSC	Human	1) Plastic-adherent when maintained in standard culture conditions;
		2) CD105 ⁺ CD73 ⁺ CD90 ⁺ CD45 ⁻ CD34 ⁻ CD14 ⁻ CD11b ⁻ CD79α ⁻ CD19 ⁻ HLA-DR ⁻ ;
		3) Differentiation into osteoblasts, adipocytes, and chondroblasts
Pericyte	Human	CD34 ⁻ CD31 ⁻ CD45 ⁻ CD56 ⁻ CD146 ⁺ PDGFRβ ⁺
	Capillary	NG2 ⁺ aSMA ⁻
	Venule	NG2 ⁻ aSMA ⁺
	Arteriole	NG2 ⁺ aSMA ⁺
	Mouse	CD31 ⁻ CD146 ⁺ PDGFRβ ⁺ NG2 ⁺
SMPC	Human	(PDGFR-β ⁺ CPM ⁺ CA12 ⁺ RAMP1 ⁺ LRP1 ⁺)?
Vascular wall SPC	Human	CD31 ⁻ CD34 ⁺ VEGFR(KDR) ⁺ TIE2 ⁺
	Mouse	C-kit ⁺
	Mouse	Lin ⁻ c-kit ^{-/low} Sca1 ⁺ CD34 ^{-/low}

Table 2

Major factors regulating the activity of vascular precursor cells

Mediators	Receptors	Function
Activators		
Ang	TIE2	EPC adhesion, migration, and homing
HIF		Master transcription factor for expression of over 60 genes related to vasculogenesis, angiogenesis, and inflammation
IL-1	IL-1R	EPC proliferation, adhesion, migration, and differentiation
IL-6	IL-6R	EPC proliferation, migration, and differentiation
MIF	CD74	EPC migration and chemotaxis
PDGF	PDGFR	Survive, proliferation, migration, differentiation, and secretion of EPCs, MSCs, and pericytes
SDF-1/CXCL12	CXCR4	Retention of SPCs in their niche; recruitment and homing of EPCs, MSCs, and SMPs; EPC proliferation and differentiation
TGF- β	T β R	Proliferation and differentiation of EPCs and MSCs; HIF expression, stability, and activity in cells
VEGF	VEGFR-1, VEGFR-2	EPC proliferation, migration, and differentiation
Inhibitors		
VEG1/TNFSF15	??, DR3	EPC survive, adhesion, migration, and differentiation
PEDF	PEDF receptor?	EPC adhesion, migration, and differentiation; expression of VEGF
IL-10	IL-10 receptor (IL-10R)	EPC differentiation
IL-18	IL-18 receptor (IL-18R)	EPC differentiation
Ang II	Ang II receptor (Ang IIR)	EPC proliferation, induction of senescence in EPCs