Engineering Functional Blood Vessels In Vivo

by

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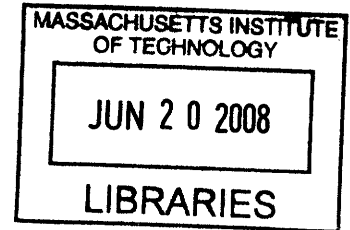

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ABSTRACT

At the present time, there are many hurdles to overcome in order to create a long-lasting and engineered tissue for tissue transplant in patients. The challenges include the isolation and expansion of appropriate cells, the arrangement of assorted cells into correct spatial organization, and the development of proper growth conditions. Furthermore, the creation of a three dimensional engineered tissue is limited by the fact that tissue assemblies greater than 100-200 micrometers, the limit of oxygen diffusion, require a perfused vascular bed to supply nutrients and to remove waste products and metabolic intermediates. To overcome this limitation, this thesis aims to pre-seed a tissue engineered construct with vascular cells (both endothelial and perivascular cells), so the vascular cells could readily form functional vessels *in situ*. Previous work in the laboratory had successfully demonstrated the formation of functional microvascular network by co-implantation of human umbilical cord vein endothelial cells (HUVECs) and 10T1/2 cells, a line of mouse embryonic fibroblasts.

To translate this concept to the clinic, we need to utilize cells that can be secured and used in clinic. To this end, we systematically replace each individual vascular cell type with a readily available source of cells. First, we investigated human embryonic stem cells (hESCs) derived endothelial cells. We demonstrated that when hESCs derived endothelial cells were implanted into SCID mice, they formed blood vessels that integrated into the host circulatory system and served as blood conduits. Second, we compared the formation and function of engineered blood vessels generated from circulating endothelial progenitor cells (EPCs) derived from either adult peripheral blood or umbilical cord blood. We found that adult peripheral blood EPCs formed blood vessels that were unstable and regressed within three weeks. In contrast, umbilical cord blood EPCs formed normal-functioning blood vessels that lasted for more than four months. These vessels exhibited normal blood flow, perm-selectivity to macromolecules and induction of leukocyte-endothelial interactions in response to cytokine activation similar to normal vessels. Third, we evaluated human bone marrow-derived mesenchymal stem cells (hMSCs) as a source of vascular progenitor cells. hMSCs expressed a panel of smooth muscle markers *in vitro* and cell-cell contact between endothelial cells and hMSCs up-regulated the transcription of smooth muscle markers. hMSCs efficiently stabilized nascent blood vessels *in vivo* by functioning as perivascular precursor cells. The engineered blood vessels derived from HUVECs and hMSCs remained stable and functional for more than 130 days *in vivo*. On the other hand, we could not detect

differentiation of hMSCs to endothelial cell *in vitro* and hMSCs by themselves could not form conduit for blood flow *in vivo*. Similar to normal perivascular cells, hMSCs-derived perivascular cells contracted in response to endothelin-1 *in vivo*. Thus, our work demonstrates the potential to generate a patent and functional microvascular network by pre-seeding vascular cells in a tissue-engineered construct. It serves as a platform for the addition of parenchymal cells to create a functional and vascularized engineered tissue.

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Chapter 1: Background and Specific Aims

Introduction

Mechanistic studies of the angiogenesis and vasculogenesis in mouse models have provided tremendous biological insight. However, mouse models may not faithfully reproduce human physiology. Alternatively, experiments can be performed *in vitro* with human endothelial cells, but there is a drawback in that the initiation and maintenance of blood flow cannot be easily mimicked *in vitro*. Therefore, there is a real need to develop a better *in vivo* model of angiogenesis and vasculogenesis with human vascular cells. Besides the gain in basic biological understanding, the ability to manipulate the growth of new blood vessels with human vascular cells will directly impact regenerative medicine.

It is clear that angiogenesis, the growth of new vessels from pre-existing blood vessels, plays a critical role in tissue repair and regeneration. Defect in angiogenesis is observed in patients with ischemic peripheral disease and delayed wound healing. In such cases, direct injection of vascular cells (both endothelial cells and perivascular cells) may offer an alternative approach to enhance the revascularization of an ischemic tissue. Besides this, the ability to create a patent microvascular network will significantly impact the engineering of functional tissue. Tissue engineering is a discipline that "applies the principles of biology and engineering to the development of functional substitutes for damaged tissue."[1] An increasing number of Americans today suffers from tissue loss or end-stage organ failure (ie. myocardial infarction and renal failure) and often times, the only viable therapeutic option is surgical repair or replacement of the defective tissues or organs. Approximately 8 million of such surgical procedures are performed annually[2]. Although tissue and organ transplants can offer dramatic improvement in quality of life

for patients, they have severe limitations. First, the demand for organs far outstrips supply. In 2005, there were 89,884 patients on the transplantation waitlist, but only 27,527 patients were recipients of transplanted organs and 7,193 patients died while on the waitlist[3]. Furthermore, allograft transplants entail a life-long regimen of immuno-suppression that often results in frequent infections and elevated risk for certain cancers[4]. One possible solution to the severe shortage of tissues and organs for transplant is to engineer such tissue artificially or to regenerate the diseased tissue *in situ*.

At the present time, there are many hurdles to overcome in order to create a long-lasting and patent engineered tissue for tissue transplant. The challenges include the isolation and expansion of appropriate cells, the arrangement of assorted cells into correct spatial organization, and the development of proper growth conditions. Furthermore, the creation of a three dimensional engineered tissue is limited by the fact that tissue assemblies greater than 100-200 micrometers, the limit of oxygen diffusion, require a perfused vascular bed to supply nutrients and to remove waste products and metabolic intermediates[5]. To overcome this limitation, a tissue engineered construct could be pre-seeded with both parenchymal cells and vascular cells (both endothelial and perivascular cells), so the vascular cells could readily form functional vessels *in situ*. Previous work in the laboratory had successfully demonstrated the formation of functional microvascular network by co-implantation of human umbilical cord vein endothelial cells (HUVECs) and 10T1/2 cells, a line of mouse embryonic fibroblasts. The goal of this thesis is to systematically replace HUVECs and 10T1/2 cells with clinically applicable sources of vascular cells and demonstrate their effectiveness in creating functional microvessels *in vivo*. This thesis aims to derive endothelial and smooth muscle precursor cells from

human embryonic stem cells (Chapter 2) and human progenitor cells from peripheral blood (Chapter 3), umbilical cord blood (Chapter 3) and bone marrow (Chapter 4).

In addition, previous data demonstrated that endothelial cells, when implanted alone, do not form stable and long-lasting vessels. This may be due to a deficiency in the recruitment of host perivascular cells during the critical early time period. This thesis aims to enhance the recruitment of host perivascular cells by genetically engineering the endothelial cells to secrete PDGF-BB, a chemotatic factor for perivascular cells (Chapter 5).

Specific Aims

Overall Hypothesis: Heterotypic interaction between endothelial and smooth muscle precursor cells directs their differentiation and provides bi-directional survival signals to form stable and long-lasting microvascular network *in vivo*.

Specific Aim 1: Differentiate endothelial precursor cells from human embryonic stem cells and demonstrate their in vivo vasculogenic capacity.

Human embryonic stem cells (hESCs) hold tremendous clinical potential due to their ability to differentiate into all the different cell types in a body. However, it is challenging to obtain a large number of lineage specific cells. This study intends to develop a simple differentiation procedure to derive a relatively pure population of endothelial precursor cells from human embryonic stem cells. Furthermore, the endothelial precursor cells will be evaluated for their capacity to form functional blood vessels in vivo.

Specific Aim 1a: Differentiate endothelial precursor cells from human embryonic stem cells.

Human embryonic stem cells will be differentiated either three dimensionally in embryoid bodies or two-dimensionally in monolayer. The efficiency of embryonic stem cell differentiation will be determined by FACS analysis of CD31+CD34+ cells at a different timepoint. Once the optimal timepoint has been identified, the endothelial precursor cells will be isolated by magnetic bead sorting for CD34+ cells. The CD34+ cells will be cultured in endothelial growth medium to promote the proliferation of

endothelial cells. The endothelial phenotype of the hESC-derived cells will be evaluated by immunostaining for endothelial markers including CD31, VE-Cadherin, and von Willebrand Factor, and uptaking of Dil-labeld acetylated low-density lipoprotein.

Specific Aim 1b: Determine the in vivo vessel forming capacity of human embryonic stem cell derived endothelial precursor cells.

The hESC derived endothelial cells will be co-implanted with 10T1/2 cells in a collagen gel in a SCID mouse installed with a cranial window. The enhanced green fluorescent protein (EGFP) labeled endothelial cells will be imaged with intravital multiphoton laser scanning microscopy. The endothelial cells will be monitored periodically to determine whether they have integrated into the host circulation.

Specific Aim 2: Determine the in vivo vasculogenic capacity of human endothelial progenitor cells.

In order to apply our technique with a source of vascular cells that is closer to clinical use, here we isolated and characterized the phenotypes of endothelial progenitor cells derived from peripheral blood and umbilical cord blood. The endothelial progenitor cells were examined for their expression of endothelial markers. The two sources of endothelial progenitor cells were then directly compared for their *in vivo* vasculogenic potential in forming stable microvascular network.

Specific Aim 2a: Isolate and culture endothelial progenitor cells from human peripheral blood and umbilical cord blood.

Mononuclear blood cells will be isolated from human peripheral blood and umbilical cord blood by density gradient centrifugation over Ficoll solution. The mononuclear blood cells will then be cultured with endothelial growth medium on a culture dish coated with fibronectin. The formation of endothelial outgrowth colonies will be assessed and the endothelial cell colonies will be subcultured. Expression of endothelial markers will be evaluated by both immunostaining and fluorescence activated cell-sorting (FACS) analysis.

Specific Aim 2b: Evaluate the ability of EPC-derived endothelial cells to form functional vasculature in vivo.

The EPC derived endothelial cells will be transduced with retrovirus to express enhanced green fluorescent protein (EGFP). The labeled endothelial cells will be incorporated with or without 10T1/2 cells, a line of murine embryonic fibroblasts, in a Type I collagen/fibronectin gel. The construct will then be implanted in a cranial window of a SCID mouse. The vasculogenic potential of the EGFP labeled endothelial cells will be monitored in real-time with intravital multiphoton laser scanning microscopy. Functional engineered vessel density will be assessed at various timepoints for the different experimental groups.

Specific Aim 2c: Determine the in vivo functional performance of endothelial progenitor cell derived blood vessels

The functional performance of engineered blood vessels will be assessed. Once the engineered vessels have stabilized, the vascular permeability, response to cytokine

stimulation and blood flow rate will be measured and compared to those of the normal mouse brain capillaries.

Specific Aim 3: Determine the capacity of mesenchymal stem cells to function as vascular progenitor for both endothelial and perivascular lineages.

Mesenchymal stem cells (MSCs) are multipotent cells that have potential to differentiate into a number of different connective tissue lineages including osteocyte, chrondrocyte and adipocyte. Data in the literature also suggest that mesenchymal stem cells may function as vascular progenitor cells with the capacity to differentiate into both endothelial and smooth muscle cells. However, the data are generally equivocal. Here we will investigate the cellular fate of fluorescently labeled mesenchymal stem cells to ascertain their vascular potential *in vivo*.

Specific Aim 3a: Define the expression of endothelial and smooth muscle markers in mesenchymal stem cells in vitro.

The expression of endothelial and smooth muscle markers in mesenchymal stem cells will be confirmed with immunostaining and polymerase chain reaction (PCR). Human umbilical cord vein endothelial cells and human aortic smooth muscle cells will be used as appropriate positive control. Mesenchymal stem cells will be induced to differentiate by co-culturing them with mouse endothelial cells. The change in the gene transcription of human endothelial and smooth muscle markers in MSCs will be quantified by real-time PCR.

Specific Aim 3b: Determine the in vivo fate of mesenchymal stem cell implanted alone or co-implanted with endothelial cells.

Mesenchymal stem cells will be transduced by retrovirus to express EGFP. The EGFP labeled MSCs will either be implanted alone or co-implanted with DsRed labeled HUVECs in a collagen gel in a cranial window of a SCID mouse. Similar to Aims 1 and 2, microvascular network derived from the EGFP labeled MSCs will be imaged by multi-photon laser scanning microscopy. The ability of MSCs to differentiate into either endothelial cells or perivascular cells will be assessed by their morphology and location in a blood vessel and their identify will be confirmed by immunohistochemistry.

Specific Aim 3c: Determine the in vivo functional performance of mesenchymal stem cell derived blood vessels.

The functional performance of the mesenchymal stem cell's derived blood vessels will be assessed. Specifically, we hypothesize that mesenchymal stem cells will differentiate into functional pericytes lining a blood vessel and they will respond to physiological stimulation. The cranial window will be superfused with a solution of endothelin-1 in order to induce the contraction of the engineered blood vessels.

Specific Aim 4: Determine the effect of overexpression of PDGF-BB in endothelial cells on perivascular cells recruitment and blood vessel stabilization

Endothelial cells when implanted alone in a collagen gel *in vivo* do not form stable microvascular network. In contrast, co-implantation of endothelial cells with perivascular precursor cells, such as 10T1/2 cells, allows for the formation of stable

blood vessels. This suggests that endothelial cells alone do not recruit sufficient amounts of host perivascular cells into a tissue-engineered construct at the critical early timepoint. To overcome such a problem, we intend to modify the endothelial cells to hasten the number and kinetics of recruitment of perivascular cells from the host. PDGF-BB has been shown to be chemotatic for pericyte/smooth muscle cells and endothelial source of PDGF-BB has been implicated in pericyte recruitment. This study intends to demonstrate an enhanced recruitment of host perivascular cells by genetically engineered endothelial cells to overexpress PDGF-BB. Furthermore, the effect of PDGF-BB overexpression on the stabilization of engineered blood vessels will be investigated.

Specific Aim 4a: Determine the effect of PDGF-BB overexpression on the proliferation and migration of pericyte precursor cells in vitro.

Human umbilical cord vein endothelial cells (HUVECs) will be transduced by retrovirus to express either EGFP or EGFP and PDGF-BB. The amount of PDGF-BB expressed in endothelial cells will be quantified by enzyme-linked immunosorbent assay (ELISA). The activity of PDGF-BB expressed in the endothelial-conditioned media will be tested by their effects on 10T1/2 cells proliferation and migration.

Specific Aim 4b: Determine the effects of PDGF-BB overexpression in endothelial cells on the recruitment of host perivascular cells and stabilization of engineered blood vessels.

PDGF-BB overexpressing HUVECs will be implanted in a collagen gel in a cranial window of a SCID mouse. Every few days the collagen gel will be imaged with multi-

photon laser scanning microscopy. The functional vessel density will be measured and compared to the control group (HUVECs expressing EGFP only). To directly observe the effects of PDGF-BB overexpression on perivascular cell recruitment, the genetically modified HUVECs will be co-implanted with 10T1/2 cells that have been labeled with DsRed in a cranial window. The kinetics and extent of perivascular cell recruitment toward the endothelial cells will be measure and quantified.

Background

The past century has seen a dramatic rise in the life expectancy of Americans from 48 years of age in 1900 to 78 years of age in 2005. Many factors have contributed to such a dramatic increase, including better diet and personal hygiene and the numerous advances in modern medicine. The leading causes of death in the early 20th century were infections. Today, the two leading causes of death for Americans are cardiovascular disease and cancer. An interesting thing connecting these two seemingly unconnected diseases is the marked functional abnormality of the blood vessels. In the case of cardiovascular disease, it is often due to an insufficient blood supply as a result of a rupture of an atherosclerotic plague or an aberrant growth of smooth muscle cells in the intimal layer of a blood vessel. One underlying cause of the atherosclerosis is endothelial dysfunction. In the case of cancer, specifically solid tumor, formation of new blood vessel drives both the growth and metastatic potential of a tumor[6]. Tumor blood vessels are also functionally, morphologically and molecularly abnormal[7]. More than three decades ago Dr. Judah Folkman proposed that tumor blood vessels might serve as a viable therapeutic target in controlling tumor growth[8]. More recently, this goal has been realized with the FDA approval of Bevacizumab, a monoclonal antibody that inhibits vascular endothelial growth factor (VEGF), for treatment of colorectal cancer and breast cancer. Clinical trials of Bevacizumab has demonstrated efficacy in controlling local tumor growth and extending patient survival when combined with chemotherapy[9]. It is clear that a better understanding of the normal physiology and pathophysiolgy of the vascular system would lead to the development of novel treatment for cancer and cardiovascular disease.

The vascular system

To maintain homeostasis, the body requires a transport network to supply nutrients, to exchange gas, and to remove waste products and metabolic intermediates from different tissues in the body. The circulatory system arose during evolution to provide such functions. The circulatory system is composed of a heart, an arterial network and a venous network (analogy: a pump, a delivery network and a return network). Working in parallel with the circulatory system is the lymphatic network that functions to drain excess tissue fluid and returns them back into the blood circulation. When the circulatory system goes awry, it can manifest in a number of different diseases. For example, when blood flow is disrupted in a tissue, the affected tissue undergoes a number of physiological and pathological changes; a process generally defined as ischemia. Ischemia induces cellular dysfunctions that thwart the normal function of a tissue, and depending on tissue type and severity of the ischemia, irreversible damage including cell death may occur. Ischemia can occur to any tissue, but its effects on the heart have the highest morbidity and mortality since the circulatory system itself is directly affected. Once a tissue is injured, it may form scar tissue, self-repair, or a combination of the two processes. Vascular remodeling is usually involved in the innate repair mechanism. Examples include the development of collateral circulation in coronary artery disease, and the angiogenic response following ischemic stroke and myocardial infarction. Unfortunately, these remodeling responses are often insufficient to overcome the disease, e.g. the extent of angiogenesis after the initial ischemic stroke is correlated with improved survival [10], but this post-ischemic angiogenic response is transient and may be completely abrogated within a few weeks [11]. In the case of myocardial infarction

(MI), significant tissue damage occurs but there may remain viable cells that can regain function if perfusion is restored. Therapeutic revascularization may facilitate tissue repair, thus allowing for partial or complete restoration of normal tissue function. This thesis focuses on developing cell-based technique to create functional blood vessels *in situ* for both therapeutic revascularization and tissue engineering.

Developmental Angiogenesis and Vasculogenesis

The cardiovascular system arises during early embryogenesis, when committed endothelial progenitor cells (known as angioblasts) are fused to form a primitive plexus of blood vessels, in an event known as "vasculogenesis". The development of the vascular system is intimately linked to the hematopoietic system. Endothelial cells and hematopoietic cells share a common origin in being derived from the mesodermal layer. Blood progenitor cells are first arisen in the yolk sac as blood islands in a process called primitive hematopoiesis. The outer surface of the blood islands is covered with angioblasts. Hematopoietic cells derived from primary hematopoiesis do not persist in the adult hood. The cells that eventually become the hematopoietic stem cells (HSCs) in the bone marrow are derived from progenitor cells in the aorta-gonad-mesoneprhos (AGM) region, and this differentiation process is known as definitive hematopoiesis [12]. The HSCs migrate to the fetal liver where they self-renew and differentiate into all lineages of cells in the hematopoietic system. After birth, the HSCs migrate from the fetal liver and homes to the bone marrow. Because of the ability of the progenitor cells in the AGM region to differentiate into both hematopoietic cells and endothelial cells, such cells are termed the hemangioblasts.

Hemangioblasts have been generated *in vitro* by differentiating mouse embryonic stem cells. The cells were shown to be Flk1 (VEGFR2) positive and a single cell can produce blast colonies that have bipotential of differentiating into hematopoietic and endothelial cells [13, 14]. The presence of hemangioblasts in adult remains controversial. No studies have yet unequivocally demonstrate a single adult stem cell can give rise to both hematopoietic and endothelial cells.

Besides hemangioblast, there may also exist a common progenitor for vascular cells (both endothelial and smooth muscle lineages). Studies in mouse embryonic stem cells have demonstrated that flk1+ or isl1+ cells are capable of differentiating into either endothelial or smooth muscle lineages depending on growth factors added in the cell culture media[15-17]. Addition of VEGF into the media selected for cells of the endothelial lineage while the addition of PDGF-BB selected for cells of the smooth muscle lineage. In addition, bone marrow-derived mesenchymal stem cells (MSCs) have also been shown to express markers of either endothelial cells or smooth muscle cells when differentiated[18, 19]. This suggests that MSCs may function as an vascular progenitor cells that exist in an adult.

Following the formation of the vascular plexus, further growth of the vascular system occurs predominantly through "angiogenesis": the growth of new vessels from pre-existing vessels. This can occur through sprouting, bridging, or intussusception (Figure 1). The vascular cells organized into hierarchal organization with larger vessels sub-divided into smaller vessels. The endothelial cells also begin to become specialized by differentiating into arterial and venous endothelial cells. This sub-specialization occurs before the onset of blood flow, thus suggesting that endothelial specialization is in part

determined by genetic pre-programming and not solely by environmental cues (ie. hemodynamics)[20].

Molecular regulators of angiogenesis

Since the blood vascular network is so critical to the health and disease of an organism, it comes as no surprise that angiogenesis is a highly regulated mechanism involving many different and interacting molecular pathways. Here, I will review three specific molecules that are involved in angiogenesis and vessel maturation: VEGF, and PDGF.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF-A, also often referred to simply as VEGF) is a master regulator of angiogenesis. There are 5 members in the mammalian VEGF family: VEGF-A, -B, -C, -D, and placenta growth factor (PlGF). The VEGF family binds to three receptor tyrosine kinases (VEGF receptor -1, -2, and 3), as well as co-receptors such as neuropilins.

VEGF-A comes in different isoforms (VEGF204, VEGF189, VEGF165, VEGF145, and VEGF121) as a result of alternative splicing. The isoforms differ in the length of their C-terminal end of the amino acid sequence. The difference manifests in varying capacity of VEGF isoforms to bind to heparin sulfate on the cell surface and in the extracellular matrix. VEGF121 is an acidic protein, does not bind to heparin sulfate and can freely diffuse in the extracellular matrix. In contrast, VEGF189 and VEGF204 are highly basic and they bind tightly to heparin sulfate. The high affinity of VEGF189 and VEGF204 to heparin sulfate results in their sequestration in the extracellular matrix. The consequence

is limited diffusion and biological action of the two isoforms. VEGF165 has intermediate properties in that it binds to heparin sulfate less tightly, thus allowing it to diffuse in the extracellular matrix. The dual actions of binding and diffusing in the ECM generate a concentration gradient of VEGF165 from a secreted source of VEGF (ie. region of hypoxic tissue). The VEGF gradient is critical in directing angiogenesis spatially. Mice that have been engineered to express VEGF specific isoform (i.e. VEGF120) succumbed to perinatal death as a result of impaired angiogenesis [21]. Thus, in using VEGF for pro-angiogenic therapy, it is critical to select the appropriate VEGF isoform or the right mixture of VEGF isoforms in generating new blood vessels.

VEGF has a multitude of effects on blood vessels, including increasing vascular permeability, stimulating endothelial cell proliferation, inhibiting endothelial cell apoptosis, and enhancing endothelial cell migration. The expression level of VEGF during vessel development is critical since either a half-fold reduction or a twofold increase in VEGF expression can lead to lethality to a mouse embryo[22, 23].VEGF expression level in the adult tissues with a few exceptions is generally low but recent findings suggest a homeostatic role of VEGF signaling in maintaining normal endothelial functions. Administering four different VEGF-signaling inhibitors in adult mice, it was shown that the vascular densities were reduced by 20 to 70% in different tissues ranging from pancreatic islets to thyroid tissue [24]. The extent of vessel regression was dependent on the particular organ, but a common feature was that the affected endothelia were all fenestrated. A previous *in vitro* study has indicated a role of VEGF in inducing endothelial fenestration [25]. Upon cessation of VEGF inhibition, the blood vessels were

able to re-grow but the vessel density remained close to 20% less than the vehicle control 40 days after the last treatment [26].

Platelet Derived Growth Factor

PDGF is a member of a super family of homodimeric cytokines including VEGF, and PlGF [27]. It was initially discovered and purified from degranulating platelets, but PDGF is now known to be expressed by many different cell types including macrophage, endothelial cells, fibroblasts, smooth muscle cells, and by a number of tumor [28, 29]. PDGF has dual functions in being both a mitogen for mesenchymal cells including muscle, bone/cartilage, and other connective tissue cells and a chemotatic agent [29, 30]. The chemotatic function of PDGF, PDGF-B in particular, plays an important role in the recruitment of perivascular cells to endothelium. Within the PDGF protein family, there are four different distinct genes (-A, -B, -C, -D) that encode structurally related polypeptide chains. PDGF-A and –B are the best characterized and they can form homo- and/or heterodimeric combination resulting in three isoforms of PDGF(-AA,-BB,-AB). The three isoforms of PDGF have different receptor affinities and thus also differ in their biological activities.

PDGF-B has been implicated to be involved in vessel maturation. *In vitro* studies suggest that PDGF-B is involved in the recruitment of perivascular cells [30]. This has been further confirmed by genetic knockout model of PDGF-B and PDGFR-β. The two knockout mice share similar phenotypes in that both mice are embryonic lethal. The blood vessels in the mice are hemorrhagic and show a reduction in the number of perivascular cells. When PDGF expression is knocked out specifically in endothelial

cells, it causes abnormality in the blood vessels in the heart, kidney and brain. This suggests that the secretion of PDGF from endothelial cells is critical during the development of matured vessels. A recent study has added a layer of complexity – it was shown that not only is the secretion of PDGF important, but a gradient of PDGF concentration is necessary for perivascular cell recruitment. PDGF-B contains, at its carboxyl terminal end, approximately 10 basic amino acid residues that are known as the retention sequence. It is believed that the retention sequence of PDGF-B interacts with the negatively charged components of extracellular matrix such as heparan sulfate on the cell surface. Normally, most of the secreted PDGF-B is retained at the cell surface, thereby limiting their actions to nearby cells only. Mice that have been genetically engineered to have PDGF-B deficient in retention sequence exhibit marked abnormality in the brain blood vessels due to a reduction in perivascular cells [31].

Structure of the blood vessels

Blood vessels follow a fairly consistent basic structure: a tube of endothelial cells surrounded by perivascular cells and extracellular matrix. The structural organization of these components depends on the size, location, and function of the associated blood vessel. For example, endothelial cells in arterioles are always continuous, and are almost completely enclosed by tightly packed smooth muscle cells, whereas capillaries are only sparsely covered by pericytes, and capillary endothelial cells may be continuous, fenestrated, or discontinuous, depending on the specific organ. Proper functional and structural association between endothelial cells, mural cells and the extracellular matrix is crucial to the stability and function of blood vessels.

Importance of perivascular cells

Although endothelial cells initiate the process of angiogenesis, they alone cannot complete the process. Endothelial cells initially form a naked tube that is highly permeable and unstable[32]. For vessel maturation and stabilization, the naked endothelial tube undergoes a series of steps to recruit and to differentiate perivascular cells to line the outer layer of the vessels[33, 34]. Perivascular cells such as vascular smooth muscle cells and pericytes are thought to provide structural integrity to the vessels, lay down the extracellular matrix, and provide necessary survival factors to the endothelial cells [35].

The importance of perivascular cells can be seen in a number of diseases that is caused by the lack of pericytes or pericyte dysfunction. Pericyte loss can lead to leaky vessels and vessel aneurysm. In diabetic retinopathy, injury to pericytes leads to the secondary change in endothelium that induces pathological angiogenesis. Damage to smooth muscle cells due to chronic amyloid deposition is also believed to be an early step that ultimately leads to hemorrhagic stroke [36]. Pericyte is an indispensable partner to endothelial cells in forming healthy blood vessels. In a model of engineered microvessels, stable and functional vessels form only in the presence of perivascular cells[37].

To date the emphasis of the pro-angiogenic therapy has been focused almost exclusively on endothelial cells, with perivascular cells either totally neglected, or treated simply as a muscular tube to maintain the blood vessel's structure. Engineered blood vessels have often been found to be immature and unstable [38], and the lack of proper perivascular cell support is likely to be one of the main reasons for this instability. Recently, our group

has shown that perivascular cells must be appropriately incorporated into the design of engineered vessels in order to form long lasting, stable vasculature [37]. By co-implanting human umbilical-vein endothelial cells (HUVECs) with 10T1/2 cells, a mesenchymal precursor cell line that is capable of differentiating into perivascular cells through heterotypic interaction with endothelial cells [39], we were able to produce small diameter blood vessels that remained patent for over 1 year with good functionality, and displayed proper coverage of perivascular-like cells.

Therapeutic Angiogenesis

Many methods designed to increase the degree of vascularization are under active investigation as treatment for ischemic diseases such as myocardial infarction [40] and stroke [41, 42], with the ultimate goal of relieving ischemia by producing long-lasting, functional blood vessels at the infarct site. Ideally, the new vessels would form rapidly (allowing for quick improvement in perfusion), and would resemble normal vessels in both structure and function. Most of the approaches in pro-angiogenic therapy can be grouped into two broad categories: 1) growth-factor based approaches where angiogenic growth factors, such as members of the VEGF or FGF families, are used to induce angiogenesis from the host; or 2) cell-based approaches where cells forming blood vessels such as endothelial cells are seeded onto scaffolds and grown under various conditions. Each approach has advantages and drawbacks.

Angiogenic factors such as VEGF can induce host angiogenesis, and can potentially be used easily "off the shelf". But growth factors alone may not be sufficient to create mature and stable vasculature. In a study with injection into normal tissue of an

adenoviral vector expressing VEGF, new blood vessels formed, but these vessels were highly disorganized, leaky and hemorrhagic [32]. Furthermore, VEGF can potentiate inflammation by increasing adhesion molecules or releasing chemokines [43-45]. The duration of the cytokine release may also be important for the formation of stable vasculature. Two weeks application of VEGF leads to transient formation of microvasculature that regressed after VEGF withdrawal. In contrast, microvasculature formed in response to 32 days of VEGF are stable and persists for months after VEGF withdrawal[46].

Delivery of a single angiogenic factor alone may not be able to induce mature vessel. Angiogenesis is a tightly controlled physiological process, it requires a constellation of growth factors expressing at the correct spatial and temporal pattern. Studies have suggested co-delivery of Ang1 and VEGF, or PDGF and VEGF can lead to vessels with higher vascular density and pericyte coverage compared to delivery of individual growth factor alone[47, 48]. In particular, sequential, controlled delivery of VEGF and PDGF-BB is able to induce a matured vascular network with vessels having a thick coat of smooth muscle cells[48]. But it takes more than 2 weeks for host endothelial cells to infiltrate to the construct; furthermore, the quality and stability of the vessels maybe low once all the growth factors have released.

Preclinical and Clinical Results of Pro-Angiogenic Therapy

Due to the critical role VEGF plays in angiogenesis, it has been actively investigated for the treatment of ischemic tissues, where there is often no effective therapy. Variety of animal models has been developed to test for the efficacy of VEGF in enhancing tissue

perfusion [49]. Two commonly used preclinical models include ischemic heart and ischemic limb that are induced by the ligation of the coronary or femoral artery, respectively. In some studies, recombinant VEGF was administered directly and in others, VEGF gene transfer was achieved by either naked DNA or adenoviral vector. In a rabbit model of hindlimb ischemia, single administration of recombinant human VEGF165 enhanced tissue perfusion and the development of collateral vessels [50]. Similar results were obtained with gene transfer of cDNA encoding VEGF isoforms [51]. Based on the encouraging preclinical data, VEGF administration has been tested in patients with limb or myocardial ischemia. The results from double-blind randomized placebo-controlled trials so far have proven that protein infusion or gene transfer with VEGF is safe and generally well-tolerated but none of the studies convincingly showed improvement in tissue perfusion or cardiac function with treatment [52-55].

The failure in the clinical trials could be related to the a number of issues including the choice and pharmacokinetics of biological agents, responsiveness of tissue to exogenous angiogenic factors, and the selection of patients [56]. All the clinical trials to date have used single agents and this fails to take into account of the complexity of the myriad molecular pathways governing vessel growth. VEGF is an initiator of angiogenesis since it guides the sprouting of endothelial tip cells from pre-existing vessels but an orchestra of molecular signals is necessary for the process of vessel maturation [39, 57]. The growth factors may need to be supply in a temporal and spatial dependent manner [31, 58]. Almost invariably, the preclinical animal models have been tested on young and healthy animals that respond robustly to the angiogenic factors. In contrast, the clinical trials were performed on old and diseased patients that are refractory to maximum

tolerated medical treatment [59]. Vascular response to angiogenic factors is reduced with age, hyperglycemia and atherosclerosis [56]. Patients with diabetes mellitus or elevated homocysteine exhibited impaired angiogenic response [60, 61]. The above factors may affect the clinical efficacy is the use of VEGF as single agent in enhancing development of collateral vessels for treatment of ischemic tissues.

Cell-based Therapy

Another strategy in forming a vascularized bed is to introduce the necessary blood vessel components-endothelial cells and/or perivascular cells directly to the tissue engineered construct. The implanted cells can potentially generate new vascular network formation in two ways: i.) directly by aggregating and anastomosing to the host vessels, or ii.) indirectly by inducing angiogenesis in the host tissue through the release of angiogenic factors. Studies have shown that implantation of human telomerase reverse transcriptase (hTERT) transduced human dermal microvascular endothelial cells(HDMECs) or Bcl-2 transduced human umbilical cord vein endothelial cells(HUVECs) subcutaneously in SCID mice can lead to functional vessel formation[38, 62]. Endothelial cells and smooth muscle cells have also been shown to express an array of angiogenic factors *in vitro*[63]. Thus, the implanted vascular cells may induce neovascularization by a combination of the two methods. Irrespective of the mechanism, new blood vessel formation is essential since blood flow is required for tissue survival. The ideal pro-angiogenic therapy would induce rapid formation of vessel thus, allowing for quick resumption of blood flow. The new vessels would resemble normal vessels in such properties as permeability, vessel geometry, vessel diameter distribution, etc.

Cell-based therapy is currently evaluated in the clinics for treatment of myocardial infarction. Generally, the procedure consists of the isolation of adult stem cells or progenitor cells from peripheral blood or bone marrow and the injection of these cells to the infracted region of a heart. A number of studies in both pre-clinical and clinical settings has demonstrated a benefit of the adult stem cell treatment by showing an improvement in cardiac functions post-MI compared to placebo [64-69]. While these results are encouraging, cellular therapy suffers from the scarcity of cell source, as well as potential unpredictability in the implanted cells' behavior.

Circulating Endothelial Progenitor Cells

The formation of new blood vessels in adult was thought to occur exclusively through the process of angiogenesis, where the newly formed vessels are derived from pre-existing blood vessels [70, 71]. The alternate process, vasculogenesis, in situ formation of blood vessel from endothelial progenitor cells, was thought to occur only during development [72]. The discovery of circulating endothelial progenitor cells along with the subsequent *in vivo* studies of their functions caused a paradigm shift.

The presence of endothelial cells in circulation has been speculated for almost 100 years, but only recently has the existence of such cells been established with the aid of modern techniques [73, 74]. In 1997, Asahara et al. isolated circulating endothelial progenitor cells from human peripheral blood and demonstrated in vitro and in vivo their endothelial characteristics [75]. CD34+ mononuclear blood cells, sorted from peripheral blood and cultured on fibronectin-coated flasks, gave rise to adherent cell clusters with cobblestone morphology. The attached cells were characterized as endothelial cells by their ability to

uptake AcLDL and the expression of a range of endothelial markers including CD31, VEGFR-2 and Tie-2. To test for the *in vivo* function of the EPCs, freshly isolated CD34+ cells were injected intravenously into mice with experimental model of ischemic limb. The CD34+ cells were shown to participate actively in angiogenesis in the ischemic tissue. However, questions remain on the contribution and duration of the injected cells to the vascular network.

The origin and nature of EPCs have remained controversial. EPCs have been defined as derived from bone marrow and that they actively participate in forming new blood vessels *in vivo*. Isolated EPCs are characterized by their expression of CD133, CD34, and VEGFR, their binding to Ulex Europeus lectin, and their ability to uptake AcLDL. However, there is a separate population of endothelial cells, circulating endothelial cells (CECs), that are in the blood besides EPCs [76]. CECs are believed to be matured endothelial cells that have shed into the blood stream under pathological settings. Recent studies have also suggested monocytes, when cultured *in vitro*, may upregulate the expression of endothelial markers and downregulate the expression hematopoietic markers [77, 78]. The *in vivo* assay thus far of injecting EPCs intravenously to home to tumor tissue or ischemic tissue left many unanswered questions. First, how readily do the *ex vivo* expanded EPCs form functional vessels when implanted *in vivo*? Second, if the *ex vivo* expanded EPCs do form functional vessels *in vivo*, how well do these vessels perform? Third, EPCs have been isolated and cultured from both adult peripheral blood (PB) and cord blood (CB); which source of EPCs is better at vascularizing tissue. Such information is both necessary and required before EPCs are to be widely used in regenerative medicine.

Alternatively, a renewable and potentially unlimited source of endothelial cells can be generated from human embryonic stem cells (hESCs). Human embryonic stem cells are pluripotent, capable of differentiating into all cell types in a body. They also have the advantage of having unlimited proliferation potential. However, there are some significant scientific challenges in using hESCs in the clinics. First, the right differentiating condition (i.e. growth factors, oxygen tension, extracellular matrix, etc.) needs to be identified for derivation for each cell type. Second, there are risks in the formation of teratoma with undifferentiated hESCs.

Bone Marrow Derived Mesenchyaml Stem Cells

Mesenchymal stem cells are multipotent cells that reside in the bone marrow, vessel wall, and adipose tissue[79]. MSCs are true stem cells in that they are undifferentiated cells with the capacity to both self-replicate and self-renew. By culturing MSCs under specific culturing conditions (varying level of hormone, and cytokines), they can differentiate into specific cell lineages of the connective tissue including adipocyte, osteocyte, and chrondrocyte[80]. MSCs can be isolated from bone marrow mononuclear cells by their propensity to adhere and grow on culture plastic[81]. Analysis of surface receptors on MSCs suggests that they express Stro-1 and CD105 but are negative for CD34 and CD31[81, 82]. Due to the multipotential nature and proliferative capacity of MSCs, they are actively being investigated for clinical use. MSCs have be used to engineer bone tissue for reconstructive surgery[83]. In animal model of myocardial infarction, injection of MSCs has been shown to limit infarct size and improve cardiac functions[68, 69]. Histological analysis reveals that MSCs have differentiated into cardiac myocytes,

smooth muscle cells, and endothelial cells[84]. MSCs may also help to revascularize the infracted heart tissue through expression of high level of VEGF[85, 86].

Potential of Mesenchymal Stem Cells as Perivascular Cells

Smooth muscle progenitor cells can be isolated from bone marrow stromal cells and they are found to be positive for PDGF-β receptor[87]. *In vitro* culture of mesenchymal stem cells shows smooth muscle cell phenotype with the expression of α-smooth muscle actin[88]. In response to TFG-β1, MSCs upregulate the expression of α-smooth muscle actin and increase in contractility [89]. *In vivo* evidence has also accumulated to support the hypothesis that MSCs have the ability to differentiate to smooth muscle cells. Recent studies have suggested the existence of a population of bone marrow cells that can give rise to circulating smooth muscle progenitor cells [87, 90]. Bone marrow derived smooth muscle progenitors have been found to contribute to the perivascular layer of blood vessels in pathological settings including age atherosclerosis and graft intimal hyperplasia[91-94]. Whether bone marrow derived cells can differentiate into perivascular cells in physiological setting remains unclear.

Potential of MSCs in Differentiating into Endothelial Cells

Mesenchymal stem cells may be a true vascular progenitor; being able to differentiate into both endothelial cells and perivascular cells. A recent report shows that culturing MSCs with high level of VEGF *in vitro* can upregulate expression of endothelial markers including VEGFR-1 and VEGFR-2[19]. The MSCs derived endothelial cells are also positive for von Willebrand factor. *In vivo* studies also suggest that MSCs may

differentiate into endothelial cells. When MSCs are injected into cardiac tissue, they become positive for CD31 and von Willebrand factor and form vascular structure[69, 84, 95]. Whether the transition of MSCs into endothelial-like cells occurs through differentiation or cell fusion[96] remains unclear.

This thesis will address the outstanding questions on the vasculogenic potential of endothelial progenitor cells, mesenchymal stem cells and human embryonic stem cells by the use of intravital microscopy coupled with fluorescence labeled cells in a chronic animal window model. In addition, this thesis examines the combined use of angiogenic factor-based and cell-based therapy in evoking a synergy in generating functional blood vessels.

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Chapter 2: Use of human embryonic stem cells

Portions of the chapter have been taken from:

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Abstract

Human embryonic stem cell (hESC) lines provide a unique system for studying human embryonic development and have enormous potential as a source of therapeutic tissues, including ES cell-derived vascular and hematopoietic lineages. However, it is difficult to generate a large-scale relatively pure population of specific lineage cells derived from hESCs. Here, we describe a novel 2-dimensional culture system without intermediate embryoid body formation for hESC differentiation into hematopoietic and endothelial cells. HESCs generated CD34+ cells that could under specific conditions differentiate into either hematopoietic or endothelial cells. Hematopoietic differentiation was induced by stem cell factor (SCF) and Flt-3 ligand (Flt3L) whereas, VEGF promoted endothelial cell differentiation. When the embryonic endothelial cells were implanted into SCID mice, they formed functional blood vessels *in vivo* that connected to the host vascular system and were durable conduits for blood for greater than 150 days. This system paves the way for further large-scale, therapeutic generation of human hematopoietic and endothelial cells derived from ES cells.

Introduction

Mouse embryonic stem (ES) cells have been studied extensively in murine embryogenesis. However, the lack of experimental cell systems has made it difficult to study many developmental processes in humans. Human embryonic stem cell (hESC) lines provide not only a unique system for studying human embryonic development, but also have enormous potential as a source of therapeutic tissues, including those of vascular and hematopoietic lineages. *In vitro*, hESCs can be triggered to undergo spontaneous differentiation by forming 3-dimensional (3D) embryoid bodies (EBs) containing many different cell types [1], including hematopoietic cells [2-4] and endothelial cells [5]. Although the EB is far less organized than an actual embryo, it can partially mimic the spatial organization in the embryo [6]. However, unlike mouse ES cells, in which a single cell is able to form an EB in specific conditions, the formation of EBs from hESCs is inefficient and usually requires a whole colony of hESCs. Dispersed single human ES cells cannot aggregate efficiently to form EBs, and a large amount of cells undergo cell death during EB formation [7]. Therefore, one of the challenges for stem cell research is to obtain sufficient differentiated cells.

Material and Methods

Human embryonic stem cell cultures

The hESC lines, H1 and H9 [8], were obtained from WiCell Research Institute (Madison, WI). HESCs (passages 29-50) were grown on mouse embryonic fibroblasts (MEFs),

which were inactivated with irradiation or mitomycin C-treatment in hESC medium containing DMEM/F12, 20% knockout serum replacement, 1 mM L-glutamine, 0.1 mM nonessential amino acid (all from Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/ml human FGF-2 (R&D Systems). The feeder-free culture on Matrigel (BD Biosciences) with MEF-conditioned medium (CM) was carried out as described [9]. MEF-CM was filtered through a 0.22 μm sterile membrane and stored at >–20°C.

HESC differentiation:

To induce 3-D embryoid bodies (EBs), the hESC colonies were treated with 2mg/ml dispase for 15 minutes at 37°C to loosen colonies; and the colonies were transferred into ultra low-attachment plates (Corning Incorporated) for EB formation. EBs were cultured in differentiation medium consisting of IMDM, 15% defined-FBS (Hyclone), 450 μM monothioglycerol (Sigma), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin for 10 days with medium change every 2-3 days. For 2D differentiation, undifferentiated hESCs were given differentiation medium as above for 10 days, and the medium was changed every 2-3 days.

After 10 days of differentiation in either 2D or 3D culture, single-cell suspensions were made from differentiated hESCs by treatment with 2 mg/ml collagenase B (Roche) for 20 minutes at 37°C, followed by treatment with cell dissociation buffer (Invitrogen) for 10 minutes at 37°C. The cells were dissociated by gentle pipetting, and passaged through a 40 μm cell strainer (BD Biosciences).

Hematopoietic and endothelial differentiation

CD34+ cells were isolated from differentiated hESCs by using MACS MicroBeads columns (Miltenyi Biotec), according to the manufacturer's instructions. As determined by flow cytometry, the purity of isolated CD34+ cells was generally 60-80% at a single column, and > 95% after the second column. Isolated CD34+ cells were seeded on gelatin-coated plates ($2x10^4$ cells/cm^2) in EGM2 medium supplement with or without hematopoietic growth factors, 100 ng/ml hSCF, and 100 ng/ml hFlt3 ligand. In some cases, the CD34+ cells were cultured in differentiation medium with the hematopoietic growth factors, 100 ng/ml hSCF and 100 ng/ml hFlt3 ligand, or the endothelial growth factor, 50ng/ml $hVEGF_{165}$ (R&D Systems) or both. After 7-10 days of incubation, the suspension cells were harvested for analyses. The adherent cells were harvested by trypsin treatment and used for analyses.

Flow cytometry and immunostaining

The cells were prepared in PBS containing1% FBS and 0.25% human IgG, and were labeled for 15-30 minutes at 4^0C with a combination of monoclonal antibodies (mAbs): CD31-PE, CD34-APC, CD45-FITC (all from BD PharMingen), Glycophorin A-PE (Immunotech), and Flk1-PE (R&D system). The samples were analyzed on a FACSCalisbur (Becton Dickson). Data analyses were performed using CellQuest and FlowJo software.

For staining, the cells were fixed with methanol for 5 minutes at -20^0C or with 4% paraformaldehyde in PBS at room temperature for 15 minutes. The fixed cells were incubated with 4% goat serum for 30 minutes to block nonspecific binding, and stained for 1 hour with the primary antibodies: SSEA-1, SSEA-4, TRA-1-60, and TRA-1-81 (all from Chemicon International Inc), CD31, VE-cadherin, respectively. The secondary antibody, FITC-conjugated rat anti-mouse antibody (BD PharMingen) was added, and the cells were incubated for 30 minutes.

For the LDL uptake assay, the cells were incubated with 10 ug/ml of diI-acetylated low-density lipoprotein (Di-LDL, Molecular Probes) for at least 4 hours. After washing twice with PBS, the cells were examined under a fluorescence microscope.

Matrigel assay

The assay was carried out essentially as previously described [10, 11]. Twenty four-well plates were coated with 200 μl/well matrigel matrix (BD Biosciences) at room temperature for more than 30 minutes. The CD34+ cell-derived endothelial cells (1-5 x 10^4 cells) were trypsinized and replated onto matrigel plates in differentiation medium at 37°C in 5%CO_2. The structures were photographed under phase-contrast microscope (Nikon) after 16 hours of incubation

Tissue engineered blood vessels

To generate hES cell-derived endothelial cells, human ES cell-derived CD34+ cells were cultured in either EGM-2 medium or differentiation medium with 100 ng/ml rhVEGF for 7 to 10 days. One million endothelial cells and 200,000 10T1/2 cells were suspended in 1 ml solution of rat-tail type 1 collagen (1.5 mg/ml) (BD Biosciences, Bedford, MA) and human plasma fibronectin (90 μg/ml) (Sigma) in 25 mM Hepes (Sigma) buffered EGM medium at 4°C. The pH was adjusted to 7.4 by using 1N NaOH (Fisher Science, NJ). The cell suspension was pipetted into 12-well plates (Falcon) and warmed to 37°C for 30 minutes to allow polymerization of collagen. Each solidified gel construct was covered by one ml of warmed EGM medium. After one day culture in 5% CO_2, a skin puncher was applied to create circular disk-shape pieces of the construct (4-mm diameter), and they were implanted into the cranial windows in SCID mice [12, 13]. Multiphoton laser-scanning microscopy was used to visualize and quantify the morphological changes of EGFP-expressing hESC-derived endothelial cells. The perfused vessels were highlighted by tail vein injection of 1% tetramethylrhodamine-labeled dextran (MW 2000,000), indicating the formation of functional engineered vessels [12, 13]

Results and Discussion

To achieve large scale differentiated cells from hESCs, we developed a 2-dimensional (2D) culture system to differentiate hESCs into hematopoietic and endothelial lineages (**Figure 2.1a**). Human ES cells were maintained on mouse embryonic fibroblasts (MEFs) in the presence of FGF-2, to maintain an undifferentiated state that was confirmed by the high expression of SSEA-4, TRA-1-60, and TRA-1-80, but not SSEA-1. It was

demonstrated that hematopoietic commitment of hESC differentiation from EBs is around day 10 [14]. To initiate differentiation, hESCs were placed in differentiation medium containing defined-FBS for 10 days without supplementation of additional growth factors. Gene expression analysis of 2D differentiation indicated that the pluripotent gene, Oct-4, was decreased, and genes for hematopoietic cells (GATA-2) and endothelial cells (CD31) were increased (**Figure 2.1b**), which suggests that differentiated hESCs contain hematopoietic and endothelial progenitors. Compared to 3D EB formation, 2D differentiation was able to generate robust numbers of differentiated cells (5- to 10-fold higher) (**Figure 2.1c**).

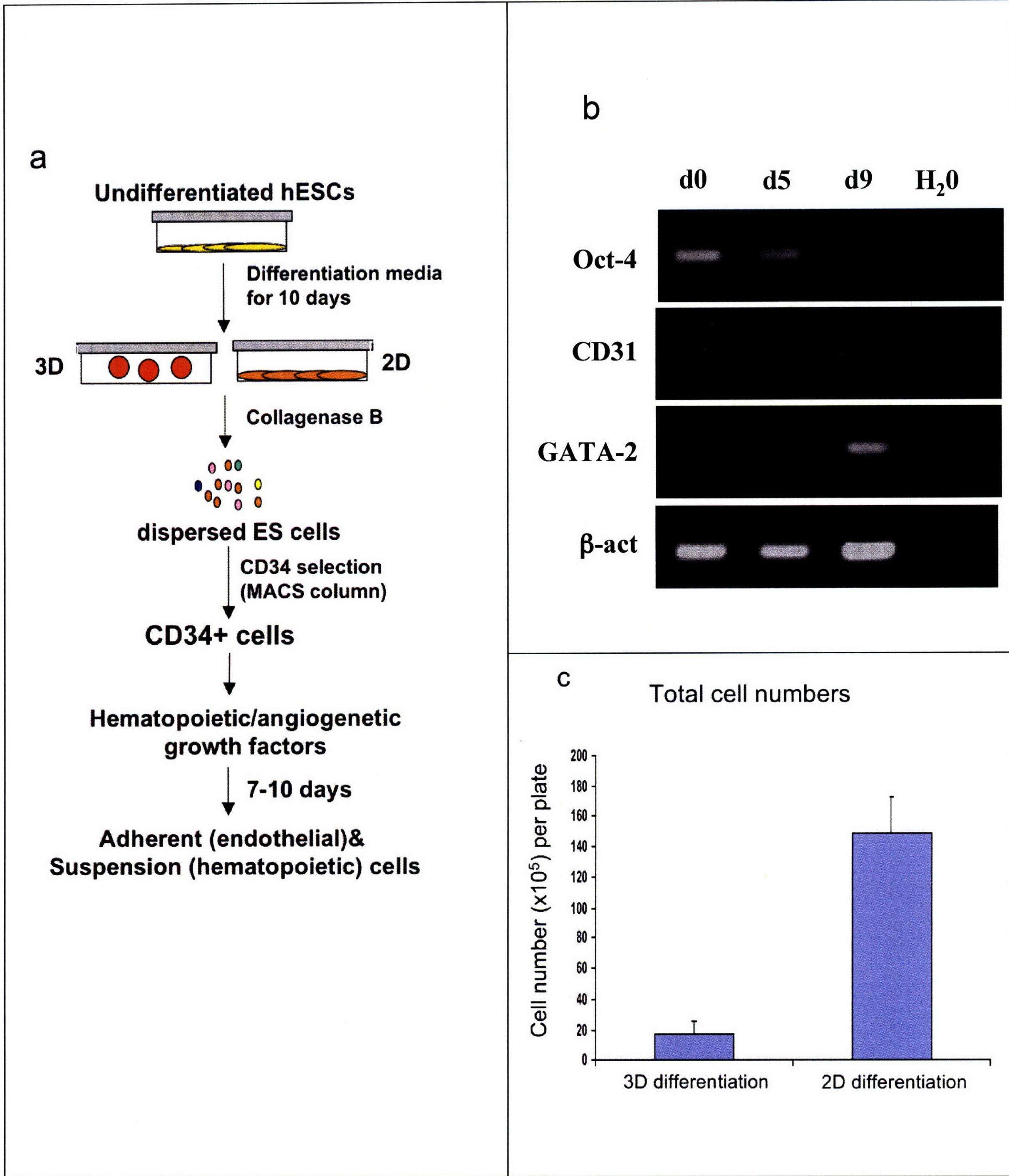

a
Undifferentiated hESCs
Differentiation media
for 10 days
3D
2D
Collagenase B
dispersed ES cells
CD34 selection
(MACS column)
CD34+ cells
Hematopoietic/angiogenetic
growth factors
7-10 days
Adherent (endothelial)&
Suspension (hematopoietic) cells
b
d0
d5
d9
H20
Oct-4
CD31
GATA-2
β-act
c
Total cell numbers
Cell number (x10^5) per plate
200
180
160
140
120
100
80
60
40
20
0
3D differentiation
2D differentiation

Figure 2.1. Differentiation of human ES cells in 2D culture.

Schematic representation of hESC culture. HESCs were cultured in IMDM differentiation medium containing 15% defined-FBS for 10 days (**a**). The CD34+ cells were isolated by MACS columns, and cultured in the presence of angiogenic or hematopoietic growth factors or both for an additional 7 to 10 days for endothelial and hematopoietic differentiation. RT-PCR analysis of CD31 and GATA-2 gene expression in differentiated hESCs (**b**). RNA samples were harvested at day 0, day 5, and day 9 of differentiation. Differentiation potential of 2D and 3D (EB) culture (**c**). Undifferentiated hESCs were induced to differentiate in either 2D or 3D cultures for 10 days. The single cell suspensions were generated as described. The total differentiated cell numbers were calculated based on the same numbers of input undifferentiated hESCs. The results reflect the mean (+/-SD) number of total differentiated hESCs from three experiments.

HESCs tend to differentiate in a heterogeneous manner. One of the scientific challenges in hESC research is to define culture conditions or growth factors to generate a single cell type from hESCs. During embryogenesis, hematopoiesis is highly coordinated with vasculogenesis. Hematopoietic and endothelial precursors originate from a cluster formation of blood islands, consisting of endothelial progenitors and hematopoietic progenitors [15-17]. These progenitors may be derived from a common precursor, the hemangioblast. In order to generate both hematopoietic and endothelial populations, we attempted to isolate hematopoietic and endothelial progenitors, and further induce their differentiation by exposing the progenitors to lineage-specific growth factors.

CD34+ cell populations in adult human peripheral blood, bone marrow, and cord blood contain both hematopoietic progenitors and endothelial progenitors [18, 19]. HESC-derived CD34+ cells, that were produced in co-culture on OP9 stromal cells, had multi-lineage hematopoietic potential [4]. Here, we investigated whether hESC-derived CD34+ cells contain both hematopoietic and endothelial progenitors. During hESC differentiation, CD34 expression increased, and ~5-8% of the cells were CD34+ by day 10, as indicated by fluorescence-activated cell sorting (FACS) analysis (**Figure 2.2a**).

Although FACS sorting usually results in purer populations of cells, the frequency of viable cells was very low when FACS sorting was employed. In order to establish a more efficient method to isolate CD34+ cells, we used Magnetic Cell Sorting (MACS) columns (Miltenyi Biotec). The cells from 10-day cultures of 2D-differentiated hESCs were treated by collagenase B, and the dispersed single cells were immunosorted by MACS MicroBead columns with the antibody to CD34 (Miltenyi Biotec). The purity of CD34+ cells ranged from ~60-80% after one column selection (**Figure 2.2a**). CD34+ cells were enriched to 80-95% after a second column selection (**Figure 2.2b**). The isolated CD34+ cells were CD45-, and the majority of CD34+ cells expressed CD31 (>60%) (**Figure 2.2a**). Even though the percentage of CD34+ cells was similar in 2D and 3D cultures, a significant increase of CD34+ cells (5-8 fold) was generated from 2D cultures because of the increase in total differentiated cells in the 2D cultures (**Figure 2.2b**). Our results suggest that neither EB formation nor co-culture with stromal cells is required to generate hESC-derived CD34+ progenitor cells.

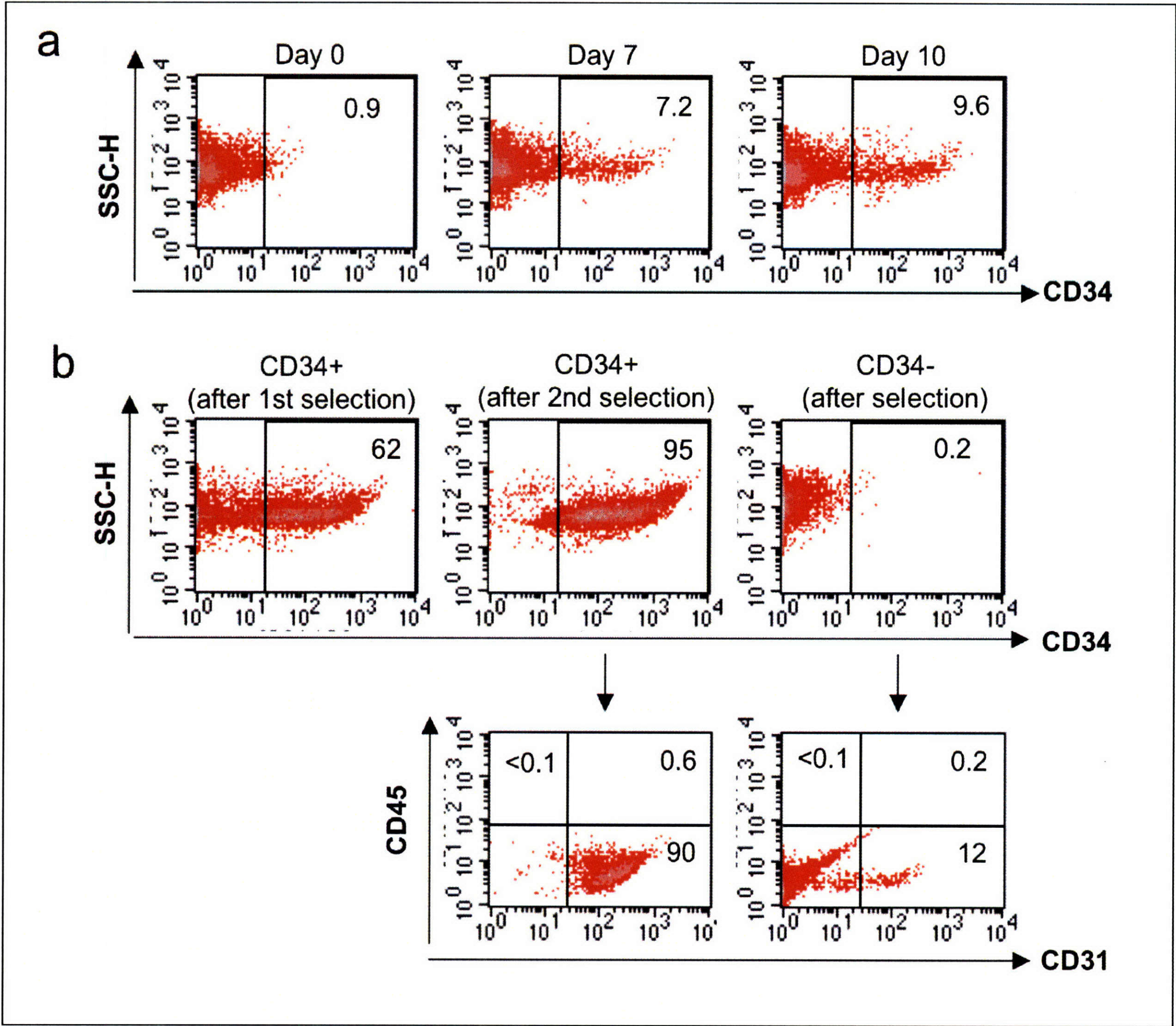
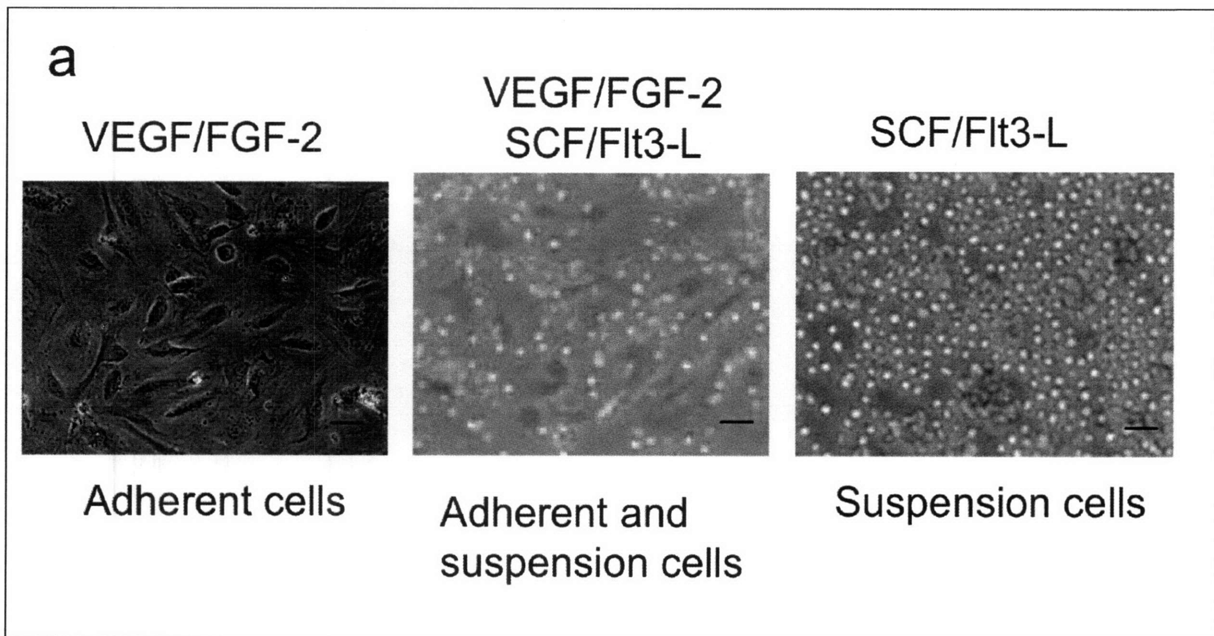

a
Day 0
Day 7
Day 10
SSC-H
0.9
7.2
9.6
CD34
b
CD34+
(after 1st selection)
CD34+
(after 2nd selection)
CD34-
(after selection)
62
95
0.2
<0.1
0.6
90
<0.1
0.2
12
CD45
CD31

Figure 2.2. Generation of hESC-derived CD34+ cells.

Flow cytometric analysis of CD34 expression in hESCs (a). Differentiated hESCs at different time points were analyzed by FACS with CD34-APC conjugated antibodies. CD34+ cells from day 10 hESCs were isolated by MACS columns (b). The purities of CD34+ cells after single and double MACS column isolation were analyzed by FACS. After double MACS column isolation of CD34+ cells, the remaining cells (CD34-) were analyzed by FACS. The expression of CD31 and CD45 from CD34+ and CD34- cells were analyzed with CD31-PE and CD45-FITC conjugated antibodies. Flow cytometric data are representative from three independent experiments.

To test whether immature CD34+ cells derived from hESCs contain a progenitor population that is capable of differentiating into endothelial and hematopoietic cells, we cultured hESC-derived CD34+ cells in the presence of hematopoietic growth factors, stem cell factor (SCF) and Flt-3 ligand (Flt3L), or in the presence of the endothelial growth factor, VEGF, or both. After an additional 7-10 days in culture, both suspension and adherent cells emerged (**Figure 2.3a**). The number of suspension and adherent cells was dependent on growth factors in the culture medium. When CD34+ cells were cultured in the endothelial medium, EGM-2, the majority of cells (90%) were adherent. Addition of hematopoietic growth factors to EGM-2 medium increased the number of suspension cells significantly (**Figure 2.3b**). Similar results were observed in IMDM culture media, in which the addition of SCF and Flt3L increased suspension cell number, and the addition of VEGF and FGF-2 increased adherent cells. To confirm the hematopoietic nature of the suspension cells, the suspension cells and the adherent cells

were analyzed on methylcellulose cultures for hematopoietic colony formation. The suspension cells generated significantly more hematopoietic colonies than the adherent cells (**Figure 2.3c**). FACS analysis indicated that the pan-hematopoietic marker, CD45, was enriched in the suspension cells, compared with the adherent cells (**Figure 2.3d**). These results suggest that the suspension cells derived from CD34+ cells are enriched in hematopoietic cells.



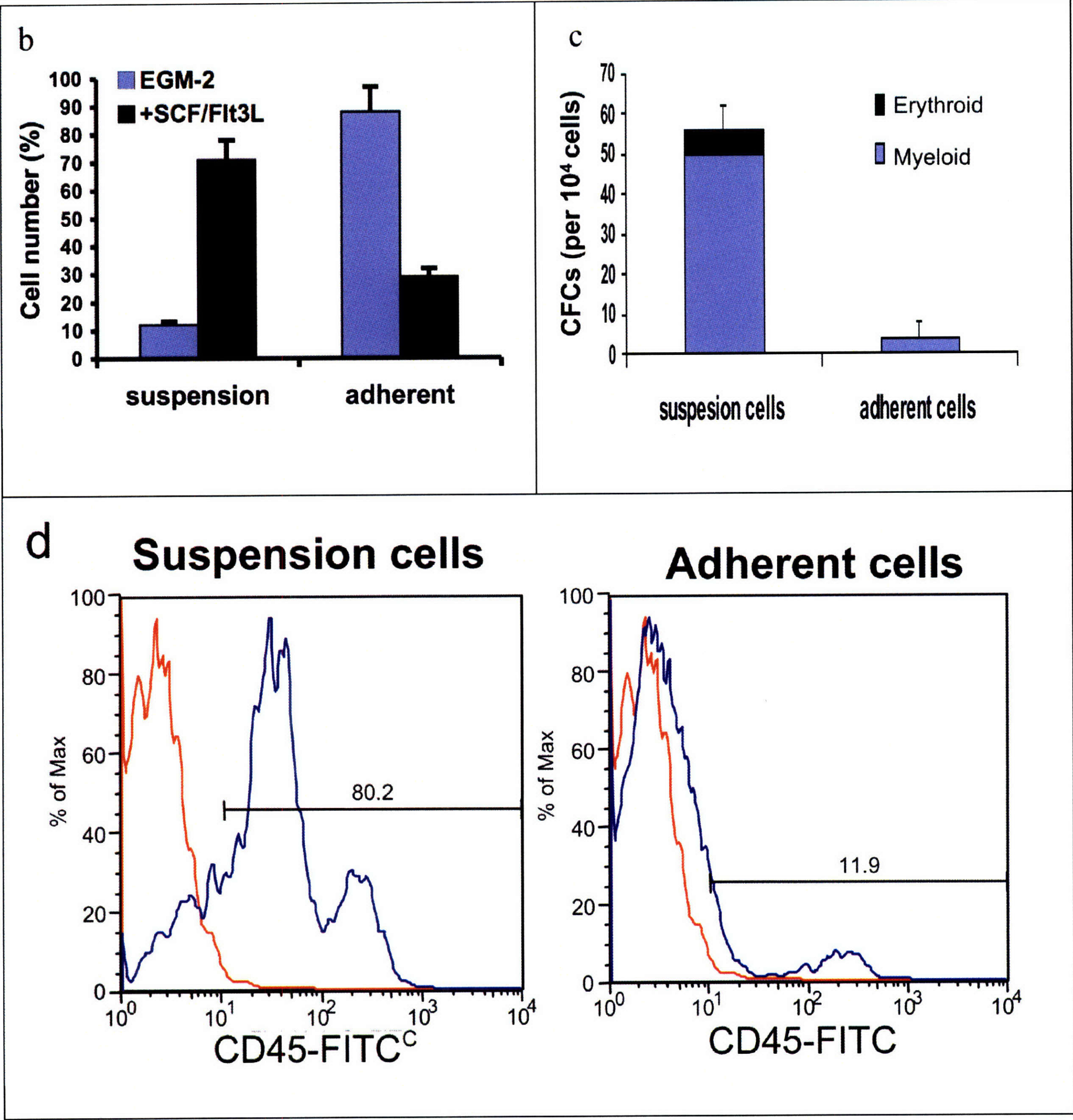
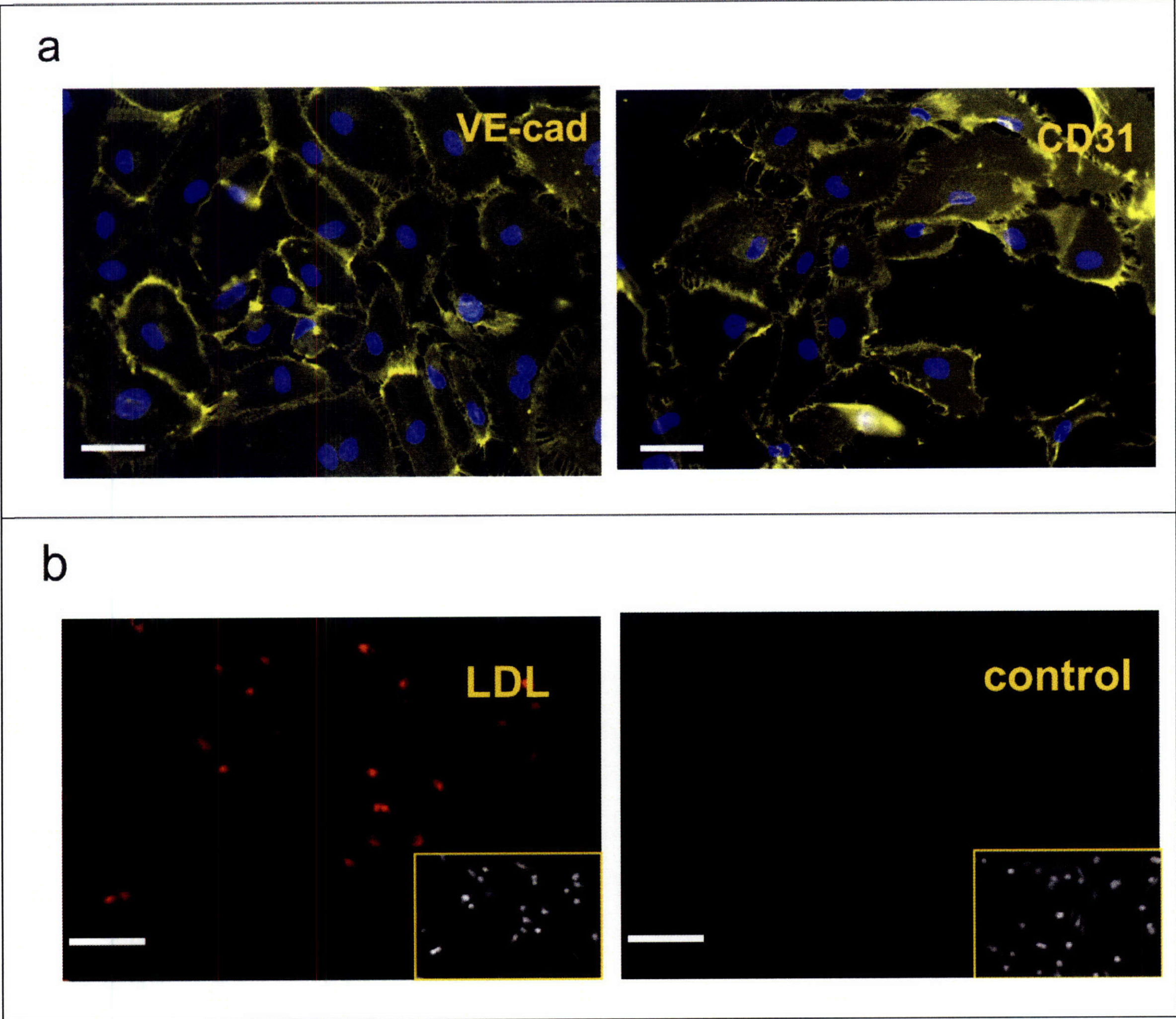

b
Cell number (%)
100
90
80
70
60
50
40
30
20
10
0
EGM-2
+SCF/Flt3L
suspension
adherent
c
CFCs (per 10⁴ cells)
70
60
50
40
30
20
10
0
Erythroid
Myeloid
suspesion cells
adherent cells
d
Suspension cells
Adherent cells
% of Max
80.2
11.9
CD45-FITC
CD45-FITC

Figure 2.3. CD34+ cells gave rise to populations of adherent and suspension cells

Differentiation of CD34+ cells is dependent on growth factors in the culture system (**a**). The addition of angiogenic growth factors, VEGF and FGF-2, promoted the growth of adherent cells with a spindle-shaped morphology; whereas the addition of hematopoietic growth factors, SCF and Flt3-L, promoted the growth of suspension cells. Scale bar = 20 μm. Cell number of adherent and suspension cells cultured in EGM-2 medium (**b**). The addition of SCF (100 ng/ml) and Flt3-L (100 ng/ml) into EGM-2 medium increased the number of suspension cells. Triplicate wells of resulting cells were analyzed on day 10 of cultures. Isolated CD34+ cells were cultured in the presence of hematopoietic growth factors for 10 days and the suspension cells and adherent cells were assayed separately for their potential to generate multi-lineage hematopoietic colonies in methylcellulose cultures (**c**). Suspension and adherent cells were analyzed by flow cytometry for CD45 expression (**d**). CD45 is a cell surface marker for hematopoietic cells. Flow cytometric data are representative from three independent experiments.

CD31 and VE-cadherin (VE-cad) are markers for human embryonic-endothelial cells [5]. To examine whether adherent cells generated from hESC-derived CD34+ cells express endothelial cell markers, the isolated CD34+ cells were cultured in the presence of VEGF for 7 days, and the adherent cells were analyzed for the expression of CD31 and VE-cad by immunohistochemistry. Both CD31 and VE-cad were present at the endothelial adherent-type junctions (**Figure 2.4a**). DiI-acetylated low-density lipoprotein (Di-LDL) up-taken assays, which have been used to characterize endothelial cells [20], were performed to confirm an endothelial-like phenotype of CD34+ cells grown in cultures.

Most of these cells incorporated Di-LDL, while neither cultured CD34- cells nor suspension hematopoietic cells incorporated Di-LDL (**Figure 2.4b**). The hESC-derived endothelial cells were then plated on Matrigel, and they rapidly formed vascular network-like structures (**Figure 2.4c**), while suspension cells were unable to do so.



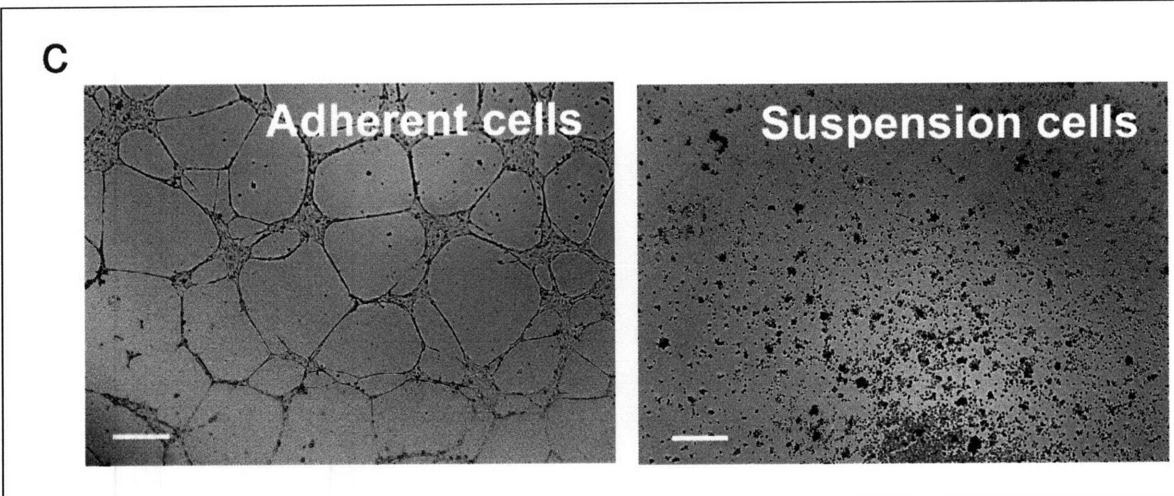


Figure 2.4. Endothelial differentiation of hESC-derived CD34+ cells.

Isolated CD34+ cells were cultured in the presence of angiogenic growth factors for 10 days. Adherent cells expressed the endothelial markers, VE-cadherin and CD31, which were determined by immunohistochemistry (**a**), were positive for Dil-AcLDL uptake (**b**), and formed vascular-like network on Matrigel (**c**). Scale bar = 100 μm (a,b), Scale bar = 125 μm (c).

Implanting a PLLA/PAGA polymer scaffold seeded with CD31+ endothelial cells derived from human ES cells into SCID mice has been shown to result in microvessels positive for human cells [5]. It was unclear how long these microvessels last *in vivo,* and whether the microvessels were connected to the mouse vascular system, though red cells were seen within them.

To test whether hESC-derived endothelial cells from CD34+ cells were able to form functional blood vessels *in vivo*, we studied their behavior in a SCID mouse model that allows us continuous observation of the engineered vascular network *in vivo* [12, 13]. Isolated CD34+ cells from GFP-H1 cells were expanded, and matured in the presence of

VEGF for 7 days to generate hESC-derived endothelial cells. These cells were mixed with rat-tail type I collagen to form 3D collagen gels, and implanted into cranial windows in SCID mice. As an initial test of endothelial cells *in vivo*, we implanted a collagen gel that contained both hESC-derived endothelial cells (GFP+) and HUVECs (without GFP). The GFP+ network-like vascular structures were observed as early as 5 days after the implantation of endothelial cells (**Figure 2.5**). After 13 days of implantation, large vacuoles appeared in the vessel tubes (**Figure 2.5**). To test whether the engineered vascular networks connected to the mouse circulatory system to carry blood, tetramethylrhodamine-labeled dextran was injected into the tail veins. By using a multiphoton laser-scanning microscopy, the perfused vessels were visualized, suggesting the formation of functional engineered vessels (**Figure 2.5**). The hESC-derived vessels in animals were continuously monitored for prolonged intervals through the cranial windows. The perfused vessels persisted, and were observed for at least 150 days.

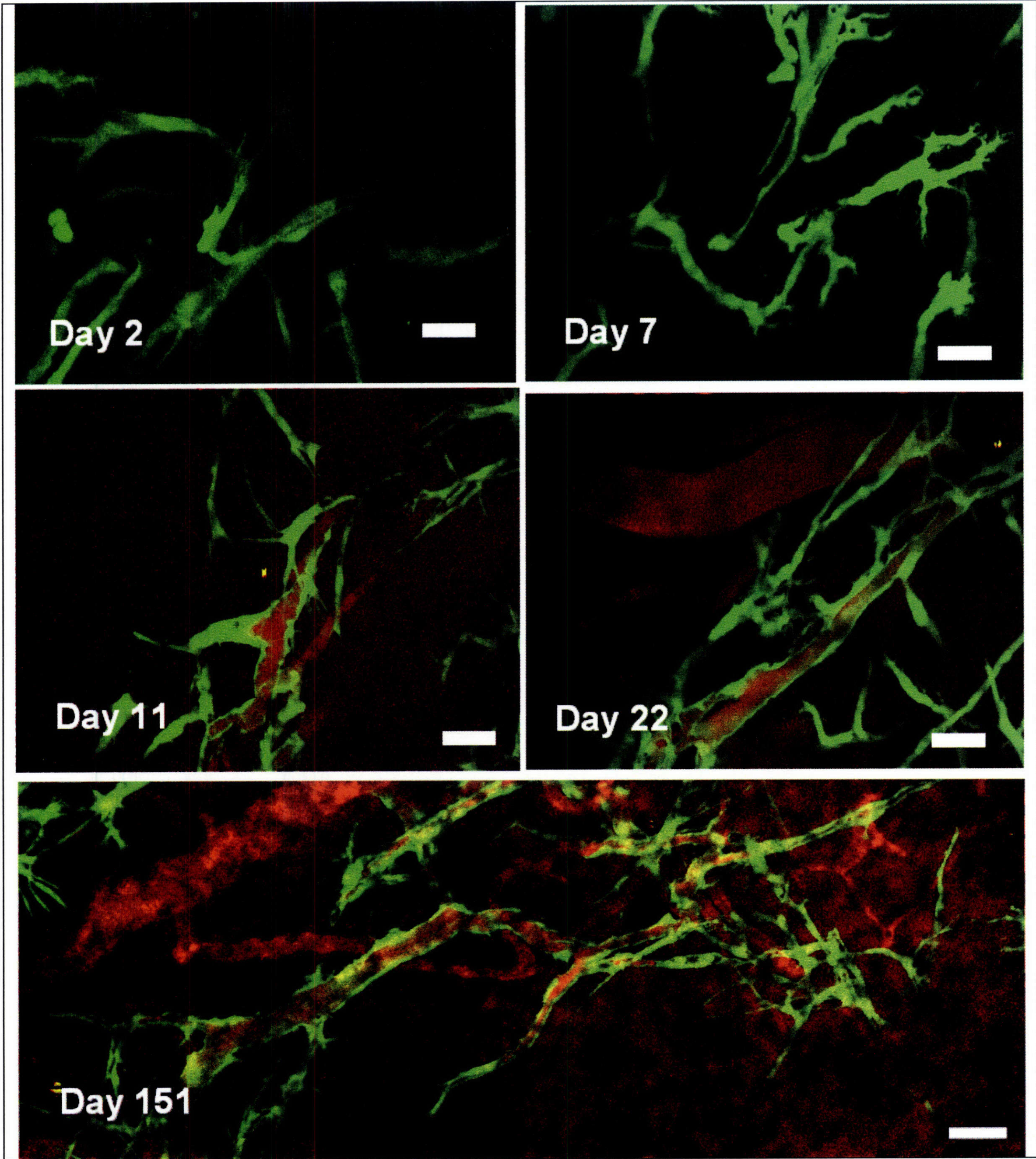

Day 2
Day 7
Day 11
Day 22
Day 151

Figure 2.5. hESC-derived endothelial cells form functional vessels *in vivo*.

hESC-derived endothelial cells (GFP+) were mixed with10T1/2 in a collagen gel, and implanted into cranial windows in SCID mice. Images were taken at day 2, 7, 11, 22, and 151 after implantation. After 11 days, rhodamine-dextran was injected into the tail vein to highlight perfused vessels. Engineered vessels were stable and functional for more than 151 days *in vivo*. Green, hESC expressing enhanced green fluorescent protein (EGFP); red, functional blood vessels contrast-enhanced with rhodamine-dextran. Scale bar = 50 μm.

One of the challenges in hESC research is to generate sufficient differentiated cells from EBs. Our results show that hESCs in 2D cultures were able to generate differentiated cells with high efficiency. HESC-derived CD34+ cells have both hematopoietic and endothelial differentiation capacity though it is not clear that this occurs at a clonal level. Therefore we cannot conclude that there is or is not a hemangioblast population in that cell fraction. Our studies clearly demonstrate that human embryonic stem cell-derived endothelial cells were capable of forming functional blood vessels in animals. This unique differentiation system may provide a renewable resource of endothelial cells for potential applications, such as engineering new blood vessels for the treatment of regional ischemia. Our next goal will be to generate hESC-derived hematopoietic and endothelial cells with animal product-free conditions to further the potential clinical application of this process.

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Chapter 3: Use Of Endothelial Progenitor Cells

Portions of the chapter have been taken from:

P. Au, L.M. Daheron, D.G. Duda, K.S. Cohen, J.A. Tyrrell, R.M. Lanning, D. Fukumura, D.T. Scadden, R.K. Jain. Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. Blood 111 (2008), pp 1302-05.

Abstract

Tissue engineering requires formation of a *de novo* stable vascular network. Because of their ability to proliferate, differentiate into endothelial cells and form new vessels, blood-derived endothelial progenitor cells (EPCs) are attractive source of cells for use in engineering blood vessels. However, the durability and function of EPC-derived vessels implanted *in vivo* are unclear. To this end, we directly compared formation and functions of tissue-engineered blood vessels generated by peripheral blood- and umbilical cord blood-derived EPCs in a model of *in vivo* vasculogenesis. We found that adult peripheral blood EPCs form blood vessels that are unstable and regress within three weeks. In contrast, umbilical cord blood EPCs form normal-functioning blood vessels that last for more than 4 months. These vessels exhibit normal blood flow, perm-selectivity to macromolecules and induction of leukocyte-endothelial interactions in response to cytokine activation similar to normal vessels. Thus, umbilical cord blood EPCs hold great therapeutic potential and their use should be pursued for vascular engineering.

Introduction

The discovery of circulating endothelial progenitor cells (EPCs) in peripheral blood—reported almost a decade ago[1]—has generated impetus for using EPCs in applications ranging from alleviation of tissue ischemia to cancer therapy. One such application is vasculogenesis– an important objective for tissue engineering. However, *in vitro* phenotypic studies have demonstrated that cells defined as EPCs consist of a heterogeneous population, containing cells with differential phenotype (i.e., endothelial and hematopoietic myeloid cells) and outgrowth potential[2, 3]. Recent reports have also shown that peripheral blood (PB)– and cord blood (CB)-derived EPCs (also referred to as endothelial colony-forming cells[2]) can form functional blood vessels when implanted *in vivo*, however the duration and functional performance of these blood vessels have not been defined[2, 4, 5]. In this report, we address two critical outstanding questions regarding the use of EPCs for vasculogenesis in tissue engineering. First, can both adult PB– and CB-derived EPCs form stable vessels? Second, if *ex vivo* expanded EPCs form durable vessels *in vivo*, how well do these vessels function compared to the pre-existing microvasculature? To this end, we utilized an *in vivo* engineered vessel model to compare the neo-vasculature generated by PB– and CB-derived EPC through vasculogenesis. We assessed the functional performance of the engineered vessels by measuring three parameters: red blood cell velocity, vessel permeability to serum albumin and leukocyte rolling in response to cytokine stimulation.

Materials and Methods

Cell isolation and culture

Adult buffy coat samples were obtained from the blood bank at Massachusetts General Hospital. Umbilical cord blood samples were obtained from the Pediatric Research Institute, University of St. Louis, MO, according to guidelines established by the Human Investigation Committee (IRB Protocols#2003-P-000588). The mononuclear blood cells (MNBCs) were isolated and cultured as described previously with minor modifications[6]. Briefly, mononuclear cells were isolated by centrifugation over Ficoll plus density gradient solution. The MNBCs were then cultured on collagen I coated dish (BD Bioscience, Bedford, MA) with EGM-2MV medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum. The medium was changed every 2 to 3 days. Once endothelial colonies were formed, the cells were then passaged and transduced with retrovirus vector encoding enhanced green fluorescent protein (EGFP) gene, as previously described[7]. All experiments were performed with cells less than 6 passages. C3H10T1/2 (10T1/2) (American Type Culture Collection, Manassas, VA) were grown and maintained in Eagle's Basal medium (BME) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine.

Immunohistochemistry

PB- and CB- EPCs were grown on chamber slides until confluent. The cells were washed with phosphate buffered saline (PBS) and then fixed by incubating them in ice-cold methanol for 30 minutes. The chamber slides were then washed with PBS to remove all traces of methanol. To block non-specific binding, the chamber slides were incubate in PBS with 3% bovine serum albumin (BSA) for 1 hour followed by overnight

incubation with primary antibodies at 4°C. The following primary antibodies were used at 1:200 dilution: CD31 (Dako), VE-Cadherin (Pharmingen), von Willebrand Factor (Dako), Tie-2 (R&D Systems), and Ki-67 (Dako). The next day, the chamber slides were washed with PBS and incubated with appropriate Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). The chamber slides were then washed and mounted in Vectashield containing DAPI (Vector Laboratories).

Mice with CB-EPCs implanted in a gel in the cranial window were sacrificed and immediately perfused with 4% paraformaldehyde intracardially to remove the red blood cells and to fix the tissue. The gels were then extracted and incubated in 4% paraformaldehyde for 3 hours and then washed with PBS. The gels were initially blocked for endogenous mouse IgG with Vector Mouse on Mouse Kit (Vector Laboratories) for two hours following manufacturer's protocol. The gels were then incubated overnight at 4°C in blocking solution containing 3% BSA, 5% normal rabbit serum, and 0.3% Triton X100 in PBS. The next day, primary antibodies (CD31 (Dako) and VE-Cadherin (Pharmingen)) were diluted in blocking solution at a dilution of 1:400 and were added to the gel. After overnight incubation with primary antibodies at 4°C, the gels were washed extensively with PBS to remove the unbound antibodies. The gels were then incubated with Cy3-conjugated rabbit anti-mouse secondary antibodies at 1:400 dilution for 3 hours at room temperature. After washing with PBS, the gels were mounted in Vectashield and imaged with confocal microscope.

Apoptosis assay

PB- and CB-EPCs were grown in chamber slides to confluent. The chamber slides were then washed with PBS to remove all traces of serum and growth factors. EGM2 basal media containing no serum or growth factors were then added to the chamber slides. After 48 hours of incubation, the chamber slides were then washed with PBS and fixed in ice-cold methanol for 30 minutes. The EPCs were then assay for apoptotic cells with ApopTag® Red In Situ Apoptosis Detection Kit (Chemicon) following manufacturer's protocol.

Preparation of tissue-engineered vessel construct

The EGFP-labeled endothelial progenitor cells (EGFP-EPCs) and 10T1/2 cells were co-cultured in a collagen gel and implanted onto a mouse cranial window (6 to 8 weeks old male SCID mice) as previously described[7]. One million EGFP-EPCs (EPC-alone group) or 1×10^5 EGFP-EPCs and 2×10^5 of 10T1/2 (EPCs + 10T1/2 co-implantation group) were suspended in 1 ml solution of rat-tail type 1 collagen (1.5 mg/ml) (BD Biosciences, Bedford, MA) and human plasma fibronectin (90 μg/ml) (Sigma) in 25 mM Hepes (Sigma) buffered EGM-2MV medium at 4°C. pH was adjusted to 7.4 by using 1N NaOH (Fisher Science, NJ). The cell suspension was pipetted into a single well of a 12 well plates and warmed to 37°C for 30 min to allow polymerization of collagen. Once the collagen gel had solidifed, one ml of warmed EGM-2MV medium was added into the well and the cell culture plate was then placed overnight in a incubator maintained at 37°C and 5% CO_2. The next day, a skin puncher (4-mm diameter) was applied to the collagen gel construct to create a circular piece and it was then implanted into a cranial window of a severe combined immunodeficient (SCID) mice.

Visualization and analysis of tissue-engineered vessels

The fate of the EGFP-labeled endothelial cells *in vivo* was tracked by intravital imaging with multi-photon laser scanning microscopy at various time points[8]. Functional blood vessels were revealed by intravenous injection of 100 μl of tetramethylrhodamine-conjugated dextran (2,000,000 MW at 10mg/ml) via tail-vein. The same region of the gel was tracked at different time points for consistency. The perfused vessel length density was quantified by manual tracing of perfused blood vessels lined by EGFP-EPCs with a macro developed in-house in Matlab. For each animal, three stacks of images (333 μm by 250 μm) were taken at 5 μm interval in the z direction and an average gel thickness around 80 μm. For consistency, we chose to analyze the middle section of the gel by projecting the maximum intensity of the middle five stacks (20 μm in thickness). EGFP-EPCs derived cord-like structure with no blood flow was similarly quantified as unperfused blood vessel density.

Vascular permeability measurement

Vascular permeability to albumin was determined by intravital microscopy as described previously[9]. Briefly, mice were injected with a bolus (100 μl) of 1% tetramethylrhodamine-labeled bovine serum albumin (Molecular Probes, Eugene, OR) in saline via the tail vein. Fluorescence intensity of the tissue was measured every two minutes for a total of 20 min by a photomultiplier (9203B, EMI, Rockaway, NJ) using a 20x objective lens. The effective vascular permeability (P) was calculated as follows: $P = (1\text{-}HT)\ V/S\ [1/(I_0 - I_b) * dI/dt + 1/K]$ where I is the average fluorescence intensity of the whole image, I_0 is the value of I immediately after the filling of all vessels by rhodamine-

BSA and I_b is the background fluorescence intensity. HT is the average hematocrit. V and S are the total volume and surface area of vessels within the tissue volume within the view field, respectively. The time constant of BSA plasma clearance (K) was 9.1×10^3 s.

Analysis of leukocyte-endothelial interaction

Leukocyte-endothelial interaction in the engineered vessels was determined as described previously[8]. Briefly, endogenous leukocytes were labeled with an intravenous bolus injection of 50 μl of 0.02% rhodamine-6G and then visualized with intravital microscopy. For each animal, 3 to 4 blood vessels were randomly chosen that resembled post-capillary venule based on width of diameter (20 to 40 μm) and blood flow pattern. Video images were recorded and 60 seconds of the recordings were analyzed offline. The number of rolling leukocytes and total flux of leukocytes was counted along a 100 μm segment of vessel. Rolling leukocytes were defined as cells interacting with and moving along the vessel wall at a velocity that was significantly lower than the centerline velocity. The ratio of rolling cells to total flux (rolling count) was used as an indicator of leukocyte rolling. Leukocyte rolling was determined at the baseline and after stimulation with IL1β (R&D Systems, Minneapolis, MN)[10, 11]. For induction of systemic inflammation, mice were injected intraperitoneally with 100 ng of IL-1β for 4 hours.

Red blood cell velocity analysis

Red blood cell (RBC) velocities along the central axis of 15-25 μm diameter vessels in both host and engineered vasculature networks were measured using the line scan modality of the MPLSM to image RBC[8, 12]. Mean RBC velocity was determined from

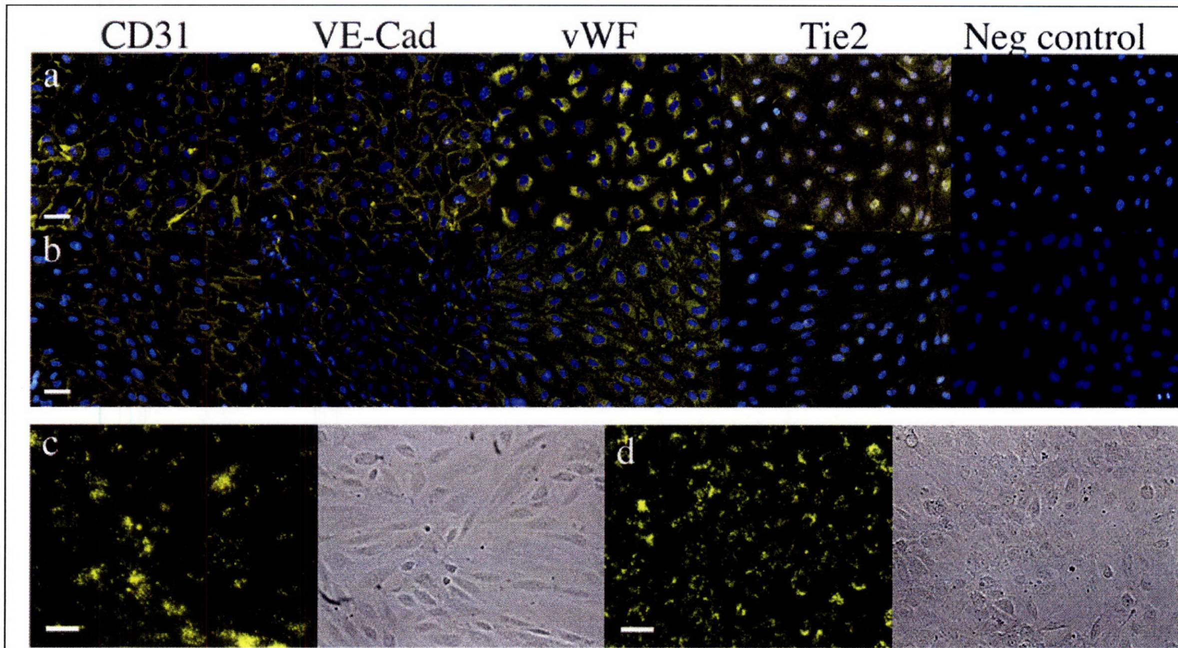
the mean orientation angle of the RBC motion using a custom image segmentation program written in Matlab (Mathworks, Natick, MA).

Statistical analysis

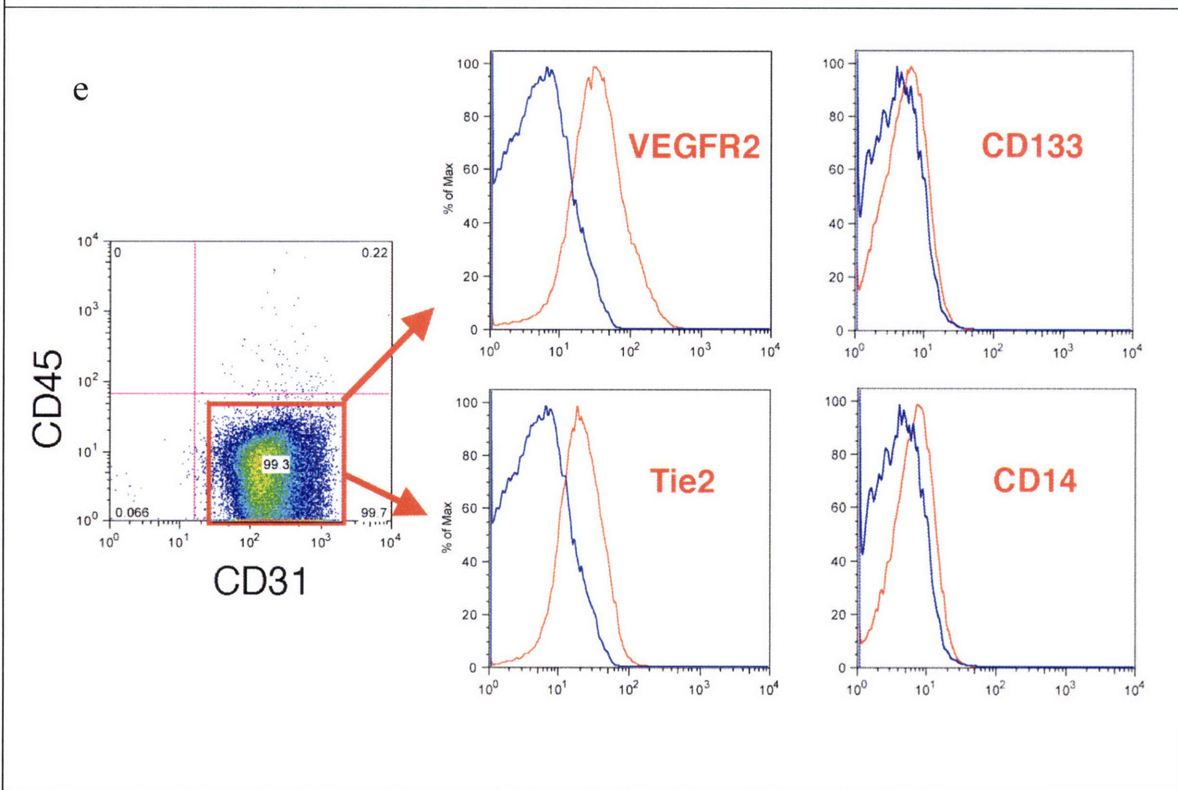
All data were analyzed by ANOVA with the Fisher post hoc test. All data are reported as mean with standard error. Statistical significance was set at $P < 0.05$.

Results and Discussion

To obtain EPC-derived colonies, we cultured PB and CB mononuclear blood cells on Type I collagen-coated dish for 14 to 21 days[6]. We confirmed the endothelial phenotype of PB- and CB-derived EPCs by immunocytochemistry and flow cytometry. EPCs from both sources expressed multiple endothelial markers, including CD31, VE-Cadherin, vWF, VEGFR2 and Tie2, incorporated acetylated low-density lipoprotein (AcLDL), and were negative for the pan hematopoietic marker CD45 and the monocyte marker CD14 (**Figure 3.1**).



CD31
VE-Cad
vWF
Tie2
Neg control
a
b
c
d



e
CD45
CD31
0
0.22
0.066
99.7
99.3
VEGFR2
CD133
Tie2
CD14
% of Max

Figure 3.1. Expression of endothelial markers in endothelial progenitor cells.

Peripheral blood derived endothelial cells (a) and cord blood derived endothelial cells (b) expressed endothelial markers including CD31, VE-cadherin, von Willebrand Factor (vWF) and Tie2, as determined by immunohistochemistry (CD31, VE-cadherin, vWF, and Tie2, yellow; DAPI, blue). PB-EPCs (c) and CB-EPCs (d) were positive for DiI-AcLDL uptake. Scale bars, 50 μm. (e) After *ex vivo* expansion of CB-EPCs, over 99% of the EPCs that are used for *in vivo* implantation are CD31+CD45- endothelial cells. These cells are positive for the endothelial-selective markers VEGFR2 and Tie2 and negative for the monocyte-specific marker CD14 and progenitor/stem cell marker CD133.

To determine if adult PB- and CB-derived EPCs are able to self-assemble into functional blood vessels, we implanted EGFP-labeled EPCs alone or co-implanted them with 10T1/2 cells in a collagen/fibronectin gel onto the pial surface in cranial windows[7]. 10T1/2 cells, a line of mouse embryonic fibroblasts, have previously been demonstrated to stabilize endothelial cells by functioning as perivascular-like cells and releasing paracrine factors[7, 13, 14]. Here, we found that both human adult PB- and CB-EPCs were capable of vasculogenesis. However, the density and persistence of the engineered neovasculature were substantially different depending on the source of EPCs.

In the case of PB-EPCs co-implanted with 10T1/2, the EPCs began to elongate and to connect to one another resulting in a mesh-like network 7 days after implantation (**Fig. 3.2a**). By day 11, some of the EPCs had joined together to form patent vascular tubes that were connected to the host circulation (**Fig. 3.2a**). However, the number of these functional engineered vessels was low and they were distributed haphazardly within the

collagen gel. Co-implantation of PB-EPCs with 10T1/2 cells did not significantly increase both perfused and non-perfused vessel densities compared to PB-EPC implantation alone (**Fig. 3.2b and Fig. 3.3a**). This is in contrast with *in vitro* data where 10T1/2 cells promote cord-like formation by the EPCs. With time, the PB-EPC derived blood vessels began to regress and disappeared almost completely 1 month after implantation, regardless of the absence or presence of 10T1/2 cells (**Fig. 3.2b and Fig. 3.3a**). Occasionally, a few of the blood vessels remained 27 days after implantation (**Fig. 3.2c**).

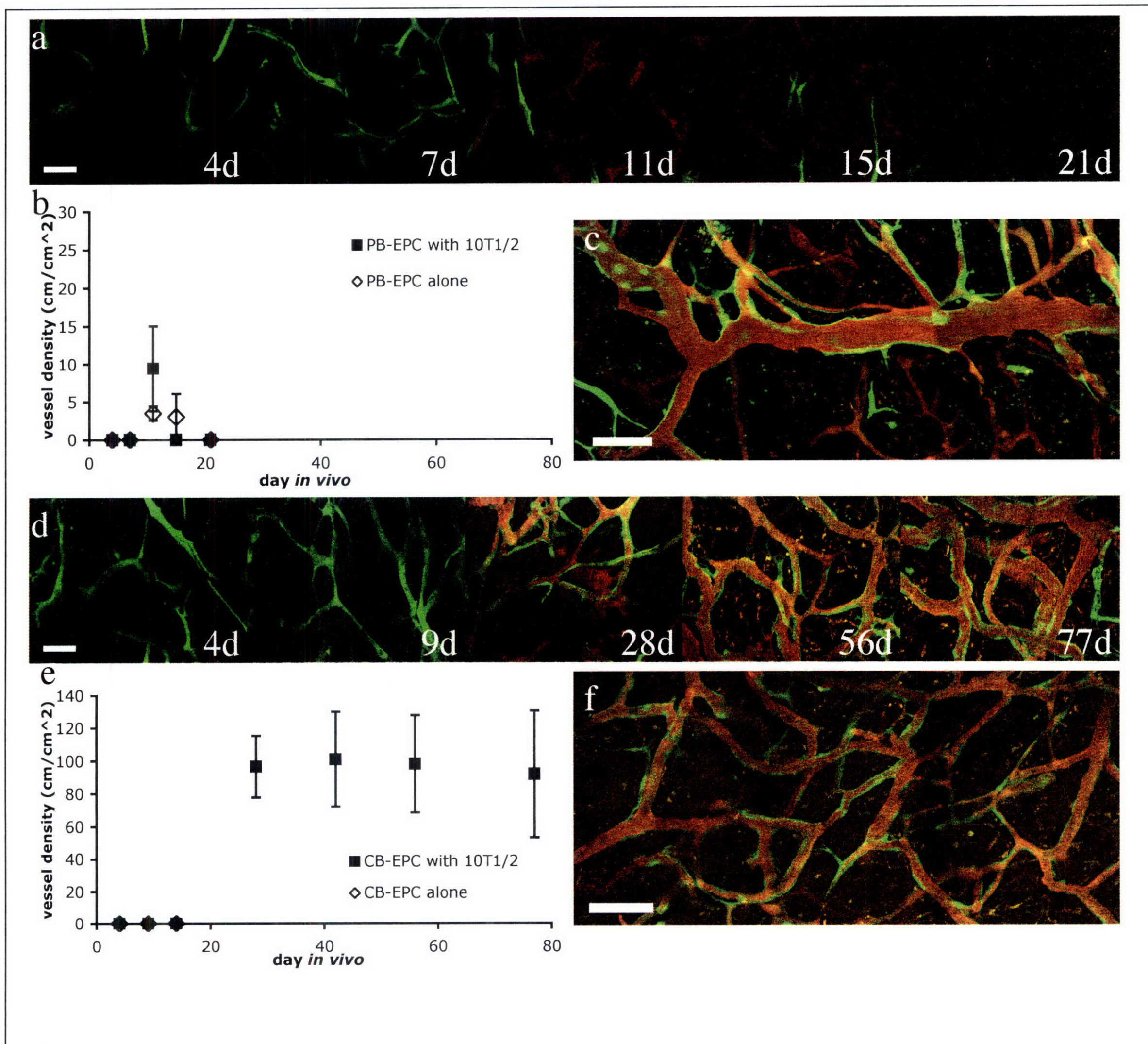


Figure 3.2. Vasculogenic potential of peripheral blood (PB)– versus cord blood (CB)-derived endothelial progenitor cells (EPCs).

PB-EPCs and CB-EPCs were mixed with 10T1/2 cells in a collagen gel, and implanted into cranial windows in SCID mice. Images were taken at periodic time points with multi-photon laser scanning microscope for *in vivo* dynamics of vascularization by the implanted endothelial cells. PB-EPCs formed vascular-like structure 4 days after implantation and some of them became perfused at day 11. The PB-EPCs derived blood vessels were transient and almost completely disappeared by day 21 (**a**). There was no significant difference in the mean (±SEM) density of functional vessels derived from PB-EPCs between groups implanted with PB-EPCs only and PB-EPCs with 10T1/2 cells (**b**) (n=4 for each group and experiments were performed with three different batches of adult peripheral blood). In some animals, there were still some sparse but functional blood vessels 27 days after implantation (**c**). In contrast, CB-EPCs formed a uniformly dense network of functional blood vessels (**d**). Implantation of CB-EPCs alone led to a rapid regression of the implanted cells while co-implantation of CB-EPCs and 10T1/2 cells resulted in a stable and functional vasculature (**e**) (n=4 for each group and experiments were performed with three different batches of human umbilical cord blood). The CB-EPC derived vascular network was stable and functional for more than 119 days *in vivo* (**f**). Green, PB or CB derived endothelial cell expressing enhanced green fluorescent protein (EGFP); red, functional blood vessels contrast-enhanced by rhodamine-dextran. Scale bars, 50 μm, **a, d**; 100 μm, **c, f**.

In contrast, CB-EPCs were able to produce a dense network of blood vessels that were distributed uniformly throughout the collagen gel **(Fig. 3.2d)**. We quantified the vascular density of the same region of collagen gel over time. In the group with CB-EPCs implanted alone, the density of engineered blood vessels was low and they quickly regressed before they could become perfused **(Fig. 3.2e and Fig. 3.3b)**. However, we found that in the group co-implanted with 10T1/2 cells, CB-EPCs formed a network of microvessels that remained stable and patent for more than 119 days **(Fig. 3.2e and Fig. 3.3f)**. We observed some host vessels infiltration into the gel, however the majority of the blood vessels were CB-EPC derived. The collagen gel was excised and whole-mount staining with human specific antibodies showed the expression of the endothelial markers, CD31 and VE-cadherin, on the EGFP-positive EPC-derived vascular endothelial cells **(Fig. 3.4a,b)**. Collagen Type IV staining revealed that the engineered blood vessels had an intact basement membrane **(Fig. 3.4c)**. The 10T1/2 cells persisted and functioned as perivascular cells *in vivo* **(Fig. 3.5a,b)**.

We next investigated whether implanting CB-EPCs at a higher density could obviate the need for 10T1/2 pericyte precursors. CB-EPCs were implanted alone at a 5-fold increase in cell density (5 million cells/ml versus 1 million cells/ml). Some of the implanted CB-EPCs aligned into functional blood vessels, however these vessels were only transiently perfused and disappeared by 23 days **(Fig. 3.5c)**. Implantation of 10T1/2 cells alone led to a minimal angiogenic response. These findings re-affirm that co-implantation of endothelial and perivascular cells is critical for vasculogenic vessel remodeling and sustenance.

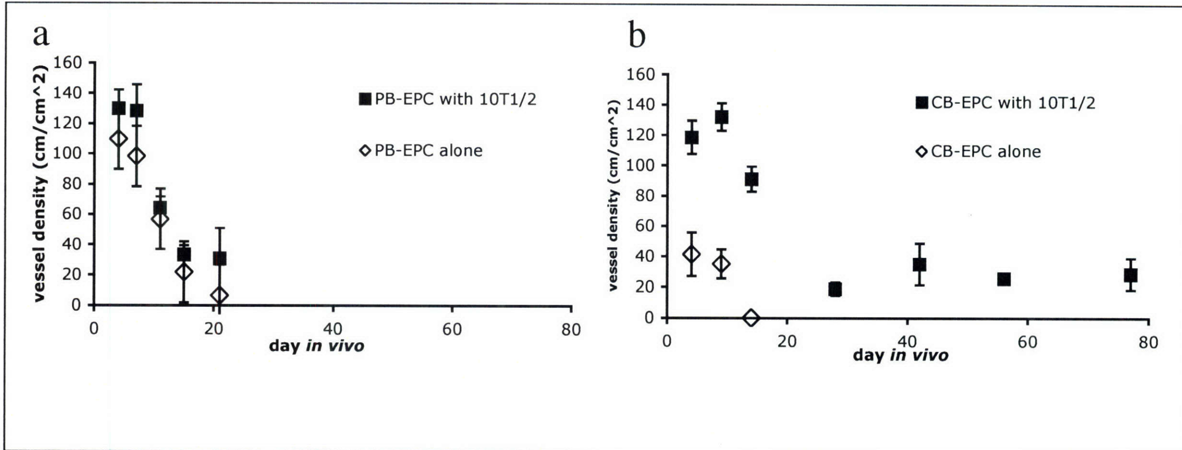
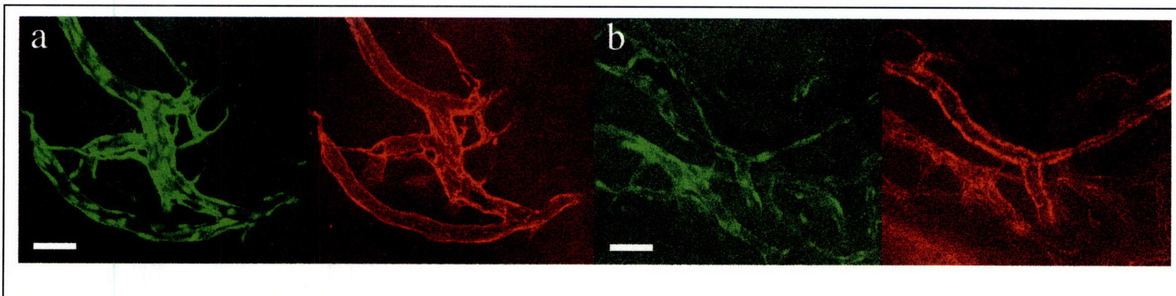


Figure 3.3. Engineered vessel density of PB-EPC and CB-EPC.

PB-EPCs or CB-EPCs were mixed with 10T1/2 cells in a fibronectin/collagen gel, and implanted into cranial windows in SCID mice. Images were taken at periodic time points with multi-photon laser scanning microscope for *in vivo* dynamics of vascularization by the implanted endothelial cells. The vessel density of unperfused blood vessels was quantified. There was a gradual reduction in the density of unperfused vessels in both groups of animals implanted with PB-EPCs only and PB-EPCs with 10T1/2 cells (**a**). There was no significant difference between the two groups. In the group implanted with CB-EPC only, there was a rapid decrease in the density of unperfused vessels (**b**). In the group co-implanted with CB-EPCs and 10T1/2 cells, there was a gradual decrease in the density of unperfused vessels and some of the unperfused vessels persisted even at late time points.



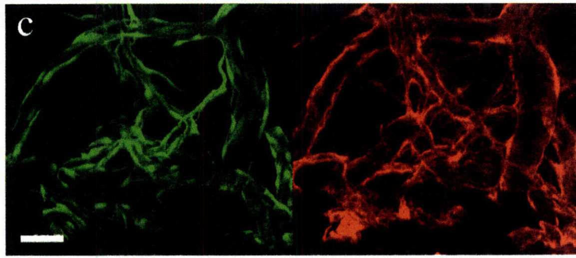
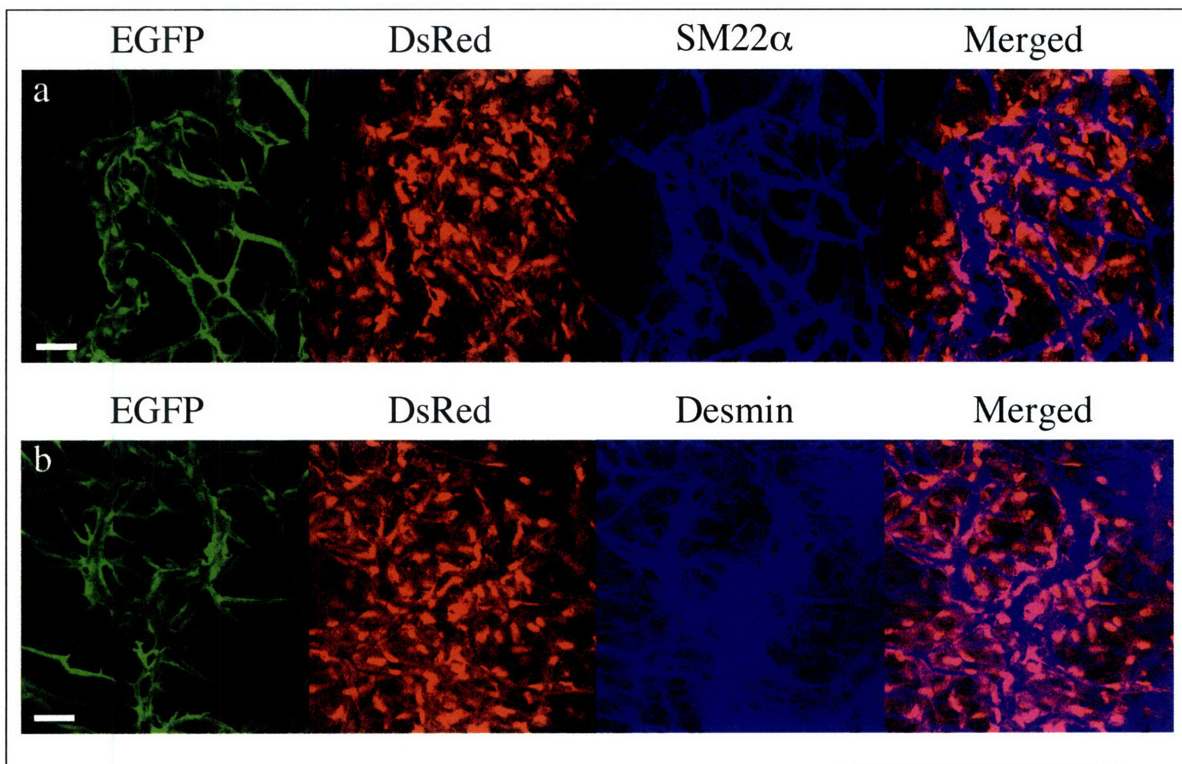


Figure 3.4. Immunohistochemistry of CB-EPCs derived blood vessels.

Whole mount staining of the implanted collagen gel revealed that the CB-EPCs (EGFP+) at day 87 after implantation maintained the expression of CD31 (**a**) and Ve-Cadherin (**b**) and had intact basement membrane of Collagen Type IV (**c**) *in vivo* (EGFP, green; CD31 and Collagen Type IV, red).



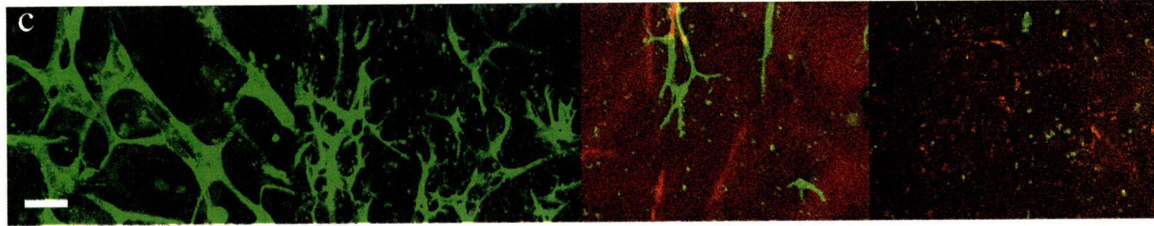


Figure 3.5. Immunostaining of smooth muscle markers in CB-EPCs derived vessels. Whole mount staining of collagen gel with CB-EPCs (EGFP+) and 10T1/2 cells (DsRed+) at day 33 after implantation. 10T1/2 cells proliferated and became part of the stroma *in vivo*. Some of the 10T1/2 cells functioned as perivascular cells around blood vessels. Only the perivascular portion of 10T1/2 cells expressed smooth muscle markers, SM22α (a) and Desmin (b) *in vivo* (EGFP, green; DsRed, red, SM22α and Desmin, blue). CB-EPCs implanted alone at high cell density (5 million cells/ml at high density vs. 1 million cells/ml at normal density) were not able to form long-lasting blood vessels (**c**).

The long-lasting functional blood vessels might be linked to a higher proliferative capacity of CB-EPCs. Indeed, Ingram *et al.* showed that CB-EPCs have a higher level of telomerase activity when compared to PB-EPCs[6]. We found a significantly higher percentage of CB-EPCs were positive for the Ki-67 proliferation marker as compared to PB-EPCs (65.9% for CB-EPCs versus 38.3% for PB-EPCs, $p<0.01$) (**Fig. 3.6a,b,e**), confirming a proliferative advantage for CB-EPCs.

In addition, PB-EPCs might have a competitive disadvantage compared to CB-EPCs due to poorer survival[15]. Cell transplantation is often inefficient. For example, it has been estimated that less than 1% of implanted cells survived 4 days after implantation into a

mouse heart[16]. We tested *in vitro* the EPCs' resistance to stress-induced apoptosis by exposing them to serum-free medium for 48 hour. After 48 hours of exposure to serum-free media, only 3.7% of CB-EPCs were apoptotic (i.e., positive for terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)) versus 13.3% of the PB-EPCs (**Fig. 3.6 c,d,f**). These findings suggest that CB-EPCs are more resistant to stress-induced apoptosis, in addition to having a proliferative advantage when compared to PB-EPCs.

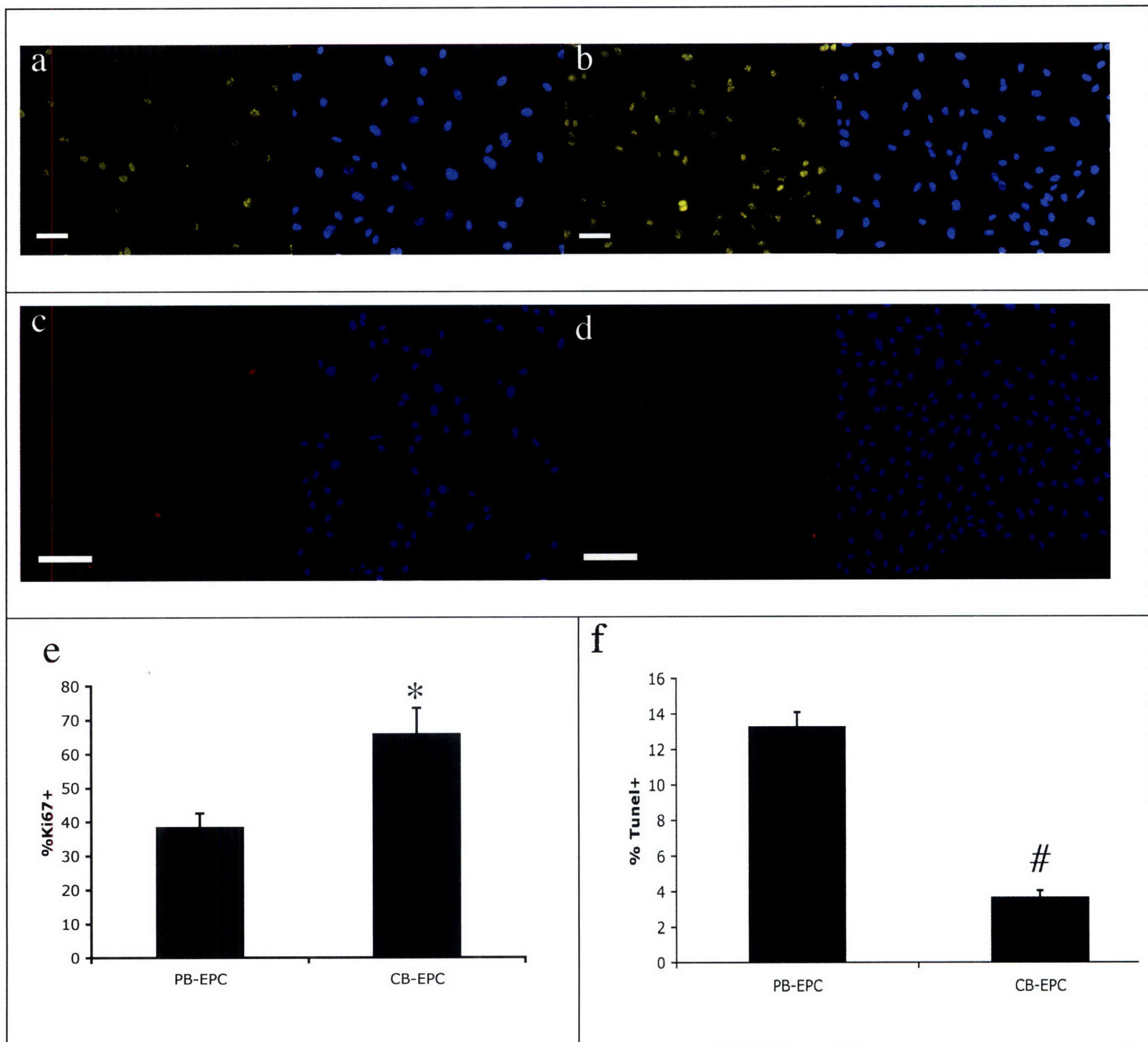


Figure 3.6. Comparison of proliferation and apoptosis in PB-EPCs and CB-EPCs. Peripheral blood (**a**) and cord blood (**b**) derived endothelial cells were stained for Ki-67, a marker for proliferating cells (Ki-67, yellow; DAPI, blue). PB (**c**) and CB (**d**) derived endothelial cells were exposed to serum free media for 48 hours and the cells were then stained for TUNEL, a marker for apoptotic cells (TUNEL, red; DAPI, blue).. Quantification of Ki-67 positive cells showed that a higher percentage of CB-EPCs was undergoing proliferation (**e**). Quantification of TUNEL positive cells showed that CB-EPCs exhibited a higher resistance to serum free medium-induced apoptosis (**f**). Scale bars, 100 μm, **a, b** Scale bars, 50 μm, **c, d**; $^*P<0.01$, $\#P<0.001$

Finally, the function of the engineered blood vessels is critical, since functional abnormalities can have serious pathological consequences[17]. For example, two major problems with immature blood vessels are the irregular flow and the increase in permeability. This leads to leakiness of plasma proteins or even hemorrhage, similar to blood vessels inside tumors[18]. We measured the permeability to serum albumin of the CB-EPC-derived blood vessels at 2 months after implantation. We found that CB-EPC-derived vessels have low vascular permeability, with values close to those of the neighboring brain capillaries (**Fig. 2e**). We have not detected any hemorrhagic episode during the period of observation. The average blood flow rate was comparable between the CB-EPC derived vessels and host pial vessels (**Fig. 2f and Supplement Fig. 9**).

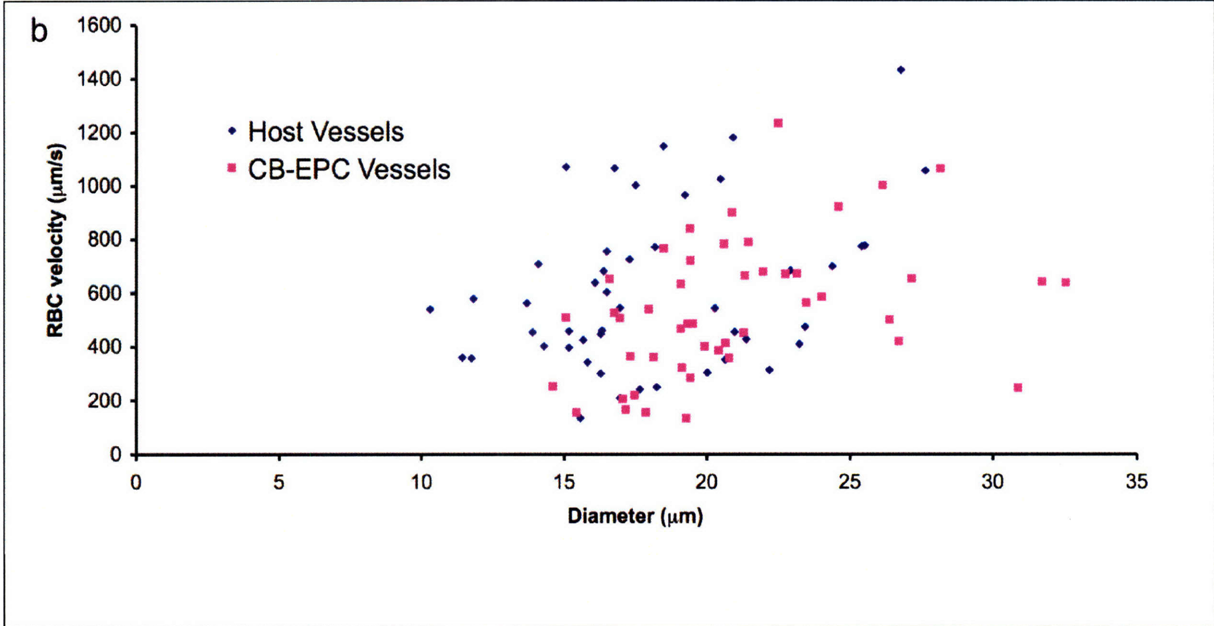
Besides serving as a conduit for blood, vascular endothelium should also have the ability to become activated to recruit leukocytes in the setting of homeostasis, infection or tissue injury. To test this in CB-EPC-derived endothelium, we induced systemic inflammation

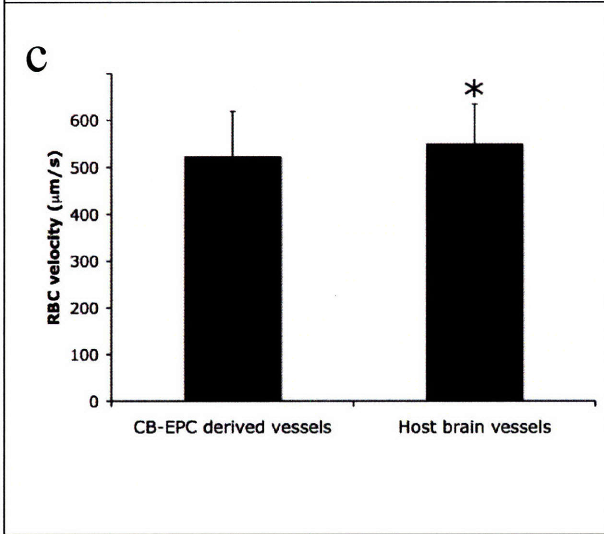
by injection of IL1β in mice with CB-EPC-engineered vessels[10]. We measured the number of rolling leukocytes at baseline and 4 hours after injection of IL1β in selected engineered blood vessels that had characteristics of post-capillary venules (based on morphology, diameter, and rate of blood flow). In these engineered vessels, we found a significant increase in the percentage of rolling leukocytes after induction of systemic inflammation suggesting that CB-EPC derived endothelium undergoes proper cytokine activation (**Fig. 2g**).

a

Table 1. Vascular permeability

CB-EPC + 10T1/2	Normal brain capillaries[7]	Tumors[7]	Type I collagen gel with VEGF[7]
$0.73 \pm 0.21 \times 10^{-7}$ cm/s	$0.3 \sim 0.6 \times 10^{-7}$ cm/s	$2.9 \sim 3.9 \times 10^{-7}$ cm/s	$2.5 \sim 4.9 \times 10^{-7}$ cm/s


b
RBC velocity (μm/s)
Host Vessels
CB-EPC Vessels
Diameter (μm)
0
5
10
15
20
25
30
35
200
400
600
800
1000
1200
1400
1600


c
RBC velocity (μm/s)
*
CB-EPC derived vessels
Host brain vessels

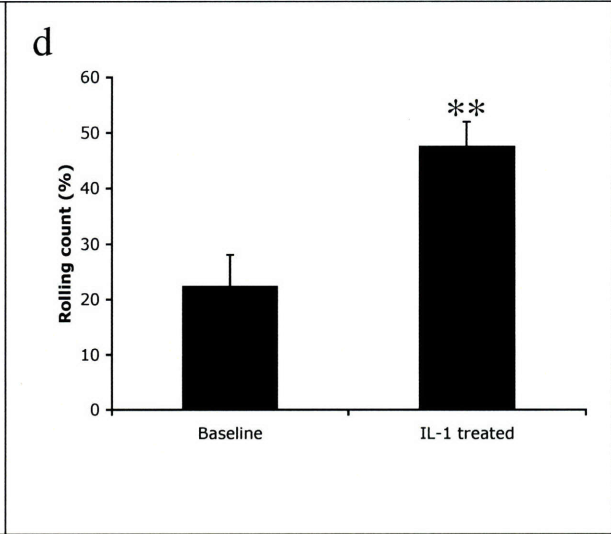

d
Rolling count (%)
**
Baseline
IL-1 treated

Figure 3.7. CB-EPCs derived blood vessels closely approximated normal vascular functions.

The vascular permeability of the CB-EPC derived blood vessels to albumin was measured (**a**). CB-EPC derived blood vessels had low vascular permeability with values similar to those of normal mouse pial blood vessels. Blood flowrate as measured by red blood cell (RBC) velocity was comparable between the CB-EPC derived vessels and mouse brain vessels (**b,c**). The number of rolling leukocytes on CB-EPC derived blood vessels was increased after induction of systemic inflammation with intraperitoneal injection of 100ng of IL-1β for 4 hours (**d**).

In summary, we found that CB-EPCs have enhanced vasculogenic ability *in vivo* compared to adult PB-EPCs. Moreover, CB-EPC co-implantation with pericyte precursors led to the formation of long-lasting and functionally normal blood vessels, providing an attractive cellular therapy platform for tissue engineering.

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Chapter 4: Use Of Human Mesenchymal Stem Cells

Portions of the chapter have been taken from:

P. Au, J. Tam, D. Fukumura, R.K. Jain, *Bone marrow derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature*. Blood (In Press)

Abstract

Vascular tissue engineering requires a ready source of endothelial cells and perivascular cells. Here, we evaluated human bone marrow-derived mesenchymal stem cells (hMSCs) for use as vascular progenitor cells in tissue engineering and regenerative medicine. hMSCs expressed a panel of smooth muscle markers *in vitro* including the cardiac/smooth muscle specific transcription co-activator, myocardin. Cell-cell contact between endothelial cells and hMSCs up-regulated the transcription of myocardin. hMSCs efficiently stabilized nascent blood vessels *in vivo* by functioning as perivascular precursor cells. The engineered blood vessels derived from human umbilical cord vein endothelial cells and hMSCs remained stable and functional for more than 130 days *in vivo*. On the other hand, we could not detect differentiation of hMSCs to endothelial cell *in vitro* and hMSCs by themselves could not form conduit for blood flow *in vivo*. Similar to normal perivascular cells, hMSCs-derived perivascular cells contracted in response to endothelin-1 *in vivo*. In conclusion, hMSCs are perivascular cell precursors and may serve as an attractive source of cells for use in vascular tissue engineering and for the study of perivascular cell differentiation.

Introduction

Human bone marrow derived mesenchymal stem cells (hMSCs) are intensively investigated for treating patients with ischemic heart disease and post-myocardial infarction. It has been hypothesized that MSCs, when injected into a failing heart, can differentiate into mature cardiomyocytes or secrete soluble factors that promote angiogenesis and enhance cardiomyocyte survival and functions[1]. Based on promising results from preclinical animal models, a number of clinical trials have been initiated to test the clinical efficacy of hMSCs injection. The early results of the clinical trial have been mixed and the exact mechanism of the cardiac improvement remains unresolved[2]. In order to maximize the therapeutic potential of hMSCs, a detailed analysis of the cellular functions of the injected hMSCs is needed.

Data from animal studies have suggested that some of the injected hMSCs can differentiate into endothelial cells or smooth muscle cells. Some of the MSCs were found to express endothelial markers including CD31 and vWF, while others expressed smooth muscle markers including α-smooth muscle actin and desmin[3, 4]. These data suggest that hMSCs may function as vascular progenitors. These results raise several fundamental questions. First, can hMSCs differentiate into endothelial cells and smooth muscle cells *in vitro*? Second, if MSCs differentiate into endothelial cells and/or smooth muscle cells, can these cells be used to form functional blood vessels *in vivo*? Third, do the MSCs-derived blood vessels respond appropriately to physiological stimulation? These are important outstanding questions that need to be addressed in order to maximize the therapeutic potential of MSCs. To address these questions, we utilized a tissue engineered

blood vessel model[5-7] and monitored the cellular fate of implanted MSCs and their vasculogenic potential by intravital microscopy.

Materials and Methods

Cell culture

Human bone marrow-derived mesenchymal stem cells were purchased from Cambrex and maintained in Mesenchymal Stem Cell Growth Medium (Cambrex, East Rutherford, NJ). MSCs were seeded at a density of 6000 cell/cm^2 and the cells were passaged at 90% confluent. All experiments with wild type hMSCs and EGFP-hMSCs were performed below 8 and 16 cell passages, respectively. Human umbilical cord vein endothelial cells (HUVECs) were obtained from Center of Excellence in Vascular Biology, Brigham and Women's Hospital and maintained in Endothelial Growth Medium (Cambrex). MS1, a mouse pancreatic endothelial cell line was obtained from ATCC (Rockville, MD). Human aortic vascular smooth muscle cells (CRL-1999) and human dermal fibroblasts (CRL-2575) were obtained from ATCC. 293ET packaging cells were a kind gift from Dr. Brian Seed (Massachusetts General Hospital, Boston, MA). All cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Retrovirus Packaging and Transduction

The retrovirus vector for transducing enhanced green fluorescent protein (EGFP), PBMN-I-EGFP was kindly provided by Dr. Gary Nolan (Stanford, CA). For retrovirus packaging, the plasmids of PBMN-I-EGFP, Gag-pol and VSVG (15μg, 7 μg, 5 μg respectively) were mixed and co-transfected into 293ET cells with lipofectamine 2000

(Invitrogen, Carlsbad, CA) per manufacturer's protocol. After overnight incubation, the 293ET cells were washed with PBS and replaced with fresh media. The next day, the supernatant containing retrovirus was collected and fresh media was added; this step was repeated 3 more times. After the supernatant was collected, it was passaged through a 0.45 μm filter (Whatman, Middlesex, UK) and was either used immediately for infection or kept at –80°C.

Co-Culture of Mouse Endothelial Cells and Human Mesenchymal Stem Cells

hMSCs were cultured either alone or co-cultured with MS1 endothelial cells in Dulbecco's Modified Eagle's Medium (DMEM) with 2% fetal bovine serum (FBS) for 2 days. Briefly, $1x10^5$ hMSCs in the hMSCs only group or $1X10^5$ hMSCs and $1X10^5$ MS1 endothelial cells in the co-culture group were added to a 6-well plate. The plate was agitated to make sure the cells were well mixed and the plate was then placed in a 37°C incubator and cultured for 48 hours. For transwell culture, $1X10^5$ hMSCs were placed in a 6 well plate and $1X10^5$ MS1 endothelial cells were added onto a 0.4 μm pore membrane insert (Corning, Corning, NY) that was placed above the 6 well plate.

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) and Quantitative Real-time RT-PCR

Total RNA was extracted with the Qiagen RNeasy mini kit, and cDNA was synthesized from RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, University Park, IL); all procedures were performed according to manufacturer's protocol. PCR for detection of endothelial and smooth muscle transcripts was performed

with 2.5x Mastermix (Eppendorf, Hamburg, Germany). Quantitative RT-PCRs were performed on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). All experiments were performed in triplicate. Final quantification of each cDNA sample was relative to human GAPDH following the manufacturer's instructions (Applied Biosystems). Species-specific primers were designed using Primer Express or Primer3 (Whitehead Institute, Cambridge, MA).

PCR primer sequence and PCR condition

Name	Forward primers	Reverse Primers	Annealing Temperature	PCR cycle
α-smooth muscle actin	ctgttccagccatccttcat	cggcttcatcgtattcctgt	52°C	30
SM22α	aacagcctgtaccctgatgg	atgacatgctttccctcctg	52°C	30
Calponin	tccaaatatgacccccagaa	cccactctcaaacaggtcgt	52°C	30
Smoothelin	ggagaactggctgcactctc	gaaacctctgcctgctgttc	52°C	30
ACLP	cgcctacggagaaagtcaag	cacaaaatcgtcatggatgc	52°C	30
Desmin	tgaagggcactaacgattcc	ctcagaaccccctttgctcag	52°C	35
Myocardin	ctcggcttcctttgaacaag	cttcccagagaatccatcca	52°C	35
VE-Cadherin	aagcctctgattggcacagt	acacactttgggctggtagg	52°C	30
PECAM1	gagtcctgctgacccttctg	cactccttccaccaacacct	52°C	30
GAPDH	catgagaagtatgacaacagcct	agtccttccacgataccaaagt	52°C	25

Transwell Migration Assay

Cell migration was assessed using Falcon HTS FluroBlok 24-well inserts (BD Biosciences, San Jose, CA) with 8-μm pores. EGFP-hMSC cells (2×10^4) suspended in 250-μl DMEM (Invitrogen) with 2% FBS were placed inside each insert while 5×10^4 per well HUVECs suspended in 800 μl EGM (Cambrex) were plated on a 24-well plate. Eight hours later, all cell culture media were changed to DMEM with 2% FBS, and then the inserts were placed in the respective wells. To examine the effect of PDGFR inhibition on cell migration, 2, 5 or 10 μM of Gleevec (Novartis, Basel, Switzerland) was added to the medium. The number of migrated cells was quantified 24 hours later as previously described[8]. Briefly, the image was threshold and the area covered by the migrated cells was quantified in ImageJ (version 1.38, http://rsb.info.nih.gov/ij/).

Mesenchymal Stem Cell Differentiation Assay

Mesenchymal stem cells were cultured in chamber slides with adipogenic or osteogenic differentiating medium. Adipogenic medium consisted of DMEM, 10% FBS, 0.5 mM isobutylmethylxanthine, 10 μM insulin, 200 μM indomethacin, and 1% peniciilin/streptamycin. Osteogenic medium consisted of DMEM, 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, 10 mM b-glycerophosphate and 1 % penicillin/streptamycin. After 30 to 40 days in culture, the cells were stained with Oil Red O and Alizarin Red or alkaline phosphatase for adipogenesis and osteogenesis, respectively.

Immunofluorescence

Mesenchymal stem cells were grown on chamber slides until confluent. The cells were washed with phosphate buffered saline (PBS) and then fixed by incubating them in ice-cold methanol for 30 minutes. The chamber slides were then washed with PBS to remove all traces of methanol. To block non-specific binding, the chamber slides were incubate in PBS with 3% bovine serum albumin (BSA) for 1 hour followed by overnight incubation with primary antibodies at 4°C. The following primary antibodies were used at 1:200 dilution: α-smooth muscle actin (Dako, Glostrup, Denmark), sm22α (Abcam, Cambridge, MA), desmin (Dako), and NG2 (Chemicon, Temecula, CA). The next day, the chamberslides were washed with PBS and incubated with appropriate Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The chamber slides were then washed and mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). For whole mount staining, collagen gels were extracted from cranial windows and incubated in 4% paraformaldehyde for 3 hours and then washed with PBS. The gels were initially blocked for endogenous mouse IgG with Vector Mouse on Mouse Kit (Vector Laboratories) for two hours following manufacturer's protocol. The gels were then incubated overnight at 4°C in blocking solution containing 3% BSA, 5% normal rabbit serum, and 0.3% Triton X100 in PBS. The next day, primary antibodies (α smooth muscle actin (Dako), Sm22α (Abcams), and Desmin (Dako)) were diluted in blocking solution at a dilution of 1:400 and were added to the gel. After overnight incubation with primary antibodies at 4°C, the gels were washed extensively with PBS to remove unbound antibodies. The gels were then incubated with Cy5-conjugated secondary antibodies at 1:400 dilution for 3 hours at room temperature. After washing with PBS, the gels were mounted in Vectashield and imaged with a confocal microscope

(Model#BX61WI, Olympus, Tokyo, Japan) using 20X/0.95 NA water objective. Image was acquired with Fluoview FV500 (Olympus).

Tissue Engineered Blood Vessel Model

The engineered blood vessel model was prepared as previously described[5]. Briefly, 1 x 10^6 HUVECs and 2 x 10^5 hMSCs cells, or 1 x 10^6 HUVECs and 2 x 10^5 10T1/2 cells, or 1 x 10^6 HUVECs alone were suspended in 1 ml of type 1 collagen–fibronectin solution. The next day, a skin puncher (4-mm diameter) was used to create a circular piece from the collagen gel. The circular piece of collagen gel was then implanted into a cranial window of a severe combined immunodeficient (SCID) mouse. The *in vivo* fate of the fluorescent protein-labeled endothelial cells and hMSCs was tracked by intravital imaging with multi-photon laser scanning microscopy at various time points[9] (modified Axioskop 50 microscope, Carl Zeiss, Germany and MaiTai Ti:Sapphire laser, Spectra-Physics, Mountain View, CA). Image was taken with 20x/0.50 NA water objective. Functional blood vessels were revealed by intravenous injection of 100 μl of tetramethylrhodamine-conjugated dextran (2,000,000 MW at 10mg/ml) via the tail-vein. The same regions in each gel were tracked at different time points for consistency. The perfused vessel length density was quantified by manual tracing of perfused blood vessels lined by EGFP-EPCs with a custom-written MATLAB macro (Mathworks, Natick, MA).

Arteriolar Contractility Assay

Arteriolar contractility was assessed by vasoactive response to endothelin-1 (ET-1). After careful removal of the cover glass, the cranial window was superfused with warm PBS.

For vessel contrast enhancement, 100 μl of 1% tetramethylrhodamine -dextran (MW 2 million) was injected i.v. The engineered vessels were monitored by multi-photon laser scanning microscopy with a 20x water-immersion objective. After baseline measurements of vessel diameter, the cranial window was superfused with 100 nM ET-1 in PBS. Then, the same region of vessels was imaged every 5 minutes over a 20 minutes period. The diameter of vessels was quantified manually with ImageJ.

Statistical analysis

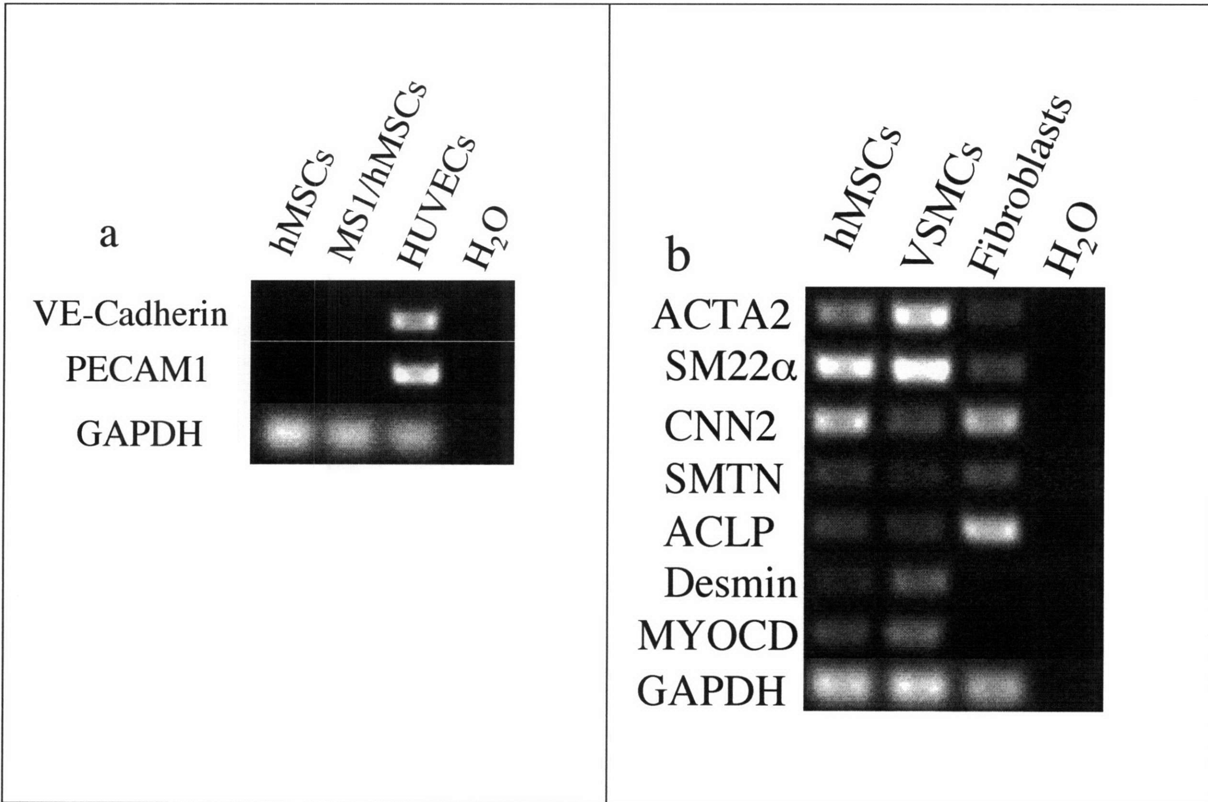
All data were analyzed by ANOVA with the Fisher post hoc test. All data are reported as a mean with standard error. Statistical significance was set at $P < 0.05$.

Results

Transcriptional profile for endothelial and smooth muscle markers

To investigate whether hMSCs exhibit endothelial phenotypes, we first checked by PCR for transcripts of endothelial markers, PECAM1 (CD31) and VE-Cadherin. These endothelial-selective markers were not detectable in hMSCs, neither at baseline nor when co-cultured with a mouse endothelial cell line (Figure 4.1a). Next, we assessed the transcription profile of a panel of smooth muscle markers in hMSCs and compared it to aortic smooth muscle cells and dermal fibroblasts (Figure 4.1b). All cell lines commonly transcribed mRNA of α-smooth muscle actin, sm22α, desmin, calponin, and smoothelin. Immunochemistry confirmed the expression of α-smooth muscle actin, SM22α, desmin and NG2 in hMSCs (Figure 4.1c-f). Myocardin was recently discovered to be a cardiac/smooth muscle specific co-activator for serum response factor in the regulation of

a number of cardiac and smooth muscle genes[10]. As expected, myocardin was transcribed in aortic smooth muscle cells. More interestingly, myocardin was transcribed by mesenchymal stem cells, but not by dermal fibroblasts. These data suggest that the expression of myocardin might potentially be a more specific marker for smooth muscle cell differentiation than the commonly used markers, such as, α−smooth muscle actin and SM22α.



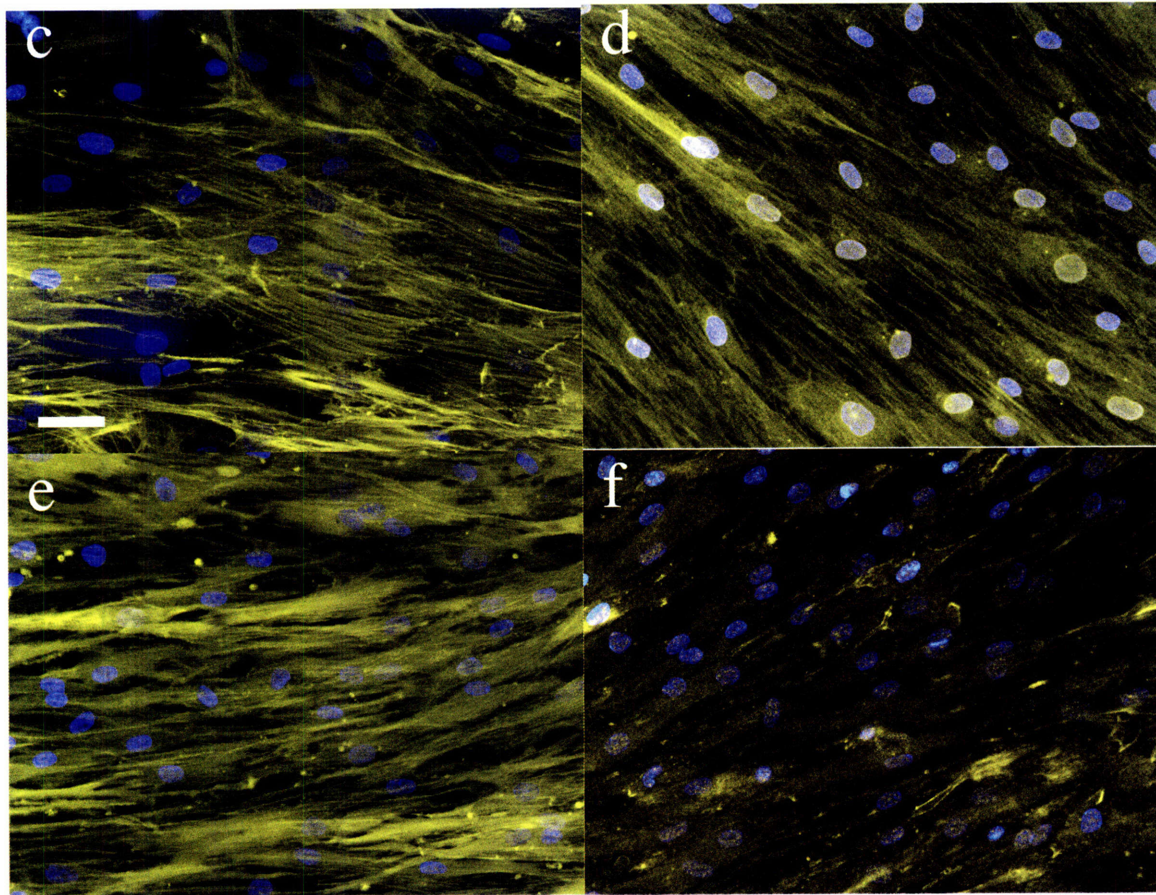
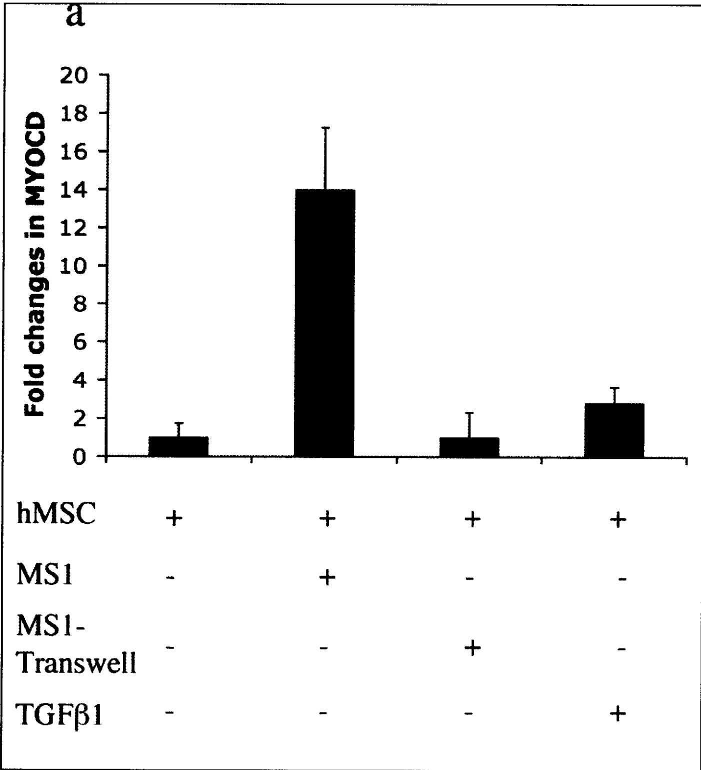


Figure 4.1. Transcriptional profile of endothelial and smooth muscle markers in hMSCs

a, RT-PCR analysis of endothelial markers (VE-Cadherin and PECAM1) in hMSCs at baseline and in hMSCs co-cultured for 3 days with MS1 mouse endothelial cell line. **b**, RT-PCR analysis of smooth muscle markers' mRNA in human mesenchymal stem cells, human aortic vascular smooth muscle cells (CRL-1999) and human dermal fibroblasts (CRL-2575). Water was used as negative control. **c-f**, Protein expression of smooth muscle markers in hMSCs was confirmed by immunohistochemistry (α-smooth muscle actin (**c**), SM22α (**d**), desmin (**e**) and NG2 (**f**), yellow; DAPI, blue). Scale bar, 50 μm.

Endothelial cells up-regulate myocardin in hMSCs through cell-cell contact

Previous studies have suggested that endothelial cells can induce the differentiation of smooth muscle cells/pericytes[11, 12]. To determine whether endothelial cells could regulate the differentiation of hMSCs toward smooth muscle lineage, we co-cultured them with a mouse endothelial cell line, MS1. Using human specific primers, real-time RT-PCR was performed to examine the transcription of myocardin in hMSCs. Co-culturing hMSC with MS1 resulted in a statistically significant up-regulation of myocardin (Figure 4.2a). TGFβ1 has been suggested to be involved in the differentiation of smooth muscle cells/pericytes[11]. We investigated whether addition of TGFβ1 could induce transcription of myocardin in hMSCs. TGFβ1 stimulation only modestly induced the transcription of myocardin and the magnitude of the induction was much less compared to the co-culture group (Figure 4.2a). To gain better understanding of the inductive signals, we checked whether the signals were mediated by soluble factors or cell-cell contact. To this end, we cultured hMSCs and endothelial cells in separate layers of a transwell culture. This allowed for the diffusion of soluble factors while preventing direct cell-cell contact between hMSCs and endothelial cells. The transwell culture did not induce myocardin transcription, suggesting that the inductive signal was provided by cell-cell contact (Figure 4.2a).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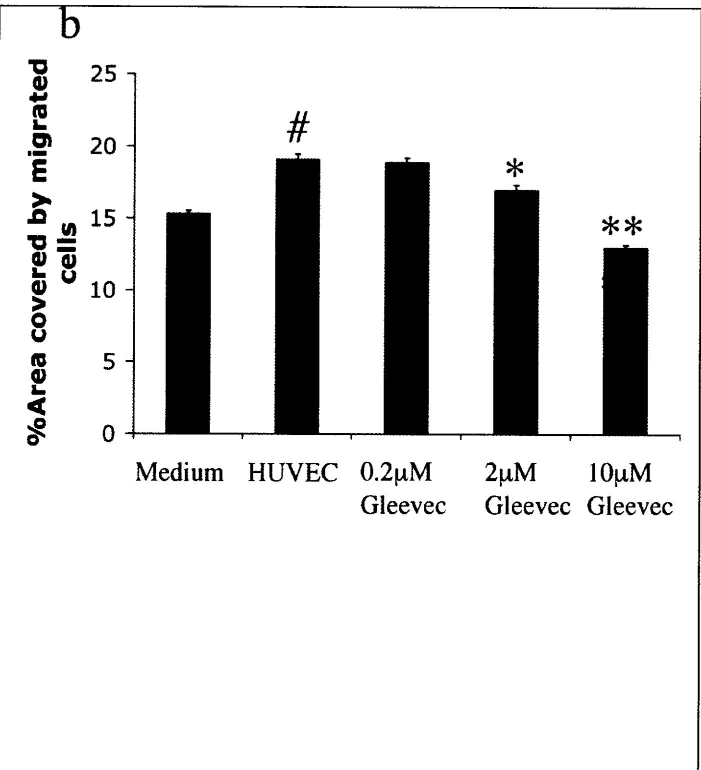
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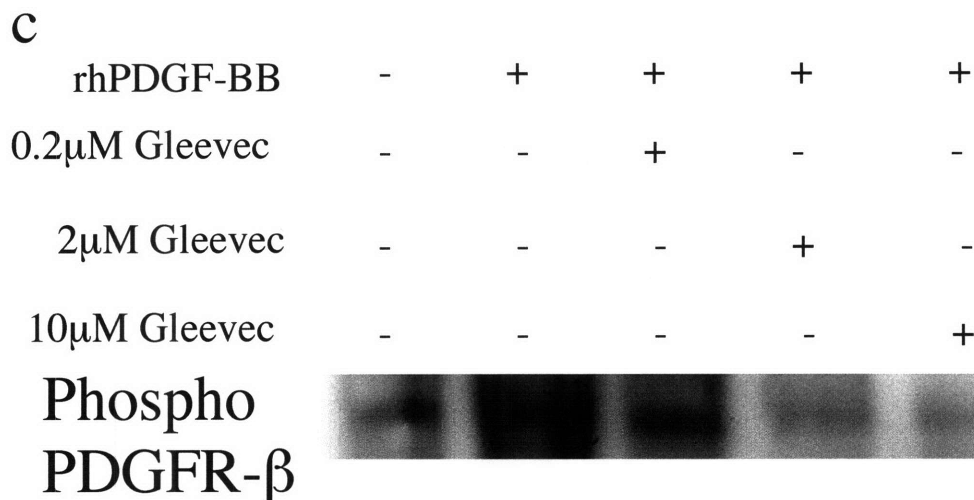


Figure 4.2. Induction of myocardin transcription and migration in hMSCs by endothelial cells

a, Quantitative real time PCR was performed to assess the induction of myocardin in hMSCs by various conditions after 2 days (hMSCs alone, hMSCs cultured with MS1 endothelial cells with contact, hMSC cultured with MS1 without contact by transwell culture, and hMSCs stimulated with TGFβ1 (10ng/ml)). Values expressed as fold increase above hMSC alone levels and normalized by GAPDH. Representative data of 3 separate experiments. **b**, Transwell migration assay was performed to assess endothelial cells induced hMSCs migration. Gleevec at 0.2 μM, 2 μM, and 10 μM was added to test for inhibition of hMSCs migration. # $p<0.0001$ (medium vs. HUVEC), * $p<0.0005$ (2 μM Gleevec vs. HUVEC), ** $p<0.0001$ (10 μM Gleevec vs. HUVEC).

hMSCs migrate toward endothelial cells in response to PDGF

A necessary step in the process of vessel maturation is the recruitment of perivascular cells to stabilize an immature vessel. Therefore, we investigated whether HUVECs could induce hMSCs migration in a transwell migration assay. In comparison to control (medium alone), HUVECs significantly increased the number of hMSCs that migrated across the transwell membrane (Figure 4.2b). Previous data suggested that PDGFR signaling mediated the process of perivascular cell migration and recruitment[11, 13]. To test whether PDGFR signaling is involved in hMSCs migration, we added Gleevec, a potent inhibitor of PDGFR, to the cell culture medium. Gleevec blocked hMSCs migration and PDGF-Rβ phosphorylation in a dose-dependent manner (Figure 4.2c).

Mesenchymal stem cells functionally stabilize engineered blood vessels

Endothelial cells implanted *in vivo* could form functional vessels, but such vessels quickly regressed unless the endothelial cells were ectopically transduced to express bcl2, an anti-apoptotic gene[14]. We previously demonstrated that co-implantation of endothelial cells with 10T1/2, a murine embryonic fibroblast cell line which differentiates into perivascular cells *in vivo*, could lead to the formation of a stable and functional microvascular network without the need for genetic modification of the endothelial cells[5]. We investigated whether hMSCs could functionally replace 10T1/2 to stabilize engineered blood vessels. EGFP labeled HUVECs were implanted alone or co-implanted with hMSCs or 10T1/2 cells in a collagen/fibronectin matrix in a mouse cranial window. The implanted endothelial cells were observed with multi-photon laser scanning microscopy to assess their ability to form functional blood vessels. When EGFP labeled

HUVECs were implanted alone, some vessels formed but they quickly regressed, similar to previous observations made by us and others (Figure 4.3a and 4.4a,c)[5, 14]. When EGFP labeled HUVECs were co-implanted with hMSCs, the endothelial cells assembled into functional blood vessels that anastomosed to the host vascular network (Figure 4.3b and 4.4a,c). The engineered blood vessel densities derived from HUVEC-hMSC and HUVEC-10T1/2 co-implantations were similar (Figure 4.3b-c and 4.4b,d). Furthermore, the HUVEC-hMSC engineered vessels were stable and remained functional for more than 130 day *in vivo* (Figure 4.4e).

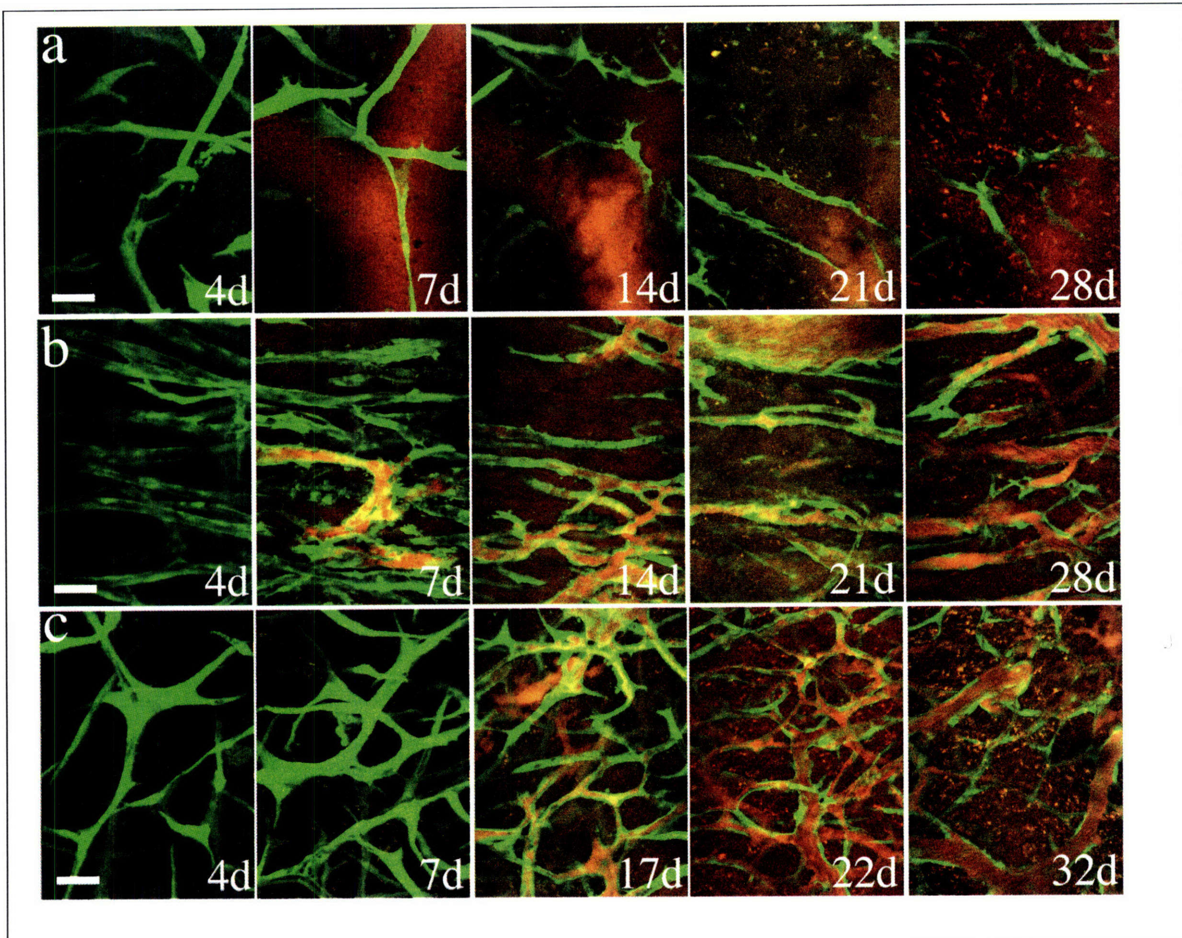


Figure 4.3. hMSCs stabilized engineered blood vessels *in vivo*

EGFP labeled HUVECs were either implanted alone (**a**) or co-implanted with hMSCs (**b**) or 10T1/2 cells (**c**) in a collagen gel onto cranial windows in SCID mice. Images were taken at periodic time points with multi-photon laser scanning microscope to monitor the *in vivo* dynamics of vascularization by the implanted endothelial cells. Green, HUVECs expressing EGFP; red, functional blood vessels contrast-enhanced by rhodamine-dextran. Scale bar, 50 μm.

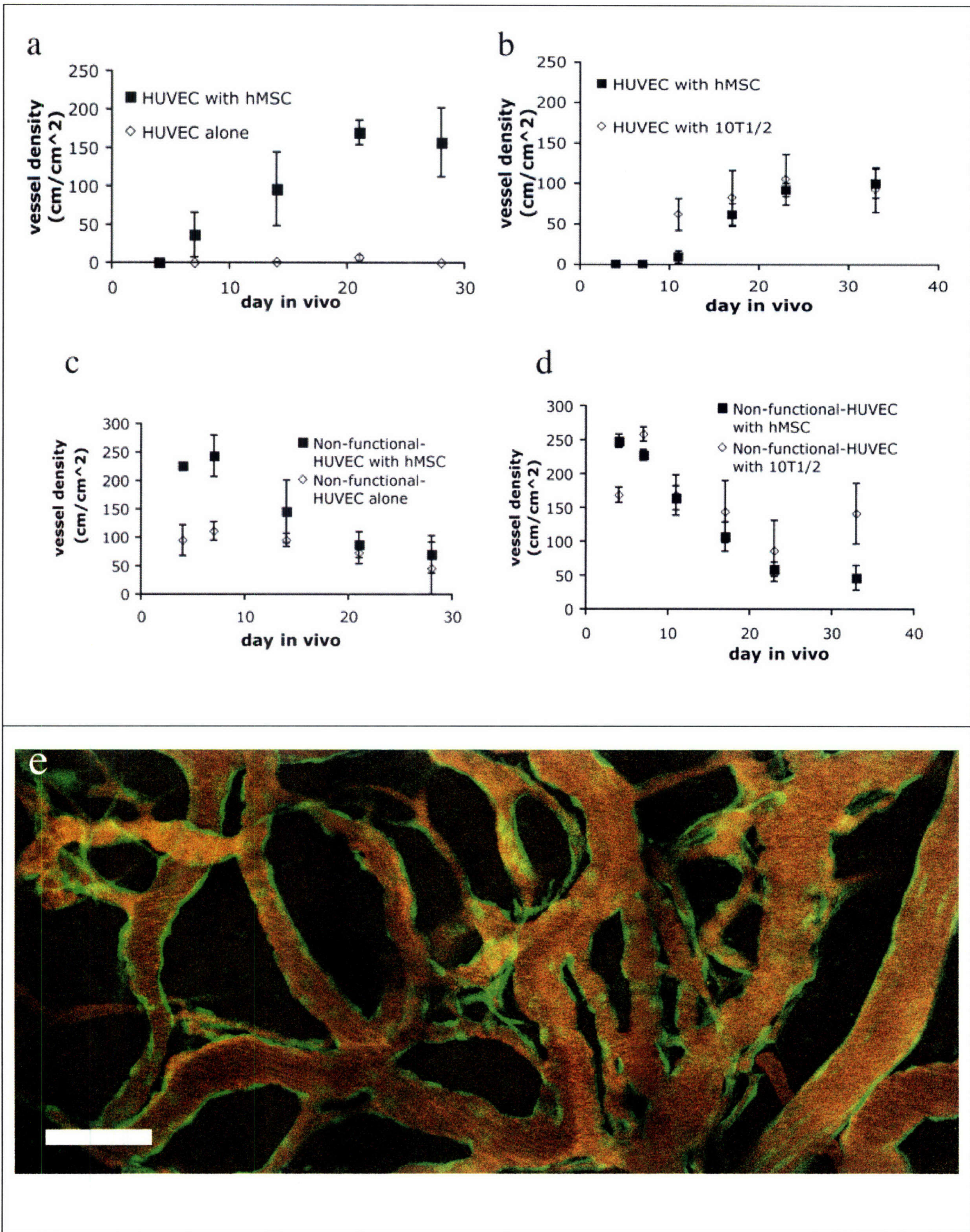

a
vessel density (cm/cm^2)
HUVEC with hMSC
HUVEC alone
day in vivo
b
HUVEC with hMSC
HUVEC with 10T1/2
c
Non-functional-HUVEC with hMSC
Non-functional-HUVEC alone
d
Non-functional-HUVEC with hMSC
Non-functional-HUVEC with 10T1/2
e

Figure 4.4. Quantification of functional engineered blood vessel density.

Quantification of functional and non-functional engineered vessels density in SCID mice implanted with HUVECs alone and HUVECs with hMSCs (**a,c**) and HUVECs with hMSCs or 10T1/2 (**b,d**) (results are mean±SEM, n=3 in (**a,c**) and n=5 in (**b,d**), different batches of HUVECs were used in study (**a,c**) and (**b,d**) resulting in variation in vessel density between the two experiments). **e**, The hMSCs stabilized vascular network remained functional for more than 130 days *in vivo.* Scale bar, 100 μm.

Mesenchymal stem cells differentiate into perivascular cells in vivo and respond to endothelin-1 stimulation

To examine the spatial orientation of hMSCs in relation to the engineered vessels, hMSCs and HUVECs were transduced with retrovirus to express EGFP and DsRed-Express (DsRed), respectively. Retroviral transduction of EGFP did not affect the multipotent nature of hMSCs as assessed by their ability to differentiate into adipocytes and osteocytes (Figure 4.5). We then investigated whether EGFP-hMSCs implanted alone could self-assemble into functional vessels. We were not able to detect lumen formation or blood flow in EGFP-hMSCs derived cells if implanted alone even after 25 days *in vivo* (Figure 4.6a). In contrast, in mice co-implanted with DsRed-HUVECs and EGFP-hMSCs, EGFP-hMSCs were observed to elongate into thin slit structures and coalesced around the HUVECs derived vessels (Figure 4.6b-f). The number of interstitial EGFP-hMSCs decreased with time and most of them were associated with blood vessels by day 83 *in vivo* (Figure 4.6g). These EGFP-hMSCs-derived cells behaved like perivascular cells with a single layer of cells wrapping around the endothelial tube. Furthermore,

EGFP-hMSC-derived pericyte-like cells were positively stained for α-smooth muscle actin, sm22α and desmin (Figure 4.7a-h).

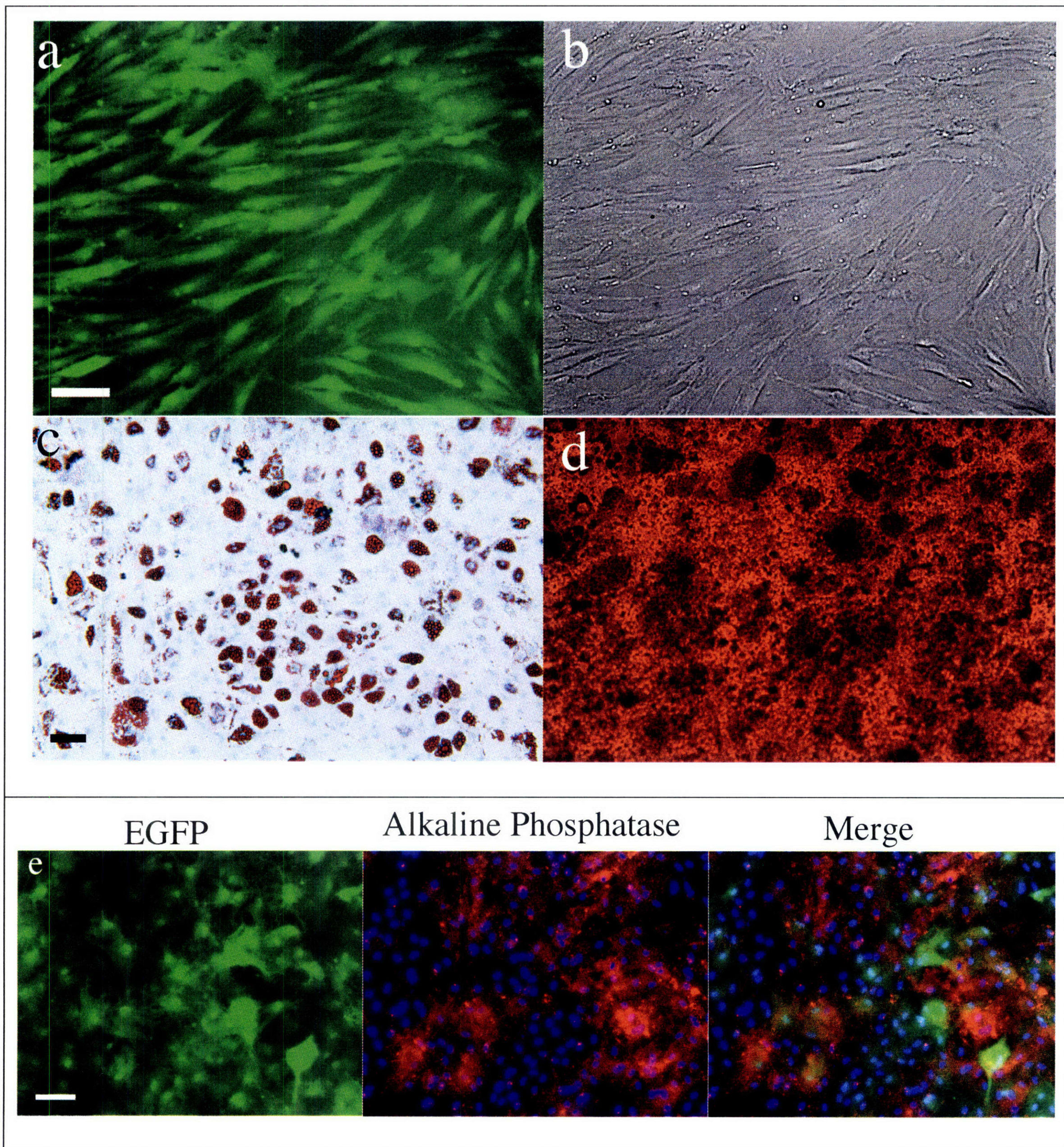
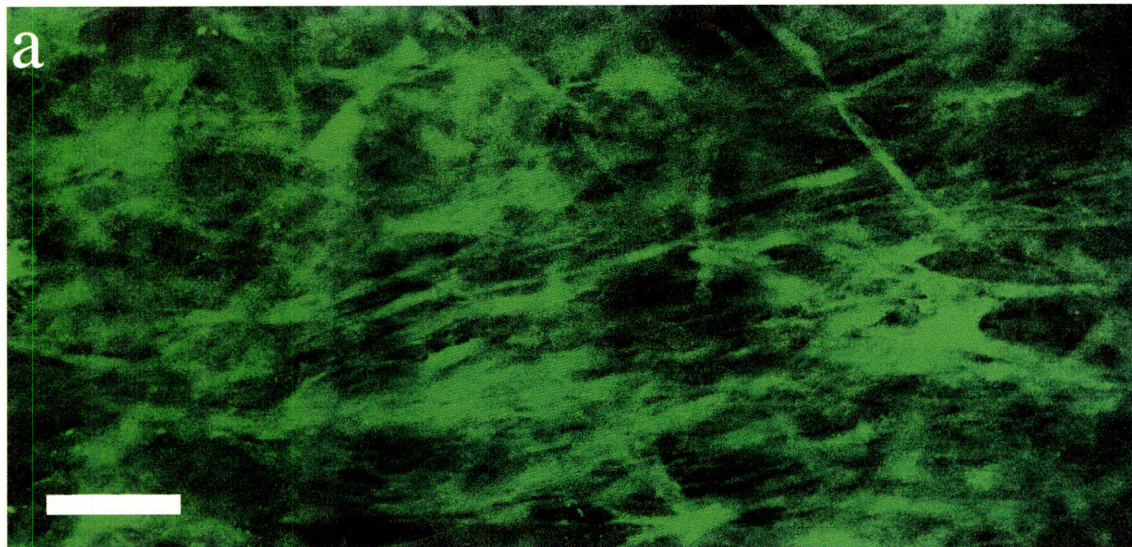
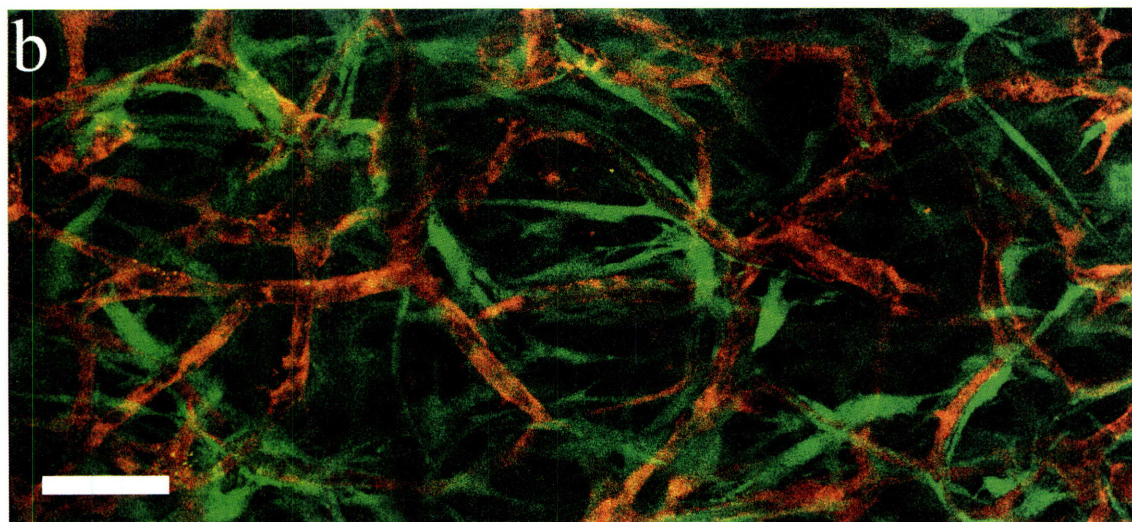
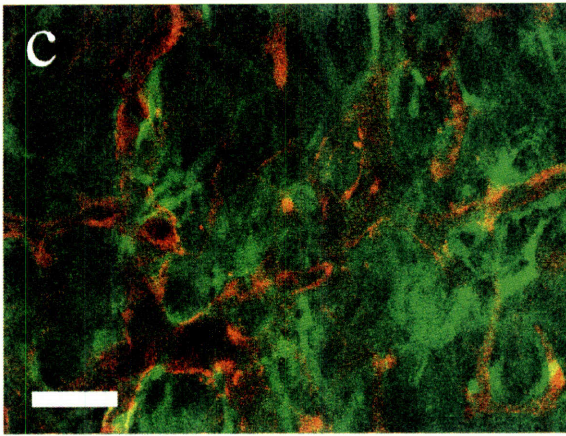


Figure 4.5. ***In vitro*** **differentiation assay for EGFP labeled hMSCs.**

EGFP transduced hMSCs were cultured in adipogenic or osteogenic medium for 30-40 days. **a,b,** Micrographs of undifferentiated hMSCs (**a**, EGFP; **b**, brightfield). ***c***, Oil Red O staining after adipogenic culture. ***d***, Alizarin red staining after osteogenic culture. ***e***, EGFP-hMSCs were stained with alkaline phosphatase for osteogenic differentiation. Scale bar, 100 μm.



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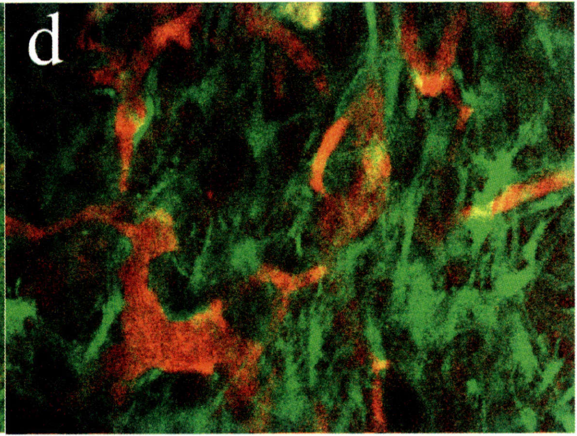
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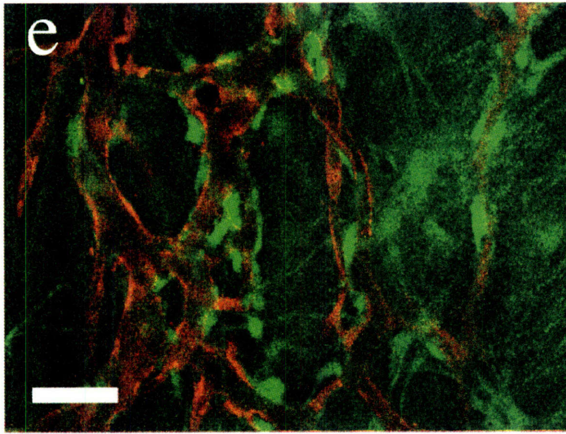
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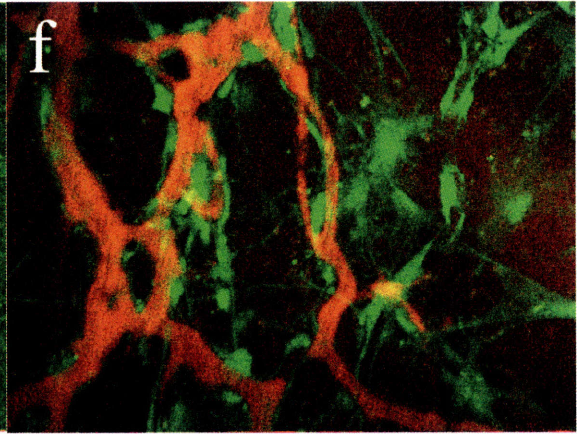
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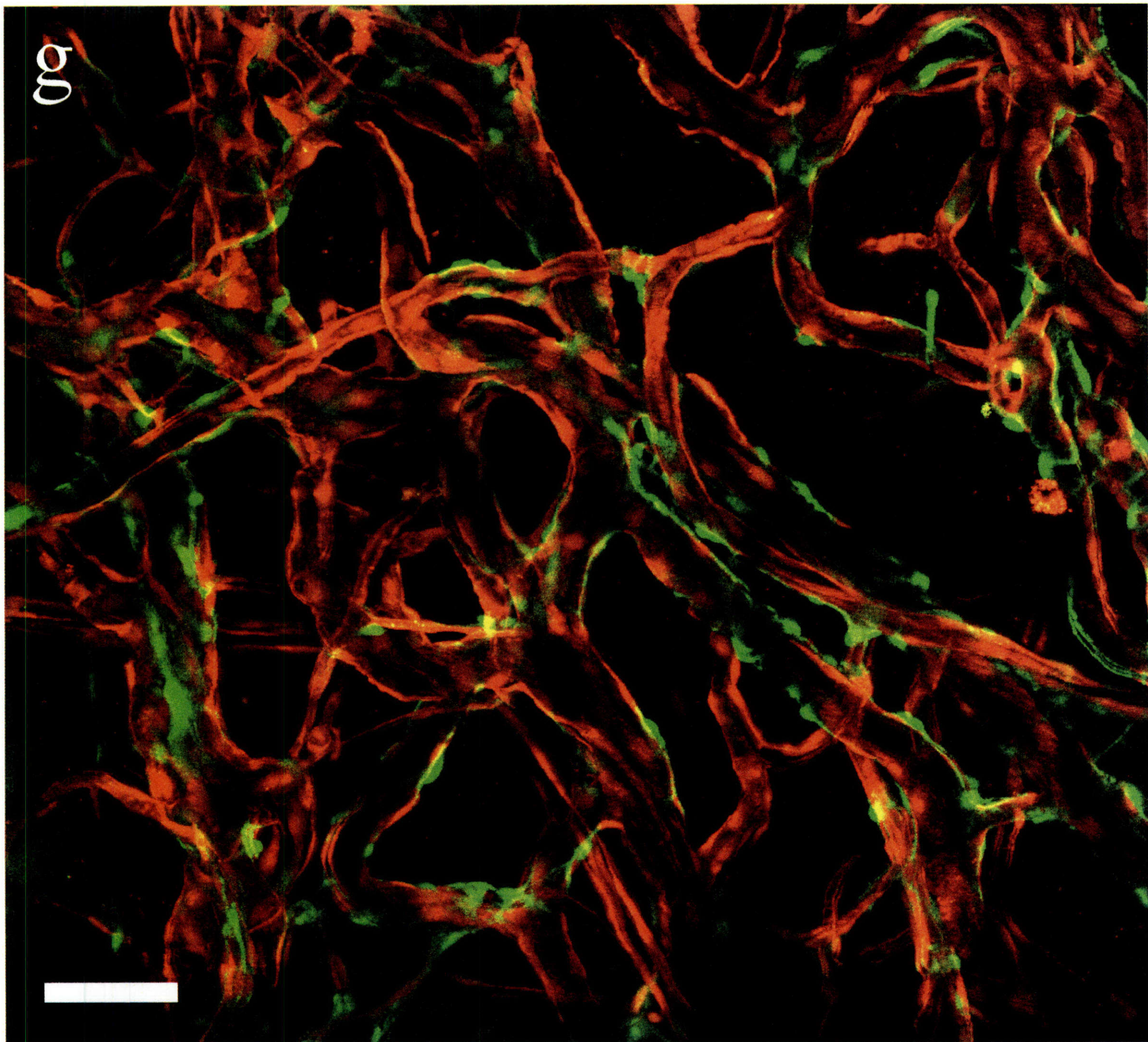
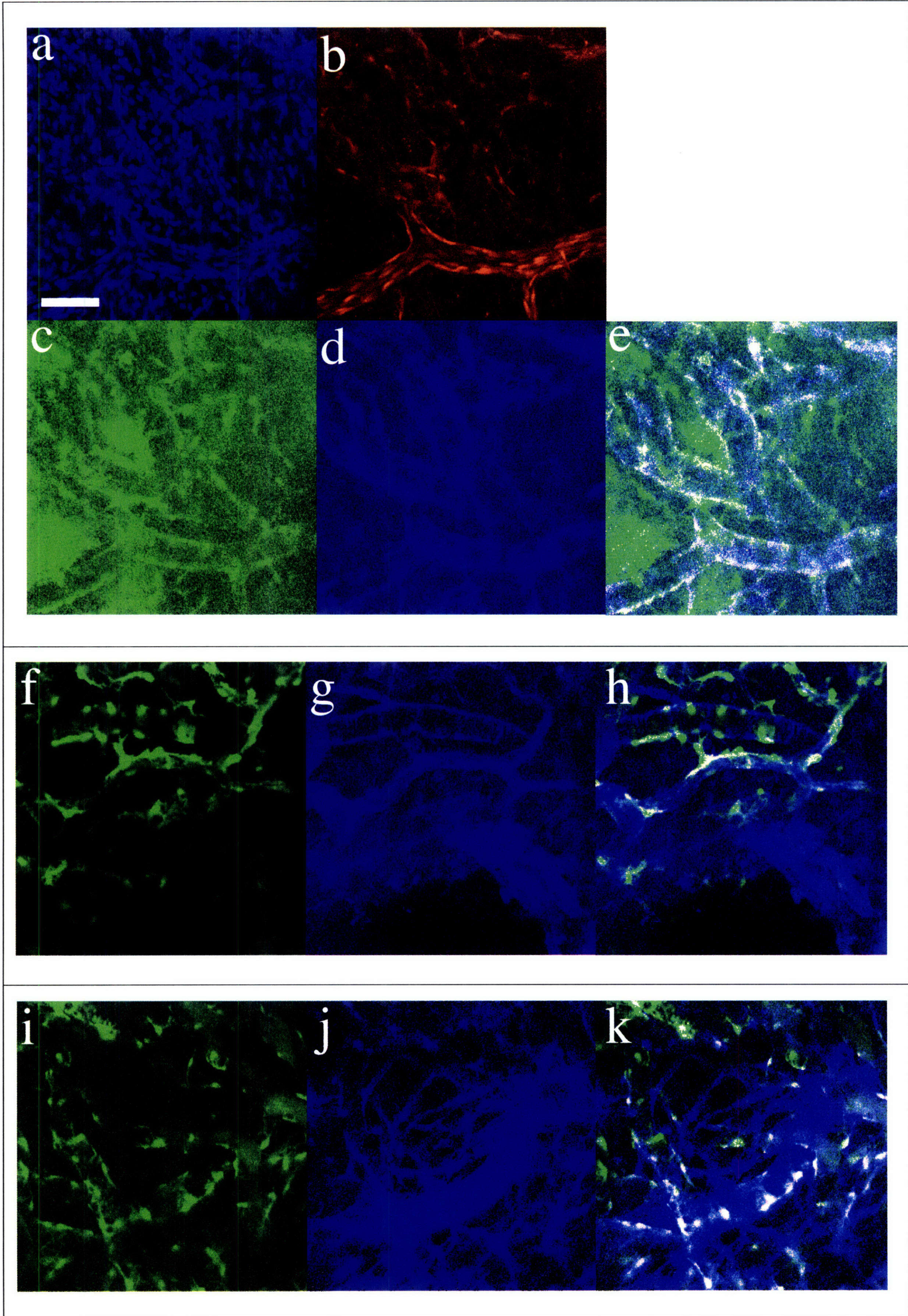


Figure 4.6. Intravital monitoring of EGFP-hMSCs in a tissue-engineered vessel model.

Fibronectin-collagen constructs with EGFP-hMSCs alone (**a**) or EGFP-hMSCs with DsRed-HUVECs (**b-g**) were implanted into cranial windows of SCID mice. Images were taken at different time points with MPLSM (**b**, day 7; **c,d**, day 19; **e,f**, day 31; **g**, day 83). Green, human mesenchymal stem cells expressing EGFP (**a-g**); red, HUVECs expressing DsRed-express fluorescent protein (**b-e,g**); red, functional blood vessels contrast-enhanced by rhodamine-dextran (**d,f**). Scale bar, **a,b,g** 100 μm; **c,d,e,f**, 50 μm


a
b
c
d
e
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g
h
i
j
k

Figure 4.7. Expression of smooth muscle cell markers in hMSC-derived cells incorporated into the tissue-engineered vessels.

Whole mount staining was performed for the extracted tissue-engineered vessel constructs. *a-h*, confocal microscopy images for DAPI staining, blue (**a**); DsRed-HUVEC, red (**b**);EGFP-hMSCs, green (**c,f,i**); α-smooth muscle actin staining, blue (**d**); SM22α staining, blue (**g**); and desmin staining, blue (**j**). Region of co-localized staining of EGFP and smooth muscle markers_are highlighted as white (**e,h,k**). a-e, f-h, i-j are the same location/construct. Scale bar, 100 μm

Pericytes have been shown to possess contractile abilities that directly regulate the blood brain barrier and local tissue perfusion[15]. To determine the function of the EGFP-hMSCs derived perivascular cells, we stimulated the blood vessels with endothelin-1, a potent contractile agent. The EGFP-hMSCs covered blood vessels constricted upon endothelin-1 stimulation (Figure 4.8a). The blood vessels narrowed within five minutes exposure to endothelin-1 and remained constricted at 20 minutes (Figure 4.8a-b). These experiments support the notion that EGFP-hMSCs have the capacity to differentiate into perivascular cells that can properly respond to physiological stimulation.

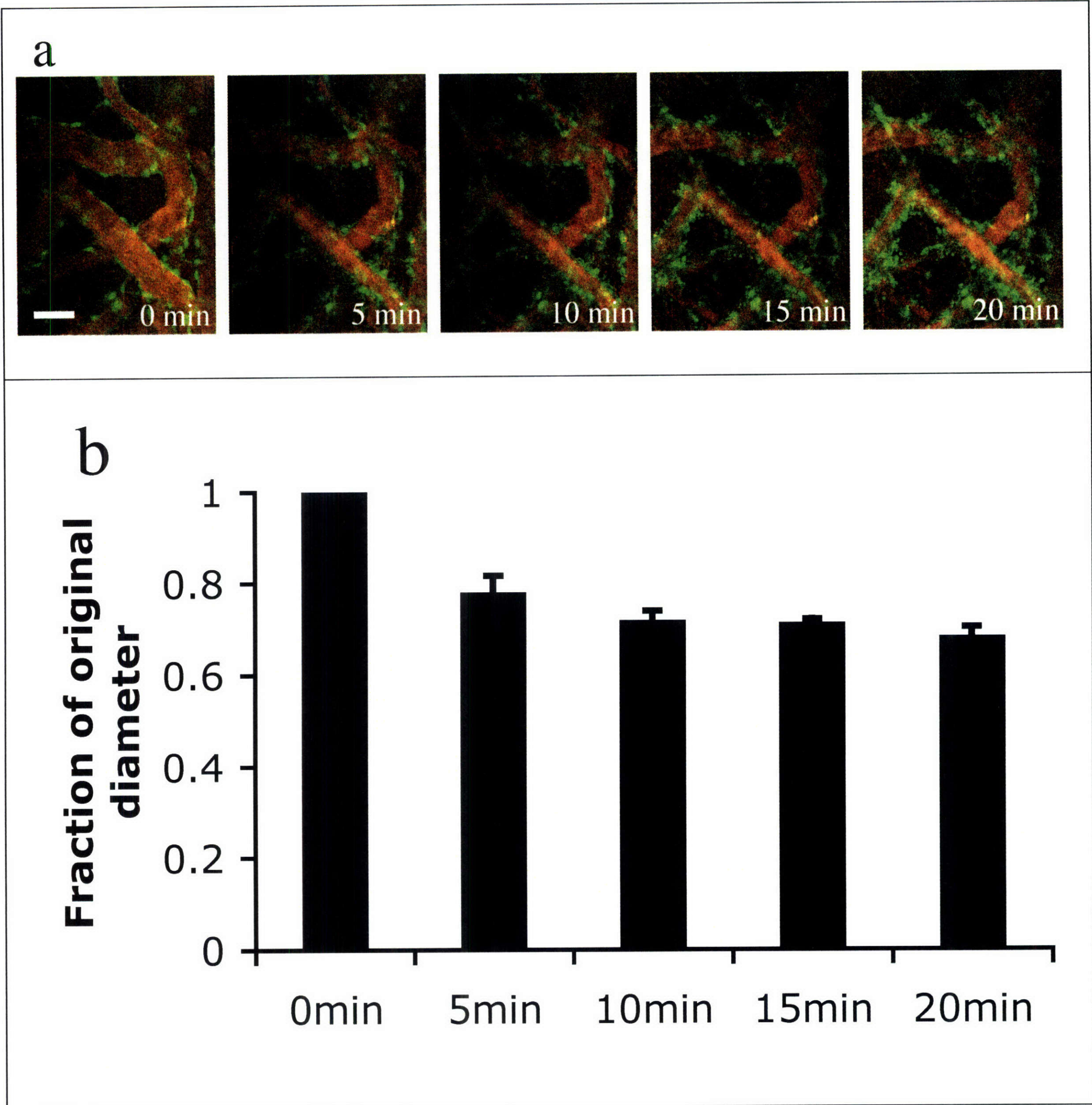
a
0 min
5 min
10 min
15 min
20 min
b
Fraction of original diameter
1
0.8
0.6
0.4
0.2
0
0min
5min
10min
15min
20min

Figure 4.8. Endothelin-1 stimulated vasoconstriction of the engineered blood vessels. HUVEC/hMSC-derived engineered blood vessels were superfused with 100nM of endothelin-1 to stimulate vasoconstriction *in vivo*. ***a*****,** representative multiphoton microscopy images during endothelin-1 superfusion. Green, EGFP-hMSCs; red, functional blood vessels contrast-enhanced by rhodamine-dextran. Scale bars, 50 μm. ***b*****,** The blood vessel diameter was quantified over a 20 minutes period (n=4). The value was expressed as a fraction of the original diameter. Scale bar, 50 μm

Discussion

Cellular therapy with hMSCs has great potential for use in regenerative medicine and is currently in clinical development. MSCs are being investigated in treatment of bone and cartilage defects, and injured myocardium after acute infarction [16]. The ability of MSCs to differentiate into several lineages of connective tissue including osteocyte, chrondrocyte, and adipocyte, is well documented[17]. Recent evidence has suggested that MSCs may also differentiate into endothelial and vascular smooth muscle cells[18]. In this report, we set out to clarify the cellular fate of implanted mesenchymal stem cells in participating in vasculogenesis *in vivo*. Our study showed that hMSCs did not spontaneously differentiate into endothelial tubes that were capable of carrying blood flow *in vivo*. On the other hand, hMSCs differentiated into and functioned as perivascular cells *in vivo* when co-implanted with endothelial cells. The hMSCs-derived perivascular cells were able to stabilize nascent blood vessels, and the engineered blood vessels remained functional for more than 130 days *in vivo*. Furthermore, the hMSC-derived

perivascular cells exhibited proper physiological function by constricting in response to stimulation by endothelin-1, an endogenous vasoconstrictive peptide.

It has long been known that endothelial cells and hematopoietic cells shared a common origin during development, but whether a common progenitor exists for endothelial cells and smooth muscle cells has been unclear[19]. The first hint of the existence of vascular progenitor cells came from an *in vitro* differentiation study with mouse embryonic stem (ES) cells[20]. Flk1 positive cells were generated and they were shown to have the potential to differentiate into either endothelial cells or smooth muscle cells depending on the growth factors in the culture medium. More recent evidence suggested such ES derived cells might even be capable of differentiating into cardiomyocytes[21, 22]. Besides the work with ES cells, other data also hinted at the close link between endothelial cells and smooth muscle cells. Under certain *in vitro* culturing conditions, endothelial cells and smooth muscle cells could take on one another's phenotypic markers, but it remains unclear whether this was due to an artifact of *in vitro* culture or a process of transdifferentiation[23]. Similarly, hMSCs have been induced to acquire endothelial and smooth muscle phenotype *in vitro*[18]. An unresolved question is whether the hMSCs-derived endothelial cells and smooth muscle cells can function properly with the acquired phenotype *in vivo*.

To systematically address these outstanding questions, we first began by evaluating the *in vitro* transcription of endothelial and smooth muscle markers. We were not able to detect endothelial markers in hMSCs at baseline and when cultured with a mouse endothelial cell line. The lack of endothelial marker expression suggested hMSCs had not

differentiated into endothelial cells with the chosen culturing condition. We could not discount the possibility that we had not identified the optimal differentiation protocol for endothelial cells. However, one difference between our study and previous studies was that we used PCR to detect endothelial markers, a more sensitive and selective method compared to the immunofluorescence based methods that were used in other studies [18].

We found that hMSCs expressed a number of smooth muscle markers. When co-cultured with endothelial cells, the cardiac and smooth muscle specific transcription co-activator myocardin was up-regulated more than 14 fold. The induction of myocardin was dependent on direct heterotypic cell-cell contact. Since TGF-β has been shown to be involved in smooth muscle differentiation and the activation of latent TGFβ requires cellular contact, we stimulated hMSCs with recombinant TGF-β1[11, 24]. The magnitude of myocardin induction induced by the given dose(s) of TGF-β1 is only a small fraction of the myocardin upregulation by endothelial cells. Interestingly, the extent of myocardin induction by TGF-β1 in hMSCs was similar to the results obtained with multipotent adult progenitor cells[25]. These data suggest other cell-contact mediators might be involved in smooth muscle cell differentiation[26].

Our data suggest hMSCs efficiently differentiate into perivascular cells when co-implanted with endothelial cells in tissue-engineered vessel model *in vivo*. The hMSC-engineered vessels may be an ideal model to study perivascular cell differentiation. While we had previously shown that 10T1/2 cells could function as perivascular cells in stabilizing engineered blood vessels, there are obvious drawbacks in using 10T1/2 cells in study of vessel maturation. 10T1/2 cells were shown to lack the expression of the

transcription co-activator myocardin even when stimulated by TGFβ1[27]. It was speculated that TGFβ stimulation of 10T1/2 cells might represent a myofibroblast response instead of true differentiation into vascular smooth muscle lineage[28]. In contrast, hMSCs expressed a number of smooth muscle markers including myocardin. These cells could be easily manipulated *in vitro*, and unlike smooth muscle cells extracted from discarded tissue, hMSCs are in a relative primitive state of differentiation. Compared to embryonic stem cells, hMSCs do not carry the potential risks of teratoma formation and difficulty in obtaining a pure population of lineage specific cells. hMSCs could potentially be genetically engineered for studying the vascular effects of embryonic lethal genes.

A previous study has investigated tissue-engineering bone by co-implantation of endothelial and hMSCs[29]. It was thought that secretion of BMP4 from endothelial cells stimulated the differentiation of hMSCs into osteocytes. In our study, we were not able to detect bone or cartilage formation *in vivo*. Most of the surviving hMSCs were associated with blood vessels, suggesting that heterotypic interaction between hMSC and endothelial cells provided a survival advantage for both endothelial cells and hMSC-derived perivascular cells. One potential reason for the lack of bone or cartilage differentiation is that EGFP transduction affected the multipotential of hMSCs. The EGFP-labeled hMSCs were able to differentiate into both osteocytes and adipocytes *in vitro*, but it is unclear how EGFP transduction might affect cell differentiation *in vivo*. Another reason for the lack of bone formation could be attributed to the mechanical property of the tissue-engineered construct. Depending on the stiffness of the matrix, hMSCs could spontaneously differentiate into different cell lineages[30]. In our study, we

used a matrix (Type I collagen/fibronectin) at a concentration with low stiffness and this might have prevented osteocyte differentiation. This finding is especially important in the setting of revascularization therapy with hMSCs since ectopic bone formation has potentially untoward effects.

In conclusion, we demonstrated that hMSCs specifically differentiated into perivascular cells that could stabilize nascent blood vessels and responded appropriately to vasoactive stimuli. hMSCs provide as an attractive cellular platform for vascular engineering and for studying perivascular cell differentiation.

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Chapter 5: Genetically Engineered Endothelial Cells to overexpress PDGF-BB

Abstract

Therapeutic revascularization induced by exogenous angiogenic growth factors or endothelial cells has yet to demonstrate efficacy in the clinic. Injection of angiogenic growth factors often produces unstable and abnormal blood vessels, and implantation of vascular endothelial cells results in unstable vascular network due to a lack of perivascular cells. Here, we tested a combination of growth factor and cellular approaches. To enhance recruitment of perivascular cells, human umbilical vein endothelial cells were genetically modified to overexpress PDGF-BB. PDGF-BB-overexpressing endothelial cells promoted the proliferation and migration of perivascular cells *in vitro*. When the endothelial cells were implanted alone *in vivo*, endothelial cells that only expressed EGFP formed transient blood vessel network that regressed completely by day 30. In contrast, PDGF-BB overexpression enhanced the survival of endothelial cells *in vivo,* however the vessel network failed to anastamose to the host circulation. Co-implantation of PDGF-BB overexpressing endothelial cells with smooth muscle precursor cells unexpectedly resulted in the rapid regression of the endothelial cells *in vivo*. These results suggest angiogenic growth factors, endothelial cells and smooth muscle cells could interact negatively and their use in combination should be tested *in vivo* for unforeseen effects.

Introduction

Angiogenesis, the growth of new blood vessels, is critical during tissue repair and regeneration[1]. In cases of insufficient or impaired angiogenesis, the injured tissue remains dysfunctional and may suffer from irreversible damage[1]. To this end, two broad approaches have been employed for enhancing angiogenesis: (i) delivery of pro-angiogenic genes (by gene transfer) or proteins (by bolus injection or controlled release devices) for endothelial and perivascular cell recruitment[2], and (ii) delivery of endothelial cells alone or together with perivascular cells[3].

Therapeutic angiogenesis requires temporally and spatially orchestrated delivery of required growth factors to achieve a functional and durable vasculature. A number of studies have shown that a single angiogenic factor may not be able to create a mature and stable vasculature. For example, injection of an adenoviral vector expressing VEGF into normal tissue leads to highly disorganized, leaky and hemorrhagic vessels[4]. Furthermore, VEGF can potentiate inflammation by increasing the expression of adhesion molecules or the release of chemokines[5-8]. The angiogenic vessels are also highly unstable and regress upon removal of the angiogenic stimuli. Vascular maturation and stabilization requires perivascular cells[2, 9]. Perivascular cells such as vascular smooth muscle cells and pericytes are thought to provide structural integrity to the blood vessels, lay down the extracellular matrix, and provide necessary survival factors to the endothelial cells[2].

One potential way to enhace vessel maturation is by sequentially delivering VEGF and PDGF-BB[10]. Such strategy was shown to induce mature vascular networks, but the

time required for host endothelial cell infiltration by this strategy might limit the amount of ischemic tissue rescued. Another limitation with angiogenic therapy is that vascular response to angiogenic factors is reduced with age, hyperglycemia and atherosclerosis[11]. Finally, patients with diabetes mellitus or elevated homocysteine may exhibit impaired angiogenic response[12, 13].

Alternatively, vascular cells can be directly injected or implanted in the ischemic site. We have previously shown that human umbilical cord vein endothelial cells (HUVECs) implanted in a collagen gel in severe combined immunodeficient (SCID) mice could form functional engineered vessels, but the vessels were only transiently perfused, did not recruit adequate number of perivascular cells from the host tissue, and regressed within a few weeks. In contrast, co-implanting HUVECs with mouse pericyte precursor cells allowed the formation of stable and functional engineered vessels[14]. Thus, providing exogenous perivascular cells overcame the inability of the implanted endothelial cells to recruit host perivascular cells within a critical timeframe for the vessels to become mature and stabilized. Nevertheless, translation of this approach will require sources of both endothelial and perivascular cells in sufficient numbers for cell-based therapy of ischemic tissues. So far, such sources remain elusive, despite promising but inconclusive results obtained with bone marrow-derived precursor cells or embryonic stem cells[15, 16].

To this end, here we tested a combination of these two approaches. We engineered endothelial cells to overexpress PDGF-BB, a mitogen and a chemoattractant for perivascular cells. We hypothesized that by increasing the levels of PDGF-BB expressed

by endothelial cells, it may be possible to hasten the recruitment of perivascular cells from the host in an appropriate timeframe to stabilize the nascent blood vessels.

Material and Method

Cell culture

Human umbilical cord vein endothelial cells were obtained from Center of Excellence in Vascular Biology, Brigham and Women's Hospital, Boston and maintained in Endothelial Growth Medium (EGM) (Cambrex, East Rutherford, NJ). 293ET packaging cells were a kind gift from Dr. Brian Seed (MGH, Boston). 10T1/2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Retrovirus Plasmid Construction

The retrovirus vector, PBMN-I-EGFP was kindly provided by Dr. Gary Nolan (Stanford, CA). Human PDGF-B cDNA was purchased from InvivoGen (San Diego, CA). Full length PDGF-B cDNA was subcloned into the retroviral vector by first using PCR to add BamHI and NotI restriction sites with the following set of primers: forward primer-GAATTCGGATCCATGAATCGCTGCTGGGCG and reverse primer-AAGCTTGCGGCCGCCTAGGCTCCGAGGGTCTC. The PCR product was cut with BamHI and NotI restriction enzymes and then inserted into the multicloning sites of PBMN-I-EGFP vector.

Retrovirus Packaging and Transduction

For retrovirus packaging, the plasmids of PBMN-I-EGFP, Gag-pol and VSVG (15μg, 7 μg, 5 μg respectively) were mixed and co-transfected into 293ET cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA) per manufacturer's protocol. After overnight incubation, the 293ET cells were washed with PBS and then given DMEM 10%FBS. The next day, the supernatant containing retrovirus was collected and fresh media was added; this step was repeated for 3 more times. After the supernatant was collected, it was passaged through a 0.45 μm filter (Whatman, Brentford, UK) and was either used immediately for infection or kept at –80°C. For the transduction of HUVECs, the supernatant was first diluted 1:1 with fresh EGM and supplemented with polybrene (8μg/ml). The diluted supernatant was then added to subconfluent monolayer of HUVECs and allowed to incubate for 4 hours. Fresh EGM medium was exchanged at the end of the incubation period and this step was repeated 2-3 times on consecutive days. After 2 to 3 rounds of infection, more than 90% of HUVECs expressed the gene of interest as assessed by the expression of EGFP. HUVECs expressing EGFP only or co-expressing PDGF-BB and EGFP will be referred to as HUVEC-EGFP and HUVEC-PDGF-BB hereafter.

Western Blot for PDGF-R and VEGFR-2 phosphorylation

For PDGF-R phosphorylation, 10T1/2 cells were starved overnight in 0.5% FBS. The cells were then exposed to conditioned media from HUVEC-PDGF-BB for 5 minutes to stimulate the phosphorylation of PDGF receptor. The cells were washed three times with PBS containing 1mM NaVO4 and 50mM NaF and then were lysed with RIPA buffer containing protease inhibitor and phosphatase inhibitor. Immunoprecipitation was

performed by the addition of 5 ml of anti-PDGFRbeta antibodies (#3162) (Cell Signaling, Danvers, MA) to cell lysates containing 0.5mg of total protein and was incubated overnight at 4° C. The next day, the antigen-antibody conjugate was precipitated with Agarose A/G. Phosphorylated PDGFR-beta was immunoblotted with an anti-phosphotyrosine antibody conjugated with HRP (clone 4G10) (Upstate, Charlottesville, VA) and total PDGFR-beta was immunoblotted with an anti-PDGFR-beta antibody (#3162) (Cell Signaling). For VEGFR-2 phosphorylation, EGFP-HUVEC and PDGFBB-HUVEC cells were incubated in serum-free medium 1 hr and then incubated with or without 50ng/ml VEGF for 2~5 min. Then the cells were scraped from plates, pelleted, and resuspended in lysis buffer. 60 μg of protein per sample was separated on a 4-15% acrylamide gradient gel (Bio-Rad). The expression of phopho-VEGFR2, VEGFR2, and Actin were detected by polyclonal antibodies against VEGF receptor 2 (1:1000) and phosphorylated VEGF receptor 2 (1:2000) (Cell Signaling), and by monoclonal antibodies against Actin (1:5000) (Sigma).

Cell proliferation assay

The activity of the ectopically expressed PDGF in HUVEC was assayed by testing its ability to induce the proliferation of 10T1/2 cells. Briefly, confluent monolayer of HUVECs that had been transduced with PDGF-BB or EGFP were incubated for 12 hours with DMEM containing 1% FBS. The conditioned medium was filtered with a 0.22mm filter and stored in –80°C until ready for use. 10T1/2 cells were plated in a 96 well plate at a density of 5,000 cells/well. The next day, the medium in each well was removed and replaced with 100ml of HUVECs conditioned medium and allowed to incubate for 24 hr.

After 24 hours, 20 ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20mg/ml, Sigma-Aldrich, St. Louis, MO) was added to each well and the plate was incubated further for 2 hours. The medium was then removed and replaced with 100ml of dimethyl sulfoxide (DMSO). The absorbance was read with a colorimetric plate reader at 550-655mm wavelength.

Protein measurements by enzyme-linked immuno-sorbent assay (ELISA)

Secreted and cell retained PDGF-BB in HUVECs were quantified with ELISA kit (R&D Systems). For measuring secreted PDGF-BB, supernatant were collected and filtered through a 0.22μm filter before measurement. For cell retained PDGF-BB, HUVECs were first washed with PBS, and then incubated with 1M solution of NaCl for 30 minutes on ice. The salt solution disrupts the charge-charge interaction between PDGF-BB and the extracellular matrix. The salt solution was then filtered with a 0.22μm filter before measurement.

Transwell migration assay

Cell migration was assessed using Falcon HTS FluoroBlok 24-well inserts (BD Biosciences, San Jose, CA)with 3-μm pores. EGFP-10T1/2 cells (2×10^4) suspended in 250-μl BME (Invitrogen) with 0.5% FBS were placed inside each insert and 5×10^4 per well HUVECs suspended in 800 μl EGM (Cambrex) were plated on a 24-well plate. The cells were kept overnight. The next day, the cell culture media of both HUVECs and 10T1/2 cells were changed to BME with 0.5% FBS, and then the inserts were placed in the respective wells. At 4, 8, and 12 hours interval, the bottom of each insert was imaged

in fluorescence using an inverted fluorescence microscope (Olympus IX70, Center Valley, PA) equipped with a motorized stage and motorized filter wheel (Improvision Inc, Lexington, MA). For endothelial cells migration, EGFP- and PDGFBB-HUVEC cells were cultured in Eagle's MEM with 1% FBS for overnight. Cells (6×10^4) were plated in each insert coated with fibronectin. The filter was placed over a bottom chamber containing 5ng/ml VEGF (purchased from R & D systems, Minneapolis, MN and Collaborative Biomedical Products, Bedford, MA) in 0.1% bovine serum albumin (BSA) in Eagle's MEM medium. The assembled chemotaxis chamber was incubated for 3 h at 37°C with 5% CO_2 to allow cells to migrate through the fibronectin-coated polycarbonate filter. Transmigrated cells were quantified using ImageJ (http://rsb.info.nih.gov/ij/) by using threshold function and measuring the area covered by the migrated cells.

Tissue engineered blood vessel constructs

One million endothelial cells and $2x10^5$ 10T1/2 cells were suspended in 1 ml solution of rat-tail type 1 collagen (1.5 mg/ml) (BD Biosciences) and human plasma fibronectin (90 μg/ml) (Sigma) in 25 mM Hepes (Sigma) buffered EGM medium at 4°C. The pH was adjusted to 7.4 by using 1N NaOH (Fisher Science, NJ). The cell suspension was pipetted into 12-well plates and warmed to 37°C for 30 minutes to allow polymerization of collagen. Each solidified gel construct was covered by one ml of warmed EGM medium. After one day culture in 5% CO_2, a skin puncher was applied to create circular disk-shape pieces of the construct (4-mm diameter), and they were implanted into the cranial windows in severe combined immunodeficient (SCID) mice[14, 17]. Multiphoton laser-scanning microscopy was used to visualize and quantify the morphological changes in

EGFP-expressing HUVECs. The perfused vessels were highlighted by tail vein injection of 1% tetramethylrhodamine-labeled dextran (MW 2,000,000), indicating the formation of functional engineered vessels[14, 17].

Cord formation assay was performed identically as above except that the tissue engineered construct was allowed to culture *in vitro*. Images of the tissue-engineered construct were taken randomly at 3, 7, and 12 days with an inverted fluorescence microscope. The formation of vessel-like network was quantified by thresholding the image and measuring the area of vessel-like structures with ImageJ.

TdT-mediated dUTP Nick End Labeling (TUNEL) Assay of HUVEC

In situ TUNEL staining (ApopTag peroxidase *In situ* detection kit, Chemicon, Temecula, CA) was used according to manufacturer's instructions to identify apoptotic cells. EGFP-HUVEC and PDGFBB-HUVEC cells were cultured in serum-free medium, in complete medium, or under hypoxia condition (1% O_2-5% CO_2-balance N_2 was used).

In vitro tube formation assay

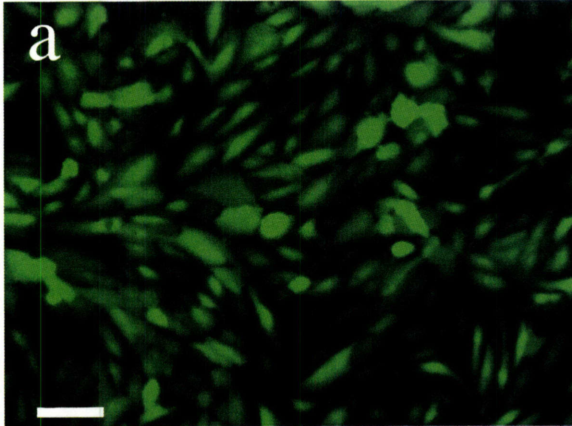
In vitro tube formation was studied using previously described procedures*. Matrigel (Collaborative Biomedical Products, Bedford, MA) was diluted with EC medium in 1:1 ratio. Next, 60 µl of the solution were added to each well of a 96-well plate and allowed to form a gel at 37°C for 30 min. EGFP-HUVEC and PDGFBB-HUVEC (20,000 cells) cells in 200 µl of complete medium were subsequently added to each well and incubated for overnight at 37°C in 5% CO_2. Under these conditions, both EGFP- and PDGFBB-HUVEC cells form delicate networks of tubes and were fully developed after 16 h.

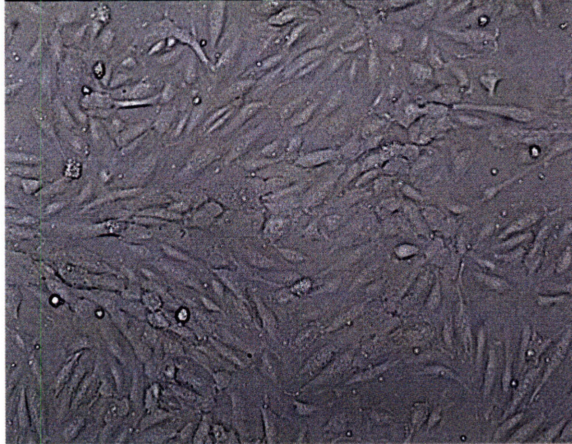
Results

Efficient retroviral transduction of HUVECs

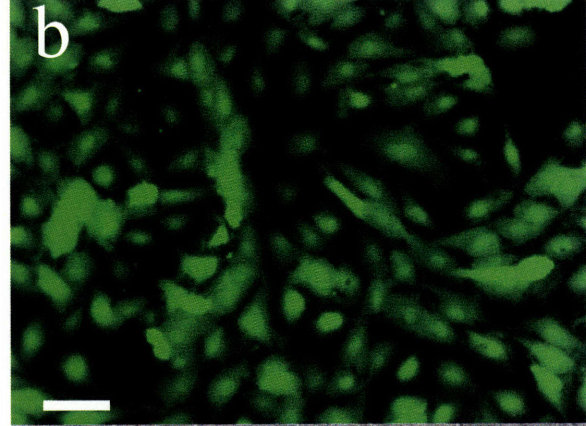
HUVECs were transduced with the retrovirus to express either PDGF-BB or enhanced green fluorescent protein (EGFP) alone. We utilized a bicistronic retroviral vector that allows for the expression of both the gene of interest and EGFP. This provides a simple method to monitor the transduction efficiency of retrovirus by quantifying the percentage of EGFP expressing cells. After two to three rounds of retrovirus transduction, close to 100% of HUVECs expressed EGFP (Fig. 5.1a,b). Next, we quantified the secretion of ectopically expressed PDGF-BB with ELISA. In HUVECs overexpressing the PDGF-BB gene, the amount of secreted PDGF-BB protein in the culture media was significantly higher than the control that had been transduced to express EGFP only (223.2 ± 1.1 versus 7.6 ± 2.2 μg of PDGF-BB) (Fig. 5.1c). To examine cell-associated PDGF-BB, HUVECs were washed with salt solution to disrupt the charge-charge interaction between PDGF-BB and the extracellular matrix. The amounts of cell-associated PDGF-BB were 98.2 ± 11.3 μg and 6.9 ± 0.2 μg for HUVEC-PDGF-BB and HUVEC-EGFP, respectively (Fig. 5.1d). In the case of HUVEC overexpressing PDGF-BB, a higher ratio of soluble to cell associated PDGF-BB was observed, possibly due to saturation of binding sites on the cell surface (Fig 5.1e).

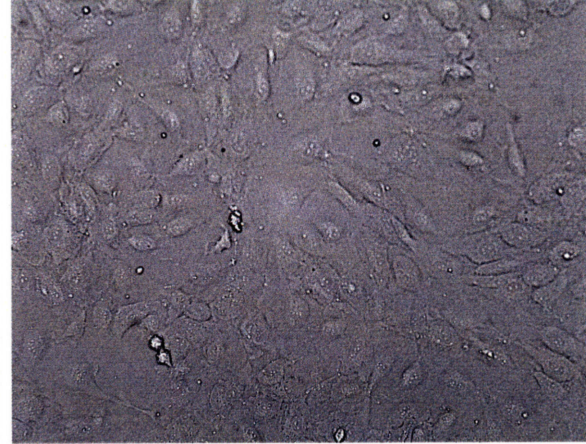
EGFP





PDGF-BB





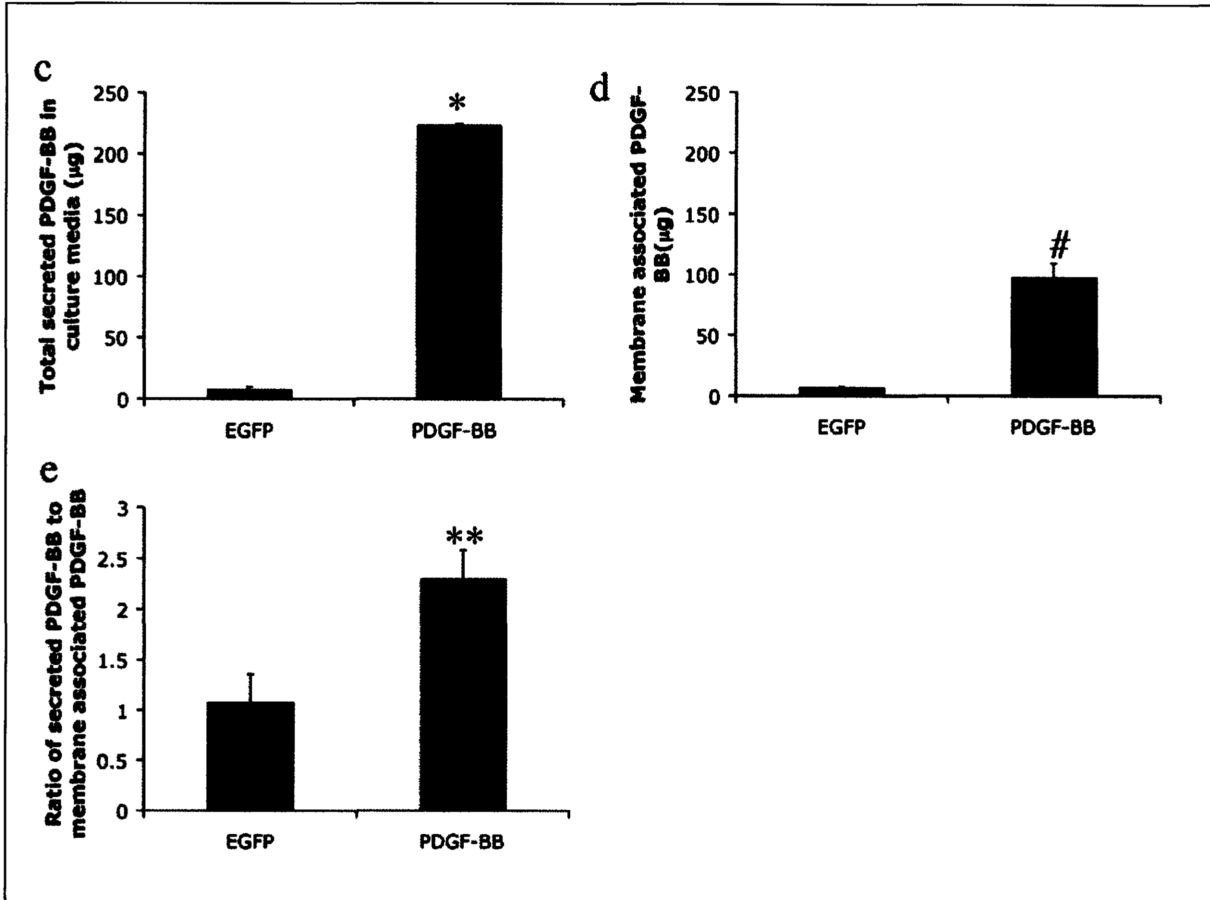
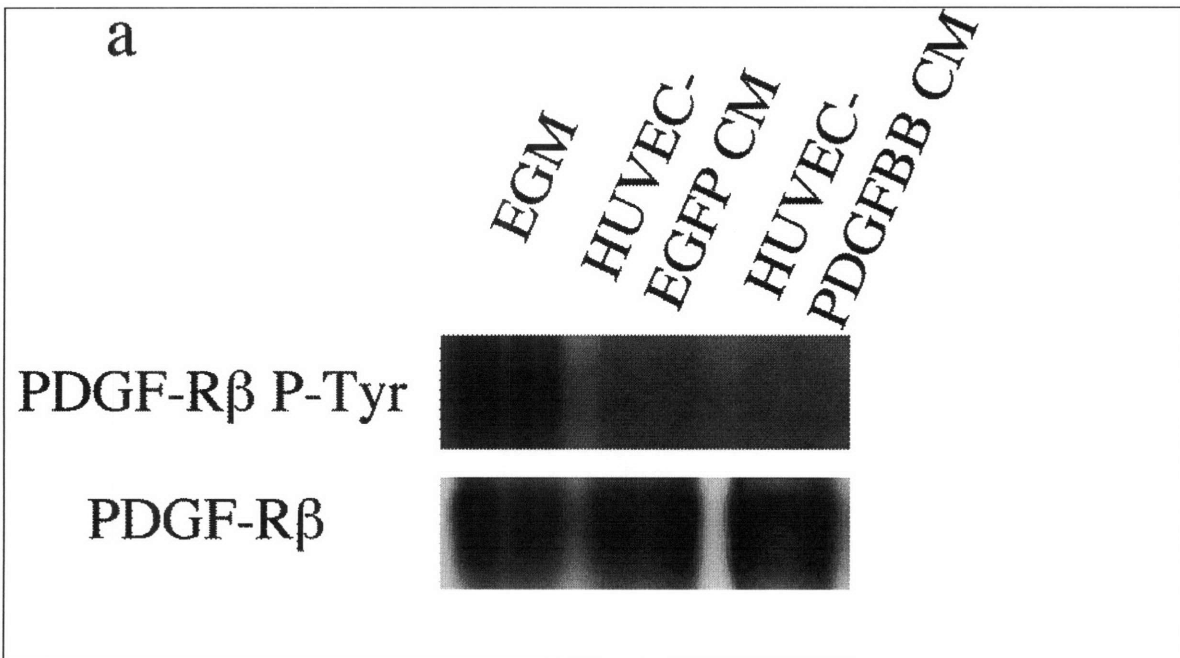


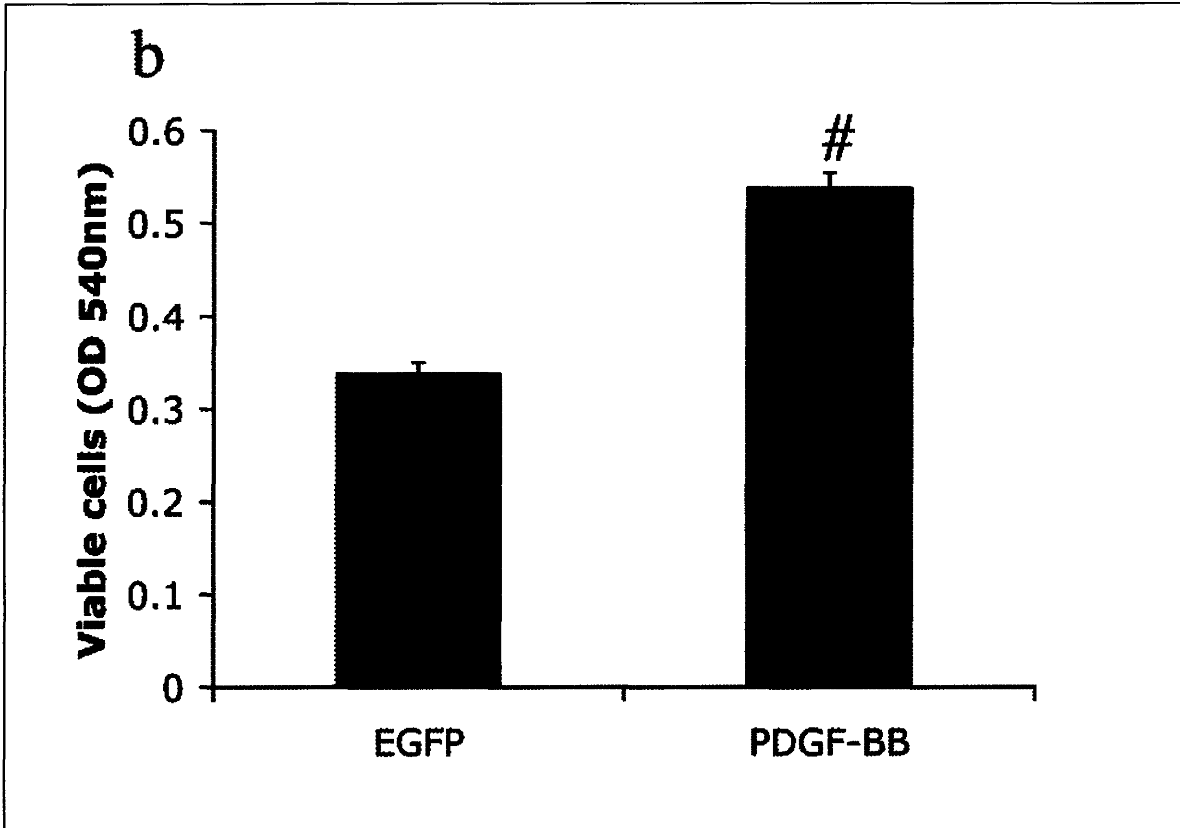
Figure 5.1. Retroviral transduction of HUVECs to overexpress PDGF-BB

HUVECs were transduced with retrovirus either to express EGFP only (a) or to co-express EGFP and PDGF-BB (b). Fluorescence image of EGFP expressing cells (green) with corresponding brightfield image demonstrate that almost all of the HUVECs expressed EGFP. The amount of PDGF-BB secretion in the culture media (c) and bound to cell surface (d) was quantified by ELISA. Ratio of secreted PDGF-BB in culture media to that on the cell surface showed more PDGF-BB was release in the media in endothelial cells overexpressing PDGF-BB (d). Scale bar, 50 µm * $p<0.0005$, # $p<0.05$, ** N.S.

Conditioned medium from PDGF-BB-overexpressing HUVECs enhances proliferation and migration of 10T1/2

To assess the function of the ectopically expressed PDGF-BB, we exposed serum starved 10T1/2 cells, a mouse mesenchymal precursor cell line, to HUVEC conditioned media. We previously showed that 10T1/2 cells could function as perivascular-like cells *in vivo*[14]. HUVEC-PDGF-BB conditioned medium stimulated significantly more phosphorylation of the PDGF-Rβ and higher proliferation of 10T1/2 cells than the HUVEC-EGFP conditioned medium (Fig. 5.2a,b). Since PDGF-BB is known to induce recruitment of perivascular cells, we next examined whether overexpression of PDGF-BB in HUVECs could promote the migration of 10T1/2 cells *in vitro*. Using Boyden chamber migration assay, we found a higher number of 10T1/2 cells migrated across a transwell in the HUVEC-PDGF-BB group than the controls over a 24 hours period (Fig. 5.2c). These findings demonstrated that ectopically expressed PDGF-BB was functionally active and that the overexpression of PDGF-BB enhanced the migration of 10T1/2 cells in *vitro*.

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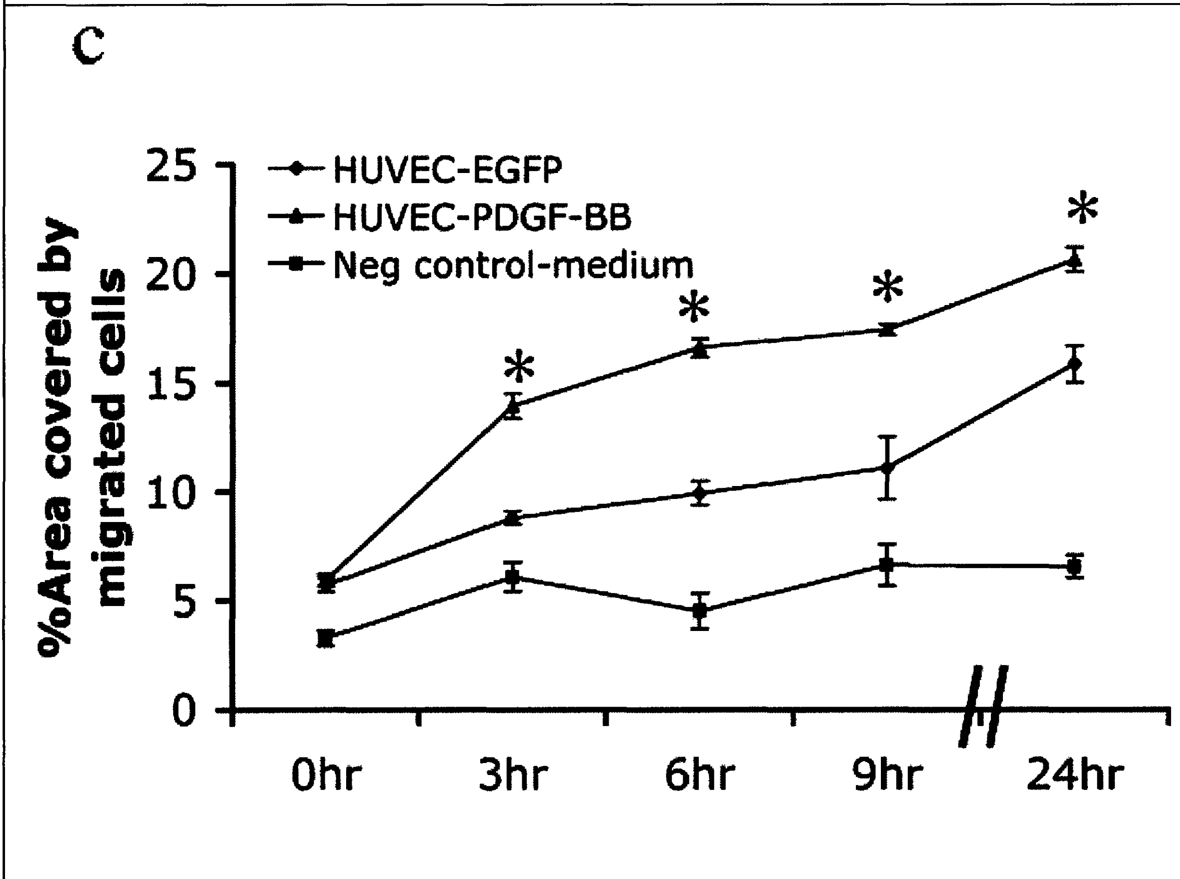
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0hr
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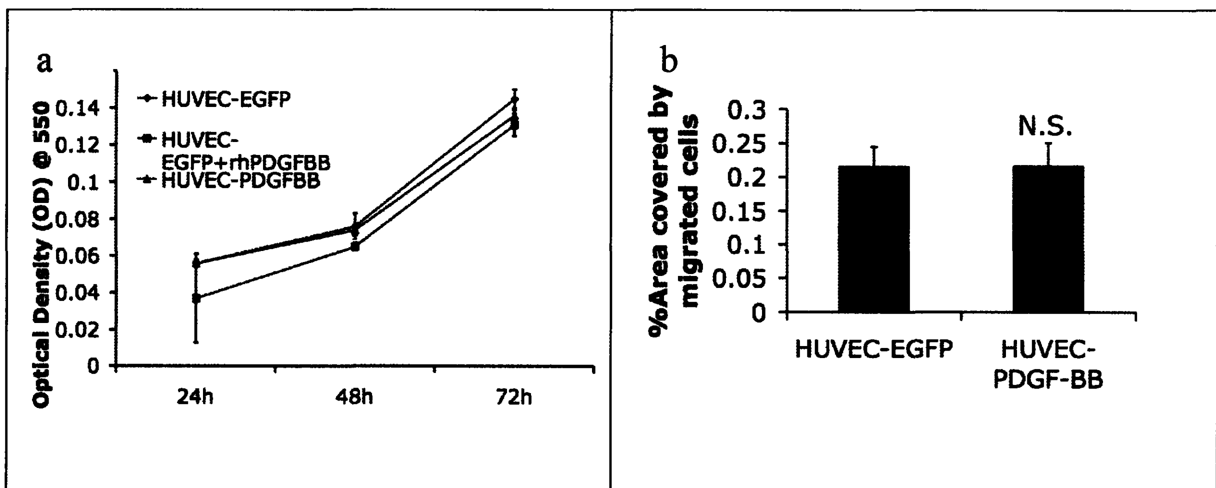
Figure 5.2. *In vitro* activity of PDGF-BB on perivascular cells proliferation and migration

HUVEC conditioned media (CM) stimulated the phosphorylation of PDGF-Rβ in 10T1/2 cells (a). Endothelial growth medium was used as control. Conditioned medium from HUVECs overexpressing PDGF-BB enhanced the proliferation of 10T1/2 cells as quantified by MTT assay compared to mock transfected HUVECs (EGFP only) (b). Transwell migration assay was performed to assess HUVECs induced migration of 10T1/2 cells (c) # $p<0.0001$, * $p<0.005$

We next checked whether ectopic expression of PDGF-BB altered the phenotype of HUVECs. The rate of endothelial cell proliferation showed no difference among HUVEC-EGFP, HUVEC-PDGF-BB, and HUVEC-EGFP supplemented with recombinant PDGF-BB (Fig 5.3a). HUVEC-EGFP and HUVEC-PDGF-BB also responded similarly to VEGF stimulated migration (Fig 5.3b). Finally, PDGF-BB overexpression in HUVECs did not affect the cells ability to form vessel-like structures on Matrigel (Fig 5.3c).



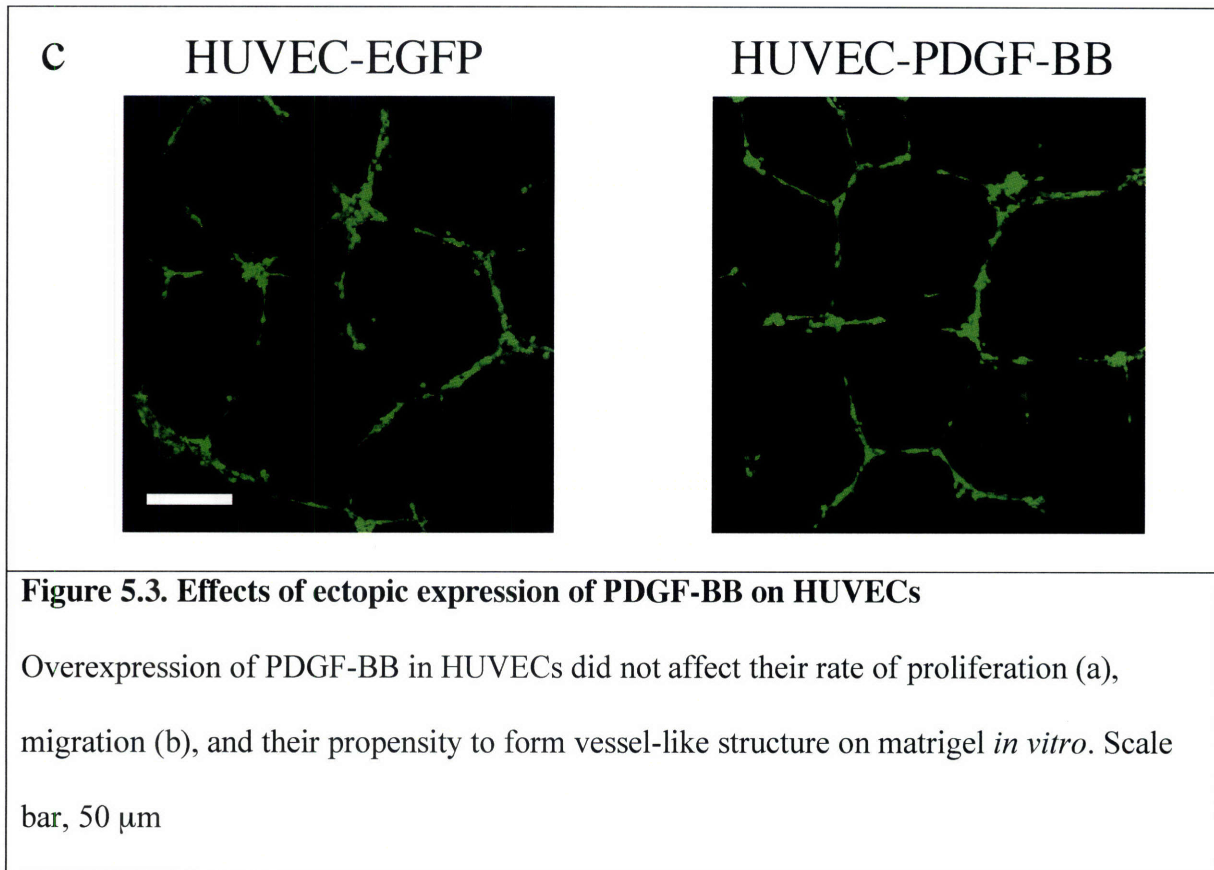


Figure 5.3. Effects of ectopic expression of PDGF-BB on HUVECs

Overexpression of PDGF-BB in HUVECs did not affect their rate of proliferation (a), migration (b), and their propensity to form vessel-like structure on matrigel *in vitro*. Scale bar, 50 μm

PDGF-BB overexpression enhanced HUVECs survival in vivo

To create engineered vessels, we implanted HUVEC-PDGF-BB cells in a fibronectin/collagen matrix onto cranial windows in SCID mice. For control, we implanted HUVEC expressing only EGFP. We tracked the functional vasculature inside the gels by multi-photon laser scanning microscopy and monitored the kinetics of blood vessel formation. In the group implanted with HUVEC-EGFP cells, the endothelial cells became elongated and interconnected, forming a mesh-like network on day 3 (Figure 5.4a). Multiple vacuoles were observed to have formed within the endothelial cells. By day 7, some these vacuoles had coalesced into luminal structure (Fig 5.4). On day 18, blood flow was seen within the lumen of the endothelium suggesting that the engineered

blood vessels had formed functional connection to the host circulatory network (Fig 5.4). Similar to previous result, engineered blood vessels derived from HUVEC only were unstable and most of the vessels had regressed by day 30 (Fig 5.4).

In contrast, in animals implanted with HUVEC-PDGF, the endothelial cells persisted even at day 30. Similar to the control group, HUVEC-PDGF-BB formed a mesh-like network with lumen inside it. However, few of these vascular structures were perfused (Fig 5.4).

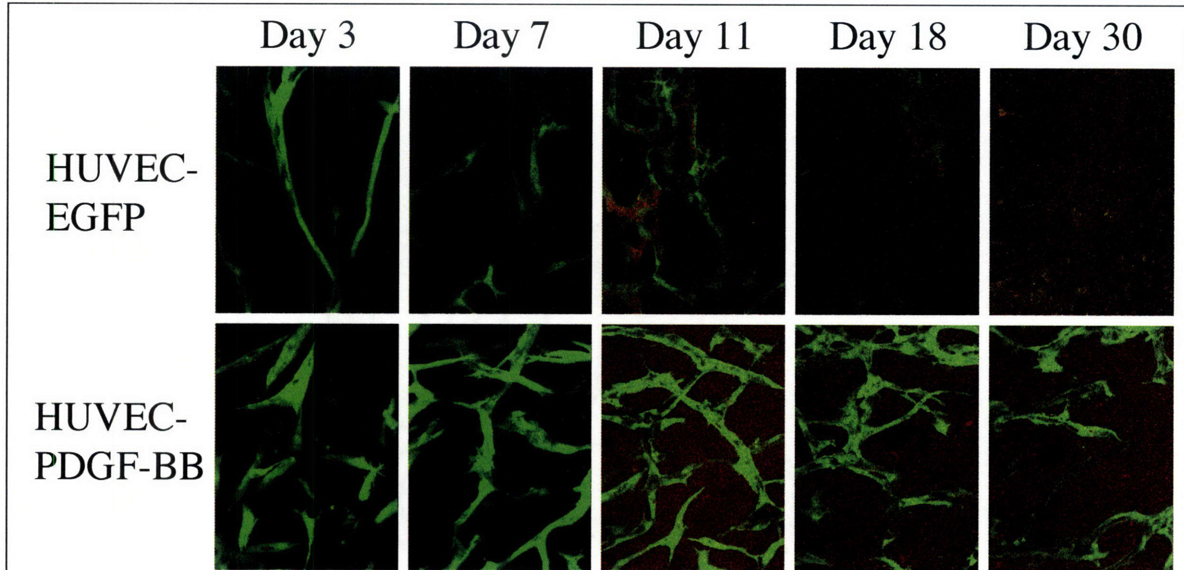
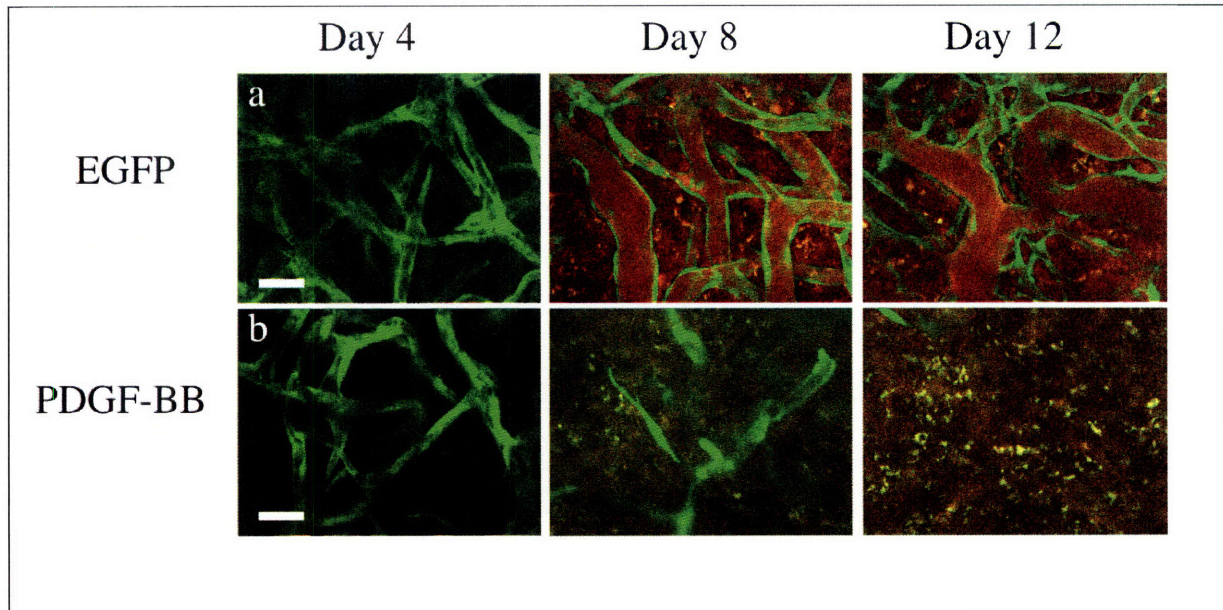


Figure 5.4. Effect of PDGF-BB overexpression on engineered blood vessels

HUVECs expressing EGFP only (a) or HUVECs co-expressing EGFP and PDGF-BB (b) were implanted in a collagen gel in cranial windows in SCID mice. Images were taken at 3, 7, 11, 18 and 30 days after implantation with multi-photon laser scanning microscope to monitor the *in vivo* dynamics of vascularization by the implanted endothelial cells (n = 5 for each experimental group).

Co-implantation of 10T1/2 cells promoted the regression of PDGF-BB overexpressing endothelial cells in vivo

Next, we co-implanted HUVECs with 10T1/2 cells to determine whether exogenously supplied smooth muscle/pericyte cells could enhance the formation of functional engineered blood vessels. In the control group with HUVEC-EGFP and 10T1/2 cells, HUVEC-EGFP formed vessel like network at day 4. By day 8, we were able to observe blood flow inside the lumens (Fig 5.5a). In marked contrast, we found that co-implantation of HUVEC-PDGF-BB and 10T1/2 cells led to an accelerated reduction in the density of HUVECs *in vivo* compared to the control (Fig 5.5b). Quantification of the total vessel length density showed a reduction in the number of HUVEC-derived vessels at day 4, and the density dropped precipitously, thereafter (Fig 5.6a). Unlike implantation of HUVECs alone, which transiently forms new vessels, we did not detect the formation of any perfused engineered blood vessels in the group implanted with HUVEC-PDGF-BB (Fig 5.6b).



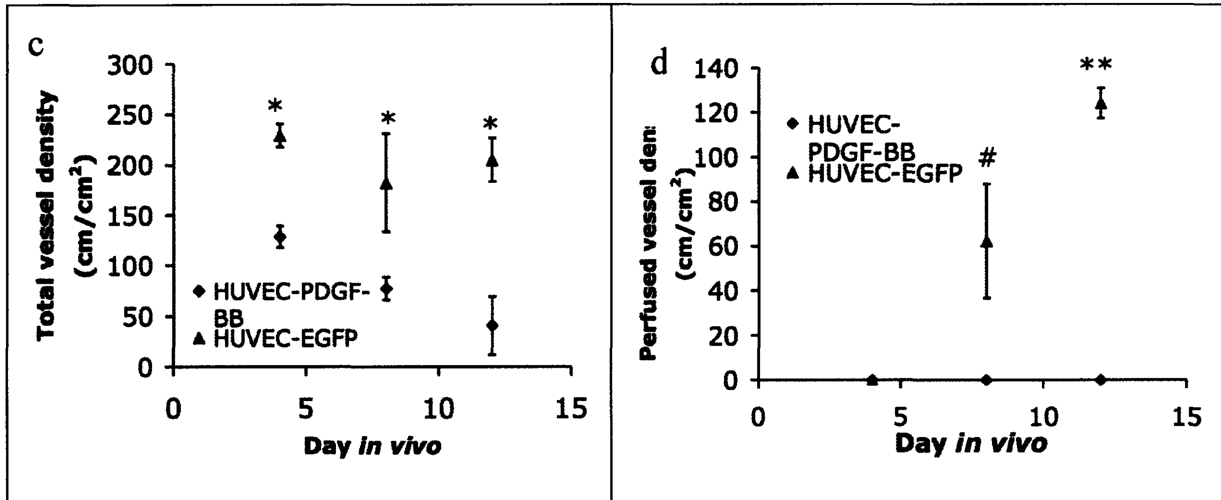
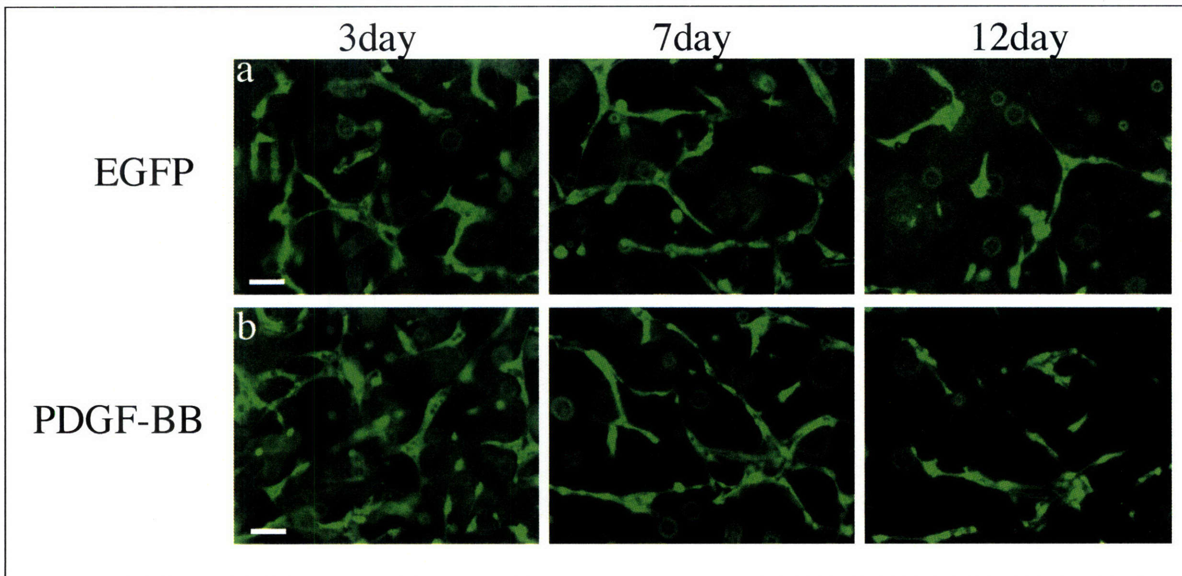


Figure 5.5. Effect of PDGF-BB expression and 10T1/2 cells on engineered blood vessels

HUVECs expressing EGFP only (a) or HUVECs co-expressing EGFP and PDGF-BB (b) were co-implanted with 10T1/2 cells in a collagen gel in cranial windows in SCID mice. Images were taken at 4, 8, and 12 days after implantation with multi-photon laser scanning microscope to monitor the *in vivo* dynamics of vascularization by the implanted endothelial cells (n = 4 for each experimental group). Densities of total engineered blood vessels (c) and functional engineered blood vessels (d) were quantified. Total engineered blood vessels denote all vessel-like structure derived from the implanted HUVECs. Functional engineered blood vessels denote HUVEC derived vessels that had blood flow as revealed by rhodamine-dextran contrast agent. Green, HUVECs expressing EGFP; red, functional blood vessels contrast-enhanced by rhodamine-dextran. Scale bar, 50 μm. * $p<0.005$, # $p>0.05$, ** $p<0.0001$

Next, we sought to determine if the *in vivo* phenotype of could be captured *in vitro* by co-culturing HUVEC-PDGF-BB with 10T1/2 cells. HUVEC-PDGF-BB and HUVEC-EGFP were incorporated with 10T1/2 cells in fibronectin/collagen matrix and cultured *in vitro*. After 3 days, we observed the formation of vessel-like structures by the HUVECs similar to those formed *in vivo* (Fig 5.6a,b). There was no significant difference in the ability of HUVEC-PDGF-BB and HUVEC-EGFP to morph into the vessel-like structures. The vessel-like structures in both groups regressed at similar rate with the progression of time and by day 12, only a few of these structures remained in the collagen gel (Fig 5.6c). These data suggest HUVEC-PDGF-BB and HUVEC-EGFP when co-cultured with 10T1/2 cells morphed into vessel-like structures with similar efficiency *in vitro*. The inability of co-implanted HUVEC-PDGF-BB and 10T1/2 cells to form stable blood vessels *in vivo* is likely secondary to altered *in vivo* microenvironment by the overexpression of PDGF-BB.



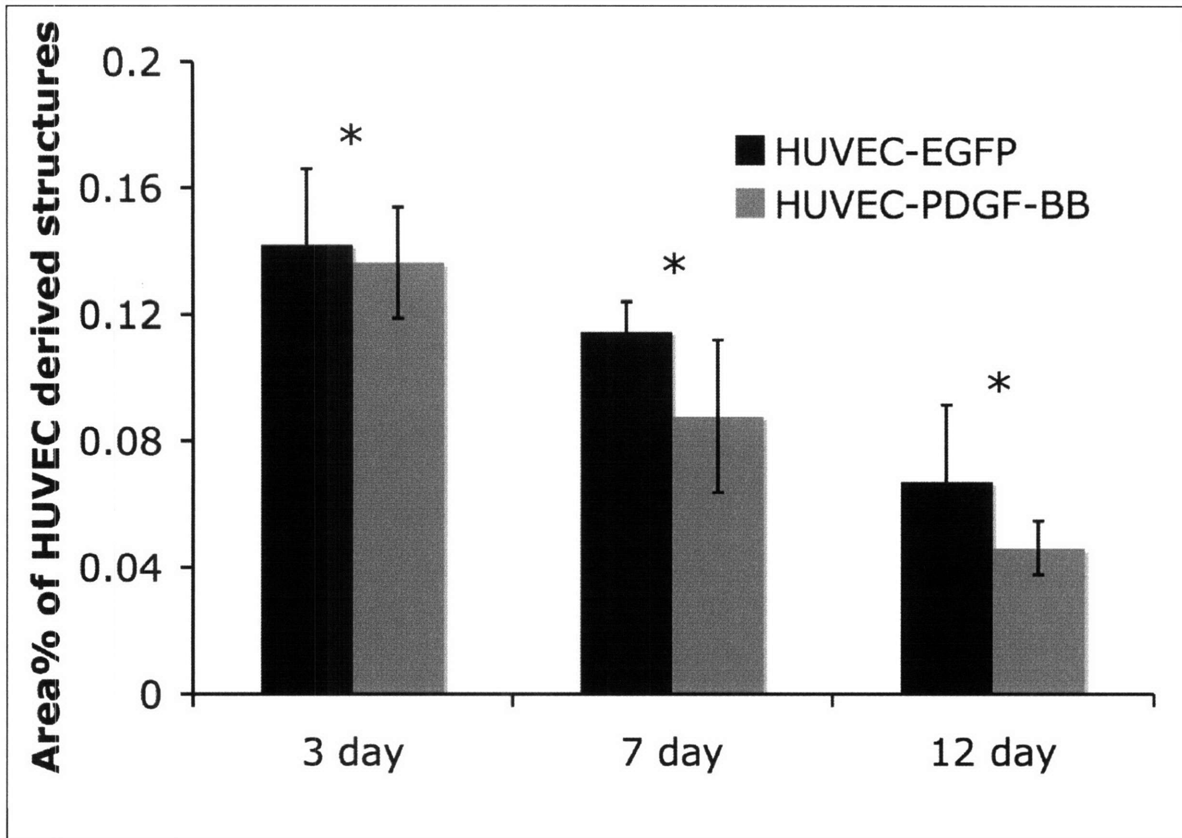


Figure 5.6. ***In vitro*** **vessel formation by endothelial cells**

HUVECs expressing EGFP only (a) or HUVECs co-expressing EGFP and PDGF-BB (b) were incorporated in collagen/fibronectin gel. Images were taken with fluorescence microscope at day 3, 7 and 12. No apparent difference was observed in the ability of HUVEC-EGFP and HUVEC-PDGF-BB to form vessel-like structure *in vitro*. The vessel-like structures were quantified (c) Scale bar, 50 μm. * N.S.

We further checked whether there's a difference in the intrinsic property of HUVEC with or without PDGF-BB overexpression. HUVEC-EGFP and HUVEC-PDGF-BB were serum-starved overnight for the induction of endothelial cell apoptosis. The two groups of endothelial cells were shown to have similar percentage of apoptotic cells as measured by TUNEL (Fig 5.7a). Finally, VEGFR-2 signaling was shown to be intact in HUVEC-PDGF-BB as assessed by VEGFR-2 phosphorylation (Fig 5.7b).

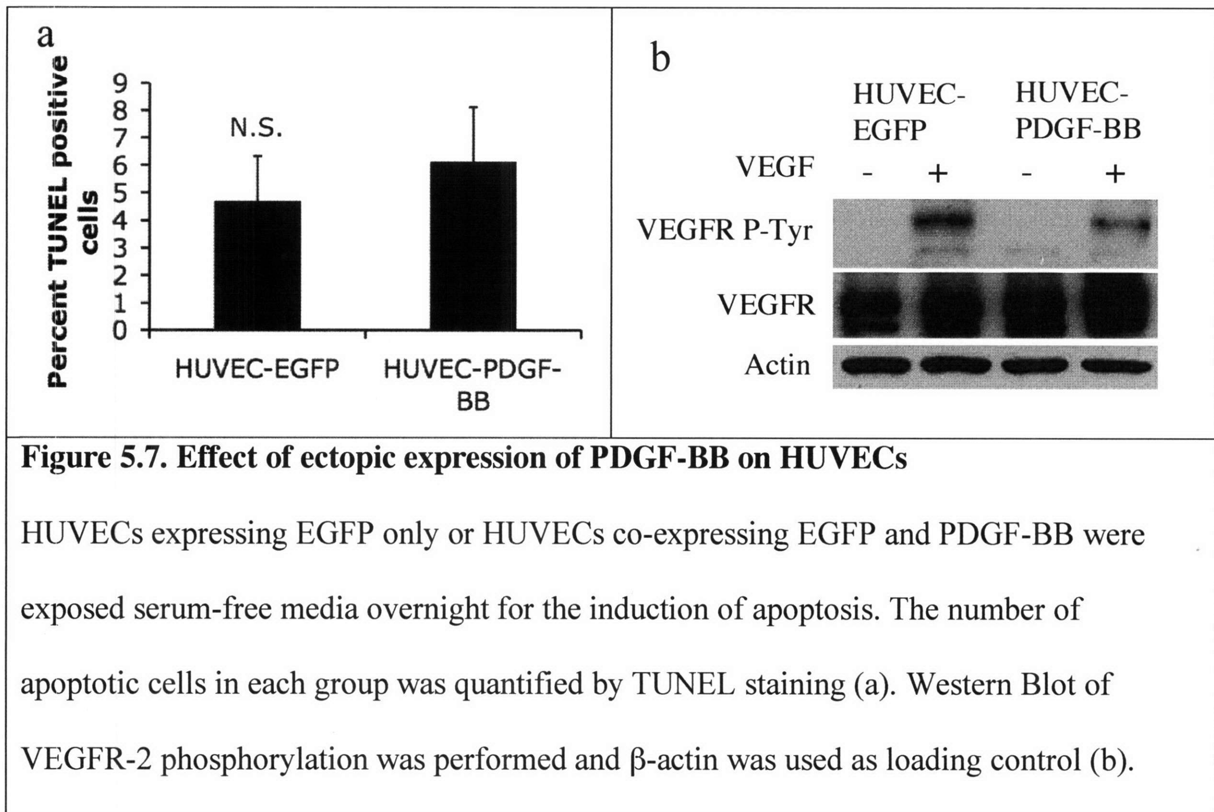


Figure 5.7. Effect of ectopic expression of PDGF-BB on HUVECs

HUVECs expressing EGFP only or HUVECs co-expressing EGFP and PDGF-BB were exposed serum-free media overnight for the induction of apoptosis. The number of apoptotic cells in each group was quantified by TUNEL staining (a). Western Blot of VEGFR-2 phosphorylation was performed and β-actin was used as loading control (b).

Discussion

Therapeutic angiogenesis has great promise in its ability to alleviate tissue ischemia and to help repair damaged tissue. Presently, a number of clinical trials are testing different angiogenic agents such as VEGF and bFGF to induce new blood vessels in poorly perfused tissue with the ultimate aim of improve tissue function[11]. In parallel, cell-

based therapy with bone marrow derived progenitor and/or embryonic stem cells is also being investigated for regenerative medicine[15, 16]. However, it is unclear whether a combination of these two approaches might yield a better result due to synergistic effects. In this report, we tested whether overexpressing PDGF-BB in endothelial cells could hasten the recruitment of perivascular cells and create a stable and long-lasting network of blood vessels. Our *in vitro* results demonstrated that PDGF-BB overexpression in HUVEC promoted the proliferation and migration of 10T1/2 cells. HUVEC-PDGF-BB when implanted alone showed an increase in the survival of vessel-like structure, however such structure rarely became perfused even at day 30. In contrast, co-implantation of HUVEC-PDGF-BB with 10T1/2 cells led to an accelerated reduction in the number of endothelial cells *in vivo*. The implanted endothelial cells did not appear to form any perfused blood vessels and by day 12, almost all the implanted cells had disappeared.

PDGF-BB has been previously implicated to be involved in vessel maturation. *In vitro* studies suggest that PDGF-BB is involved in the recruitment of perivascular cells[18]. This has been further confirmed by genetic knockout model of the ligand (PDGF-BB) or the receptor (PDGFR-β)[19]. These two knockout mice share similar phenotypes in that both mice are embryonic lethal. The blood vessels in the mice are hemorrhagic and show a reduction in the number of perivascular cells. When PDGF-BB expression is knocked out specifically in endothelial cells, it causes abnormality in the blood vessels in the heart, kidney and brain[20]. This suggests that the secretion of PDGF from endothelial cells is critical during the development of matured vessels. A recent study has added a layer of complexity – it was shown that not only is the secretion of PDGF-BB important,

but a gradient of PDGF-BB concentration is necessary for perivascular cell recruitment[21, 22]. PDGF-BB contains, at its carboxyl terminal end, a sequence of positively charged amino acids that is termed the retention sequence. It is believed that the retention sequence of PDGF-BB interacts with the negatively charged components of extracellular matrix such as heparan sulfate on the cell surface. Normally, most of the secreted PDGF-BB is retained at the cell surface, thereby limiting their actions to nearby cells only. Mice that have been genetically engineered to have PDGF-BB deficient in retention sequence exhibit marked abnormality in the brain blood vessels due to a reduction in perivascular cells[21].

Based on these observations, we reasoned that incorporating PDGF-BB directly in the tissue-engineered construct might disrupt the spatial distribution of PDGF-BB. Disabling the local chemotatic gradient of PDGF-B has been shown to reduce vascularization[23]. Therefore, we engineered the implanted endothelial cells to overexpress PDGF-BB in order to establish a local chemotatic gradient of PDGF-BB that is critical for pericyte recruitment in nascent vessels[24]. Indeed, *in vitro* results suggested that HUVEC-derived PDGF-BB increases pericyte precursor cell proliferation and migration. The unexpected finding of our study was that the *in vivo* phenotype of HUVEC-PDGF-BB was dependent on the presence of 10T1/2 cells. In the absence of 10T1/2 cells, HUVEC-PDGF-BB survived better than HUVEC-EGFP *in vivo*. However, when co-implanted with 10T1/2 cells, *in vivo* overexpression of PDGF-BB in HUVECs led to an accelerated regression of the nascent blood vessels. The exact mechanism for the reduction in the number of the endothelial cells is unclear; however, saturation of binding sites by overexpressed PDGF-BB may disrupt gradient of PEGF-BB at the close proximity of

endothelial cells (Fig. 1e). Ratio of endothelial cells and pericyte precursor cells may also play an important role. 10T1/2 cells may overwhelm endothelial cells due to significant increase in their proliferation by overexpressed PDGF-BB. In fact, it has been shown that 10T1/2 cells suppress HUVECs number when equal number of HUVECs and 10T1/2 cells are co-cultured[25].

PDGF-BB is a known dedifferentiating agent for smooth muscle cells. Stimulation of smooth muscle cells with PDGF-BB reduces the expression of smooth muscle markers including α-smooth muscle actin and SM-MHC[26]. Constitutive expression of PDGF-BB in HUVECs might have inhibited the differentiation of perivascular cells that is needed to stabilize the blood vessels. Moreover, PDGF-BB stimulation has been shown to induce VEGF and TGFβ1 in 10T1/2 cells *in vitro*[27, 28]. VEGF is a pro-survival factor for endothelial cells by activation of the Akt pathway[29]. On the other hand, TGFβ1 has been shown to inhibit proliferation and promote apoptosis of endothelial cells *in vitro* [30]. Interestingly, VEGF was shown to act in concert with TGFβ1 to induce endothelial cell apoptosis [31]. It is thus possible that endothelial cells overexpressing PDGF-BB induced the expression of VEGF and TGFβ1 in 10T1/2 cells and these two growth factors in turn promoted endothelial cell apoptosis *in vivo*.

In summary, our findings show the complexity of *in vivo* systems in which well-characterized cells and growth factors are used in combination for therapeutic angiogenesis, and demonstrate the need to determine optimal dosage, spatial and temporal distribution of these cellular and molecular elements *in vivo*.

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Chapter 6: Conclusion

One fundamental barrier in creating functional artificial tissue is the lack of a blood supply to deliver oxygen, nutrients, and to remove metabolic waste. Numerous strategies have been attempted to solve this challenge. The two main approaches include: 1. The release of angiogenic factors by mean of delivery of proteins or DNA plasmids to induce host blood vessels infiltration, and 2. incorporation of cells that directly differentiated into blood vessels. Each approach has its own advantages and drawbacks. In this thesis, I mainly focused on the cellular approach and then extended it by investigating a combination of angiogenic and cellular approaches.

The inspiration of this thesis stems from an intriguing finding. When Bcl2-transduced HUVECs were implanted subcutaneously in a SCID mouse, the HUVECs formed functional conduit that connected to the host circulation as demonstrated by the presence of red blood cells inside the lumen[1]. In that study, Schechner *et al.* demonstrated that the expression of Bcl2, a proto-oncogene that inhibits apoptosis, was critical in the survival and maintenance of the HUVEC derived vessels. When untransduced HUVECs were implanted in a mouse, the endothelial cells quickly disappeared.

These data suggested to us that it was feasible to engineer functional microvessels *in vivo* by implantation of endothelial cells. However, the necessity of ectopic expression of Bcl2 poses a significant drawback due to the potential risks for transformation. Thus, we explored an alternative strategy to enhance the survival of the engineered blood vessels without the need for genetic modification. Based on the knowledge our laboratory has accumulated over the years with tumor blood vessels, we hypothesized that co-implantation of endothelial cells with an exogenous source of perivascular cells would stabilize the engineered blood vessels *in vivo*.

Tumor blood vessels are noted for their marked abnormality as a consequence of the excess amount of pro-angiogenic factors secreted by the tumor cells or the tumor stroma. This imbalance between the pro-angiogenic and anti-angiogenic signals drives the morphological and functional abnormality of a tumor blood vessel. Tumor blood vessels are often tortuous and leaky and the pericytes around a vessel are often absent or loosely attached to the endothelium. Anti-angiogenic therapy can "normalize" the tumor blood vasculature and helps to improve the clinical efficacy of radiation and chemotherapeutic treatment by enhancing the delivery of oxygen and chemotherapeutic [2]. How does this knowledge of tumor blood vessels help us to create and improve an engineered blood vessel? One important finding discovered in our laboratory was that anti-angiogenic treatment with anti-VEGFR2 antibodies prunes away immature blood vessels while leaving the pericyte-covered blood vessels intact and/or by actively recruiting pericytes to the immature tumor vessels [3, 4]. Mature blood vessels were defined as those that were circumscribed by pericytes. This result suggests that pericytes play a critical role in regulating the survival of endothelial cells *in vivo*. The presence of pericytes was able to overcome the anti-vascular effects of VEGF blockade.

With this in mind, we decided to co-implant HUVECs with 10T1/2 cells, a line of mouse embryonic fibroblasts. Previous studies had suggested 10T1/2 cells might function as pericyte progenitor cells in supporting the maintenance of vascular-like network *in vitro* [5]. Our experimental result confirmed this finding *in vivo*. We were able to demonstrate that HUVECs when co-implanted with 10T1/2 cells were able to form long-lasting and stable blood vessels [6]. Some of the HUVEC derived vessels maintained their integrity for close to one year.

However, in order to translate this concept to the clinic, we need to utilize cells that can be secured and used in clinic. To this end, we systematically replace each individual cell type with a readily available source of cells. In collaboration with Dr. David Scadden's laboratory, we explored the derivation of endothelial cells from human embryonic stem cells (**Chapter 2**). We were able to induce the differentiation of human embryonic stem cells to endothelial lineage and demonstrated that these hESC derived endothelial cells were fully capable of participating in vasculogenesis and to anastomose with a mouse vascular network when implanted *in vivo*. However, there remained significant issues regarding the differentiation efficiency, culture condition, and line of human embryonic stem cells.

While our differentiation protocol was able to generate enough endothelial cells for *in vivo* testing, it remained wholly inefficient. The percentage of CD31+ or CD34+ cells generated was quite variable ranging from 7% to 30% of total number of cells. Some of the variability can be attributed to different lots of fetal bovine serum used in the study and different starting size of embryonic stem cell colonies. In the 2D differentiation protocol, the hESCs were cultured and differentiated with a feeder layer of murine embryonic fibroblasts (MEFs). The presence of the MEFs may negatively influence the differentiation process through the release of paracrine factors. Since different batches of MEFs were used in the study, this may also have contributed to the variability in the experiment.

Our experience suggests that a more defined and optimized cell culture and differentiation protocol is needed to increase reproducibility. How to optimize the differentiation of endothelial cells from hESC? First, a chemically defined cell culture

medium should be used for both maintenance and differentiation of human embryonic stem cells. This will minimize the variability caused by fetal bovine serum and MEFs. Another interesting possibility is to couple the expression of a reporter gene (e.g., EGFP or luciferase) to an endothelial promoter (ie. VE-Cadherin or Tie2). Under such a system, endothelial differentiation will drive the expression of a reporter gene allowing for easy identification of endothelial cells. When coupled with a high throughput screening system (e.g., microfluidic culture), the reporter gene will aid in the testing of different combination of growth factors (e.g., BMP4, VEGF) to induce the differentiation of endothelial cells. With this strategy it may be possible to systematically define the optimal differentiation protocol for derivation of endothelial cells from human embryonic stem cells.

Another problem we experienced with hESC differentiation is the growth of non-endothelial cells within the population CD34+ cells. After two weeks of culturing, we began to see colonies of non-endothelial cells with fibroblastic morphology. These cells quickly outgrew the endothelial fraction and eventually became the dominant cell type. This raises the possibility of a population of vascular progenitor within the CD34+ population and this is supported by a recent study [7]. Alternatively, the endothelial cells may undergo a process of endothelial-mesenchymal transition and becomes a population of fibroblast-like cells [8]. However, per Ochkam's razor, the most likely scenario is cell impurity during the isolation of the CD34+ cells. Even after 2 rounds of magnetic bead selection, the purity of CD34+ cells only reached 80 to 95% as determined by FACS analysis. This problem may be partially overcome by use of a selection marker (i.e. neomycin or puromycin resistance gene) driven by an endothelial promoter (i.e. a Tie2 or

VE-Cadherin promoter). This concept has already been validated in mouse embryonic stem cells to genetically select endothelial cells[9].

In our study, we utilized human embryonic stem cells that were derived from discarded embryos. Besides the associated ethical concerns, there remains a problem of immuno-compatibility. An exciting development that bypasses both of these concerns is the recent demonstration of induced pluripotent stem (iPS) cells derived from human adult fibroblast cells. Simply infecting adult fibroblasts with retrovirus to ectopically express three to four genes, the fibroblast cells were converted into a population of embryonic stem cell-like cells[10, 11]. iPS cells hold tremendous promise for regenerative medicine since they provide a source of renewable and tailored cells that are completely immuno-compatible with their recipients. An important next step will be to derive endothelial cells from iPS cells and to demonstrate their *in vivo* vasculogenic potential.

Since the successful use of ES or iPS-derived cells will likely to take some time, we investigated an alternative source of endothelial cells, endothelial progenitor cells that are already being tested for clinical use (**Chapter 3**). Endothelial progenitor cells can be isolated from umbilical cord blood and peripheral blood. However, there was a dearth of data regarding the *in vivo* persistence and performance of the endothelial progenitor cell derived blood vessels. We demonstrated that cord blood EPC derived blood vessels persisted much longer *in vivo* than those derived from adult peripheral blood EPCs. This result is not entirely surprising since accumulating evidence suggests the organismal age of a cell directly impacts cellular function[12].

The outstanding question arising from this project is the differential *in vivo* survival of endothelial progenitor cells. *In vitro* study suggests that cord blood EPCs were more resistant to serum starvation induced apoptosis than peripheral blood EPCs.

However, this result did not provide a causal link. We attempted by PCR Array to identify a difference in the transcription level of apoptosis-related genes. Both sources of EPCs dramatically up-regulated anti-apoptotic genes (e.g., Bcl2) upon serum starvation. One difference between PB-EPCs and CB-EPCs is the expression of telomerase. Interestingly, there is data in literature that link telomerase activity with angiogenesis and anti-apoptosis in endothelial cells. Therefore, it would be interesting to down-regulate the expression of telomerase in CB-EPCs with RNA interference and then test for the CB-EPCs *in vivo* survival and vasculogenic capacity.

Our experiments with human bone marrow derived mesenchymal stem cells suggest that hMSCs facilitated the engineering of stable and long-lasting microvasculature by differentiating into perivascular cells (**Chapter 4**). One intriguing result from this study was that there appears to be a crosstalk between the hMSCs and endothelial cells for their survival *in vivo*. The majority of the hMSCs that persisted *in vivo* were associated with blood vessels. *In vitro* data further suggested that cell-cell contact mediated the differentiation of hMSCs. It is unclear to us at this time whether both survival and differentiation signals were provided by the same molecule. We explored some candidates that may mediate the cell-cell contact dependent signals. We utilized neutralizing antibodies against N-Cadherin and alpha4beta1 integrin, but we were not able to detect changes to myocardin transcript level in hMSCs by real-time PCR. It's possible that sub-optimal concentration of antibodies was used in this study. The

molecular mechanisms for the cell-cell contact dependent hMSC differentiation and survival awaits further elucidation.

The combination of cell and growth factor based approach offers an exciting new way to induce the formation of functional blood vessels *in vivo*. We genetically engineered endothelial cells to constitutively secrete PDGF-BB in the hope that it would hasten the recruitment of host perivascular cells (**Chapter 5**). However, we observed that the endothelial cells quickly died once they are implanted *in vivo*. This effect was only observed *in vivo,* suggesting PDGF-BB over-expression indirectly induces the regression of endothelial cells through an unknown mechanism. At this time the molecular mechanism of endothelial cell death remains unclear to us. However, this work emphasizes the complex nature of the *in vivo* microenvironment and its interaction with the implanted cells. Care must be taken in the combined use of cell and growth factor based approaches in inducing blood vessel growth, since it is likely that unintended effects may arise.

This thesis has served as a necessary first step in utilizing clinically relevant sources of vascular cells to create functional microvasculature *in vivo*. It systematically defines the optimal source of endothelial cells and perivascular cells for vascular engineering. It has exposed the difficulty and limitation in the use of human embryonic stem cell derived endothelial cells. It further illustrates the unintended effects of genetically engineered endothelial cells. A major barrier in engineering functional tissue is the lack of a functional microvasculature. We hope that in some small measure this work provides a guide to overcome this problem and helps to propel the field of tissue engineering forward to its eventual clinical application.

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