HEMANGIOBLASTS: FROM HEMATOPOIETIC STEM CELLS TO ENDOTHELIAL PROGENITOR CELLS AND THEIR EFFECTOR MOLECULES

By

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This work is dedicated to my mother, Bernadette Guthrie, and my father, Edwin Guthrie.

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Research in the field of stem cell has received much attention in the past few years. Stem cells hold tremendous potential for treating many debilitating conditions and diseases. My study describes how the hematopoietic stem cell is plastic, or capable of producing non-hematopoietic tissue in addition to all of the expected blood lineages. Specifically, the hematopoietic stem cell is capable of producing endothelial cells of blood vessels. I describe this through a series of experiments where I transplanted a single hematopoietic stem cell into a lethally irradiated recipient and reconstituted all of the blood lineages. This single cell was then able to produce endothelial cells under conditions of injury and ischemia in an attempt to relieve the ischemic pressure. I found that the hematopoietic stem cell can function as a hemangioblast, capable of producing not all of the blood lineages and also blood vessels. This activity suggests the possibility of modulating this hemangioblast activity.

I determined that two genes play a role in blood-pressure maintenance and immune responses in the Nitric Oxide Synthase pathway. These genes are also able to modulate hemangioblast function in mice. This ability to alter blood vessel formation would be extremely useful in conditions of pathologic blood vessel growth such as diabetic retinopathy, the leading cause of blindness worldwide, or tumor blood vessel growth where decreasing the blood supply could starve the cancer cells. Conversely, wound healing, and therapy for conditions such as stroke or cardiac ischemia, would benefit from increased blood vessel growth. This knowledge can be directly applied by using pharmacological agents that either inhibit or upregulate the Nitric Oxide Synthase genes to modulate blood vessel formation for therapies useful in human patients.

CHAPTER 1
INTRODUCTION AND BACKGROUND INFORMATION

The discovery of the ability of stem cells to differentiate along alternative developmental fates heralded a new tool for the treatment of many debilitating diseases. The ability of exogenous cells to home to areas of injury, take up residence, and reprogram themselves to new tissue types allows for functional repair of dysfunctional tissues. While in some tissue types this has been known to occur, such as vasculature reperfusion in wound healing, the exact cells contributing to the endothelial tissue were identified only recently. Elucidation of the contributing cell to certain types of vascular repair, viz. hematopoietic stem cells, now allows exploration of the molecules that play parallel roles in both hematopoiesis and blood vessel formation. Indeed, tailoring of the hematopoietic stem cell's hemangioblast activity could improve currently limited palliative care for conditions such as diabetic retinopathy or could provide an improved targeted approach for tumor growth suppression and elimination. The potential for clinical therapies is profound.

The unifying goal of my study was to further describe the characteristics of the hematopoietic stem cell in relation to its plastic ability to produce the endothelial tissue lining the blood vessel walls. I do this immersed in the current environment of sanguine skepticism towards stem cell plasticity highlighting how this work addresses the controversy. I begin by outlining the backdrop for current research and provide a barometer to measure the current stem cell climate. I outline the limitations to stem cell research in relation to the hematopoietic exploration along with the methods by which

they were addressed. Chapter 2 describes the development of a novel, robust, and reproducible model for inducing hematopoietic stem cell hemangioblast activity thereby promoting an alternative developmental fate along the endothelial lineage. Chapter 3 underscores how this model was applied to the critiques of stem cell plasticity and how the hematopoietic stem cell functions in conditions of injury. There are many biological molecules that can modulate hematopoiesis and neovascularization. In chapter 4 I describe how nitric oxide has the ability to play a significant role in hematopoietic stem cell derived hemangioblast activity. Finally, in chapter 5 I will identify some of the limitations of stem cell based research and therapy including both biological and ethical implications.

Hematopoiesis and Vasculogenesis During Embryonic Development

The rapid growth of the early embryo necessitates conversion from a mechanism where simple diffusion provides the necessary nutrients and removes metabolic byproducts for the ever-increasing cell number to a mechanism of circulated transport. The developing blood and vasculature provide this circulation. During murine development, hematopoiesis and vasculogenesis begin as early as Day 7 in the region of the yolk sac.[1, 2] Endothelial cells are derived from mesodermal precursors in the yolk sac and begin to constitute the primary vascular system in parallel with initiation of premature hematopoiesis.[3-5] This vasculogenesis begins with a cluster of cells, called blood islands, composed of a "nucleus" containing hematopoietic stem cells (HSC) surrounded by more differentiated angioblasts, the cells which will form blood vessels, on the periphery.[6] The close proximity of the two precursor cells and the developmental relationship between the formation of blood and blood vessels suggest a shared parent cell from which both are derived: the hemangioblast.

Until the Day 10 of development, the yolk sac remains the primary site of hematopoiesis. Around Day 12 the liver which then becomes the primary site of hematopoiesis.[7] However, there are other regions of potential hematopoiesis in the para-aortic splanchnopoeura (PAS) from Day 8.5 to 10, and the aorta-gonad-mesonephrous (AGM) region from Day 10.5 through Day 12.[7-11] The potential of these areas was determined through a series of transplantation studies where cells isolated from these regions are able to rescue lethally irradiated recipients.[12-14] This hematopoietic rescue capability defines the first location from where functionally defined HSC arise. Endothelial cells on the ventral surface of the aorta are derived from the PAS/AGM regions, and HSC are also found nestled in the endothelial floor of the aorta, again suggesting the that this area contains cells which are have the capabilities of the hemangioblast.[12]

Formation of Blood Vessels in Adults

Vasculogenesis and angiogenesis are two distinct roles of the hemangioblast. Vasculogenesis is defined as the *de novo* generation of blood vessels via the recruitment of undifferentiated progenitor cells to the site of vessel formation where they differentiate into vascular endothelium.[11] During embryonic development, the vascular system is formed through vasculogenesis. After development is complete, new blood vessel formation is attributed to the process of angiogenesis where vessels are formed by sprouting from the pre-existing vasculature.[15] Until 1991, angiogenesis was thought to occur by the proliferation of resident endothelial cells at the site where new vessels are forming, but George *et al.*[16] showed that endothelial cells circulate in the blood. They found that peripheral blood contained endothelial cells by staining blood samples with the

endothelial cell specific antibody S-Endo 1 and analyzing these cells by Fluorescence Activated Cell Sorting (FACS). The discovery of circulating endothelial cells unvaryingly leads us to question where these cells are derived.

There are two possibilities of circulating endothelial cell parentage: the existing vasculature where cells extrude themselves from blood vessel walls and enter the circulation; the bone marrow itself, via an endothelial cell progenitor (EPC) intermediate. Several studies describe endothelial cells which derived from the bone marrow.[17-22] If this is the case, the HSC and EPC populations could possible be distinguished through their cell surface marker expression, or through "tagging" of the parent cell. No studies have yet directly addressed this question; however there is significant indirect evidence linking endothelial cells to the EPC and its involvement in adult neovascularization. One such study described several cell surface antigens present on the EPC, such as CD133 and CD34, that are also present on the HSC.[23] However, there are differences in the two populations, namely that fetal liver kinase-2 (VEGFR-2) expression is only found on committed progenitors.[24] This is one of the first hints that EPC may be a more differentiated or committed HSC daughter cell. CD 34 positive cells can phenotypically function as endothelial cells after several days of culture on fibronectin. They are capable of incorporating acetylated LDL, producing nitric oxide when stimulated with VEGF, and express of PECAM-1 and Tie-2, both of which are specific to endothelial cells.[25] CD133 positive cells appear to be a more immature subgroup of the CD34 population. The CD133 positive cells are able to repopulate the bone marrow compartment of radioablated sheep, and evidence shows that a subset of cells which are CD34, CD133 and VEGFR-2 positive may be EPC.[26-28] CD133 and CD34 positive cells

are believed to be more primitive EPC because they lack VE-cadherin or Von Willebrand expression. Only 3% of these cells express VEGFR-2.[27] CD34 negative, CD133 positive, and VEGFR-2 positive cells may represent a more mature or further differentiated population of endothelial cells.

The exact markers and phenotype of EPC are not known, and the conditions under which these cells are stimulated to proliferate, circulate, and home to sites of injury are poorly understood. There is disparity in the amount of neovascularization occuring in certain vascular beds with some tissue-types experiencing significantly more vessel formation in relation to others. In addition, the wide range of ischemia models utilized for study have been found to induce different levels of neovascularization. Crosby *et al.*[29] have shown that up to 11% of endothelial cells contributing to neovascularization are EPC derived. This contribution occurred during injury and was not observed under normal physiologic conditions. Grant *et al.*[30] demonstrated that circulating endothelial cells, specifically endothelial cells which contribute to the formation of blood vessels during injury repair, arise from the HSC through an EPC intermediate. This finding lends to the possibility of regulating vessel formation at a precursor level through a molecular mediator. The ability to orchestrate formation of blood vessels is highly desired for conditions in which pathological vascular growth, or lack of growth, and leads to damaging conditions ultimately decreasing the quality of life.

Regulation of Neovascularization

Vascular endothelial cells maintain a tight border between the circulating blood and the outside tissue. This monolayer of cells acts as a non-adherent surface where circulating cells cannot interact and adhere without the presence of certain surface markers, such as the integrins or selectins of the cellular adhesion molecule family.

While this boundary must necessarily remain intact, mechanisms exist in which cells within the blood can extravasate into the surrounding tissue in order to fight infection or provide repair. Conversely, mechanisms exist by which cells in tissue can enter the bloodstream illustrated by bone marrow cells ability to proliferate in the bone marrow compartment, migrate to the inner marrow vessels, and enter the circulation. Endothelial cells generally have a very low level of apoptosis and thus a low turnover rate. Cells in certain organs, such as the eye, can live for years without being replaced.[31] As a result, there are infrequent endothelial cells circulating in healthy adults usually numbering 1-3 per milliliter of blood.[32] This emphasizes how the steady state of endothelial cells is non-dividing unless stimulated by injury when mechanisms to upregulate endothelial mitosis stimulate proliferation.

Positive regulators are growth factors frequently detected in adult tissues in which there is apparent angiogenesis and include Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF).[33] *In vitro*, it has been found that VEGF and bFGF upregulate many endothelial cell functions, including proliferation, migration, extracellular proteolytic activity, and tube formation.[34] This has led to the notion that these factors act directly on endothelial cells to upregulate their activity. Indeed, VEGF is increased in tumors when the transformed cells begin to recruit blood vessels for growth.[34] Conversely, a method must exist that can limit the amount of neovascularization occurring so as to not produce pathologic vasculature. Endothelial quiescence is thought to be maintained by the presence of endogenous downregulators such as Tumor Growth Factor-beta (TGF-ß) and Tumor Necrosis Factor-alpha (TNF-α).[35] Unlike to VEGF and bFGF, angiogenic downregulators may act directly on

endothelial cells, or indirectly by inducing the production of inflammatory and other non-endothelial cell regulators.[36, 37] TGF-ß and TNF-A inhibit endothelial cell growth *in vitro* and have therefore been considered as direct acting negative regulators.[35] Unexpectedly, TGF-ß and TNF-α are angiogenic *in vivo*, and it has been demonstrated that these cytokines induce angiogenesis indirectly by stimulating the production of stromal and chemoattracted inflammatory cell positive regulators.[38]

Other cytokines that have been reported to regulate angiogenesis *in vivo* include HGF, EGF/TGF-, PDGF-BB, interleukins (IL-1, IL-6, and IL-12), interferons, GM-CSF, PlGF, proliferin, and proliferin-related protein.[39-41] Chemokines that regulate angiogenesis *in vitro* have also been identified including IL-8, platelet factor IV, and groß.[41-43] Angiogenesis can also be regulated by a variety of noncytokine or nonchemokine factors, including enzymes (angiogenin and PD-ECGF/TP), inhibitors of matrix-degrading proteolytic enzymes (TIMPs), plasminogen activator inhibitor-1 (PAIs), extracellular matrix components, coagulation factors or fragments (thrombospondin, angiostatin, hyaluronan, and its oligosaccharides), soluble cytokine receptors, prostaglandins, adipocyte lipids, and copper ions.[39, 42-45] This plethora of cytokines demonstrates the complexity of regulating of the angiogenic process, and justifies assessing their role in stem and progenitor cell governance of neovascularization. These positive and negative regulators often coexist in tissues in which endothelial cell turnover is increased. Although this has yet to be proven *in vivo*, the current working hypothesis is that the angiogenic switch of tumors involves either the induction of a positive regulator and/or the loss of a negative regulator.

Stem Cell Transplantation

The adult bone marrow (BM) is a rich reservoir of tissue specific stem and progenitor cells. BM cells may be a source of EPC. Therefore tapping into BM in combination with neovascularization regulators may provide significant and manageable therapy. Stimulation of angiogenesis may be of benefit in wound healing and fracture repair. Therapeutic growth will also be beneficial in the treatment of ischemia, and substantiated by extensive experimental data.[46-49] Pesce *et al*.[49] demonstrated that under ischemic conditions, transplanted umbilical cord cells gave rise to enhanced arteriole length and density along with skeletal muscle fibers. Another group transplanted early bone marrow cells into nonirradiated, aged mice and found a contribution to vasculature from subsequently transplanted neonatal myocardium.[48] In addition, Orlic *et al.*[50] demonstrated that bone marrow cells can differentiate into myocytes and vascular structures. They also mobilized bone marrow cells with stem cell factor and granulocyte-colony stimulating factor and found that marrow cells could home to infarcted regions of the heart, replicate, differentiate, and ultimately promote myocardial repair.[51] This could lead to significant alterations and improvements in treatment for cardiac ischemia.

Current therapy for myocardial ischemia relies on drugs that reduce myocardial oxygen demand, mechanical endovascular revascularization procedures (angioplasty), or bypass surgery.[52] However, compensatory neovascularization is an important physiological process that occurs in chronic myocardial ischemia.[53] It has recently been demonstrated in experimental models of myocardial ischemia and infarction in the pig and rat that VEGF and VEGF receptors 1 and 2 are increased in chronically ischemic myocardium and also in regions of ischemia surrounding an area of infarction.[54-56] Those studies demonstrated that the VEGF ligand is upregulated in cardiomyocytes and its

cognate receptors exhibited increased expression in endothelial cells. Further studies have revealed that hypoxia is a potent inducer of VEGF in cultured cardiac myocytes.[57] Correspondingly, escalated bFGF activity has been shown in myocardium after coronary artery ligation.[58] This occurs in parallel with an increase in collateral blood flow in dogs, and elevated levels of bFGF (but not VEGF) have been detected in the pericardial fluid of patients with unstable angina.[52] These observations on the molecular mechanisms of physiological angiogenesis in ischemic myocardium led to the notion that cell based therapy or pharmacological stimulation of angiogenesis may augment or even replace more conventional forms of therapy. As will be described next, this notion has recently received considerable experimental support in animal models.

Vascular healing may be mediated in part by the recruitment of EPC. In several studies, genetically marked bone marrow-derived EPC were recruited to the ischemic limbs of mice.[11,17] In addition, transplantation of mature endothelial cells (EC) derived from *in vitro* generated, human bone marrow-derived, multipotent adult progenitor cells has facilitated revascularization of various tissues.[59] The physiologic significance of EPCs and EC in neovascularization was further underscored when thoracic aorta from adult dogs previously transplanted with haploidentical bone marrow,were replaced with Dacron grafts impervious to the ingrowth of established EC. In 3 month old grafts, the newly established EC layer were determined to arise from donor derived cells from the bone marrow.[58] These findings indicate that EC derived from the EPC of bone marrow origin can contribute to new blood vessel formation.

EPC for Neovascularization

This low number of EPC in the circulation increases dramatically under conditions such as acute stress or injury to vasculature walls where there is a large apoptotic event of

EC. Normal replacement of the EC is usually accomplished by the surrounding local endothelial cells which increase their proliferation and migrate to the areas of ischemia. The terminally differentiated EC, however, are not able to proliferate considerably and may not have the capacity to provide for the demand for new vessels. As described in numerous stuidies, researchers have isolated circulating cells that are bone marrow derived yet have endothelial potential—the EPC. These EPC are capable of lessening the ischemic pressure of injured organs by revascularizing injured areas and restoring organ function.

Our current understanding of the neovascularization process is founded on the classical light-microscopy observations made by Clark and Clark in 1953.[60] They were among the first to reveal the sequence of events leading to the formation of new capillary blood vessels in the translucent tails of amphibian larvae. These and later observations in nondevelopmental settings provided a detailed histological account of new blood vessel formation.[61, 62] On these pioneering results our current knowledge was founded. Clark and Clark described a local angiogenic stimulus that causes endothelial cells of preexisting capillaries or postcapillary venules to become activated. Although the precise molecular consequences of this activation process remain to be clearly defined, activated blood vessels are vasodilated, have increased vascular permeability, and experience accumulation of extravascular fibrin as well as proteolytic degradation of the basement membrane of the parent vessel.[46-48] The endothelial cells then extend thin cytoplasmic arms which direct migration into the surrounding matrix towards the angiogenic stimulus. Migrating endothelial cells elongate and align with one another to form a capillary sprout, and endothelial cell division, which occurs proximal to the migrating tip, further

increases the length of the sprout. The solid sprout gradually develops a lumen proximal to the region of proliferation. Contiguous tubular sprouts fuse at their tips to form a functional capillary loop in which blood flow is soon established. Vessel maturation is accomplished by reconstitution of the basement membrane and recruitment of mural cells.[49] These cellular functions contribute to the formation of patent, endothelium-lined, blood vessel structures.

Nitric Oxide as Potential Regulator of Vascular Formation

The process of angiogenesis in the adult is a complex sequence of growth factor release, vasodilation, and recruitment or proliferation of endothelial cells to build the vessels. These events are heralded by EC activation, most notably vasodilation, which facilitates growth by granting access for cells to enter the area and remove any damaged and dead cells/debris, increases nutrient depositing and breakdown of existing extracellular matrix, and allows cells to establish permanent residence. One of the molecules which has been shown to play an extensive role in vasodilation is Nitric Oxide (NO).

NO has been used in nature for over 250 million years, longer than mammals have existed. The horseshoe crab uses NO to prevent blood cell aggregation, and this function is still retained in mammals. Other kingdom and phyla also utilize NO including fireflies for their flashes, and plants that use NO's cytotoxic effects to fight infection. Victorian physicians recognized its vasodilatory effect, even if they did not understand its mechanism, and its medicinal value was written in a Sherlock Holmes story.[130] The medical uses for NO continued into World War I where doctors noticed that factory workers in ammunition plants had lower blood pressures. This led directly to the nitroglycerine tablet still used today to treat angina. The gas molecule itself, however,

was considered only a pollutant until recently. In the early 1990s the journal *Science* named it molecule of the year. During this time over 250 articles per month were written further characterizing NO and its effects. Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad received the Nobel Prize in Medicine in 1998 for their work on "nitric oxide as a signaling molecule in the cardiovascular system." One historic irony is that Alfred Nobel made his fortune by making dynamite from nitroglycerine, a known NO donor.

NO is unique among physiologic substances in the body as it is the only gas produced in mammals that has a biological effect. This singular messenger molecule is involved in the regulation of diverse physiologic functions including central and peripheral nerve cell neurotransmission, promotion of the cytotoxic actions of immune cells, and preventing/increasing leukocyte adhesion.[63-67] It also has profound vasomotor regulatory affect on vascular beds, specifically the regulation of smooth muscle contractility and thus vasodilation.[63, 64]

Three distinct isoforms of the enzyme that synthesizes NO (NOS) have been identified, all of which share a 50-60% homology.[67] Two isoforms are constitutively active: the form expressed primarily in neuronal tissue (nNOS) and the form first found in vascular endothelial tissue (eNOS). The third form's activity can be induced in a variety of cell types usually in response to inflammatory signals and bacterial products, and has been named inducible NOS (iNOS). Each of the three isoforms require homodimerization for activity. The C-terminal portion of the NOS protein closely resembles the cytochrome P-450 reductase possessing many of the same cofactor binding sites.[68] The extreme C-terminus contains an NAPDH binding region, conserved in all three isoforms, that exactly aligns with the binding region of the cytochrome P-450.[68]

Following this is a flavin adenine dinucleotide and flavin mononucleotide consensus sequence that is self-sufficient, unlike the P-450 enzyme, in that the oxygenation of its substrate L-arginine occurs at the heme site in the N-terminal region.[69] NO is generated via a 5-electron oxidation of a terminal guanidinium nitrogen on L-arginine.[68]

Most of the physiologic actions of NO are brought about by the activation of soluble guanylate cyclase. Binding of NO to the heme moiety of the enzyme causes a conformational change that upregulates the activity over 400-fold resulting in the formation of the intracellular second messenger cyclic GMP.[70] NO has numerous angiogenic effects, including (but not limited to) increasing matrix metalloprotinase (MMP) expression and tyrosine phosphorylation of proteins in sprouting tips of capillaries.[65] Inhibiting NO production has been shown to decrease capillary formation in rats with portal hypertension.[66] In addition, DNA synthesis can be impaired by the inhibitory effect of NO on ribonucleotide reductase which addresses the cytotoxic and cytostatic effect of NO during an immune response. In the aqueous environment of the cytosol, NO interacts with water to form the free radical peroxynitrate.[67] Peroxynitrate interacts with DNA leading to oxidation and initiation of a complex series of transformations involving base damage or strand breaks as well as reactions with the deoxyribose portion of the DNA.[71] The DNA damage itself, along with the cell cycle arrest as repeated and costly DNA repair occurs, ultimately leads to apoptosis.

Role of NOS in vessel formation

The process of angiogenesis can be divided into two components: endothelial cell proliferation and blood vessel tube formation. The potent angiogenic agent VEGF stimulates NO release from endothelial cells.[72] VEGF-induced NO release has been shown to modulate angiogenesis both *in vitro* and *in vivo*.[73, 74] The adult mouse model we

have developed utilizes the angiogenic influence of VEGF as we artificially increase local expression of this growth factor in the retina mimicking the pathophysiology that occurs in diseases associated with retinal neovascularization such as Diabetic Retinopathy and Retinopathy of Prematurity. The established resident vascular endothelial cells, the endothelial cells found in the circulation, and those derived from HSC all respond to VEGF and influence local NO concentration. NO is crucial for the myriad of physiological vascular functions, and its inappropriate production and release has been linked to several pathologies.[75] Consequently, agents which modulate NO activity could find beneficial use in a therapeutic setting. As has been shown, NO plays an integral role in blood vessel formation, and consequently makes a good starting candidate for manipulating hemangioblast function.

The two isoforms which have a direct influence over endothelial cells are the iNOS and eNOS isoforms as nNOS is found only in neuronal tissue.[67] The role of eNOS in angiogenesis is complex. Brooks *et al.* have demonstrated that eNOS deficiency, either through gene disruption or through pharmacological inhibition, significantly protects the developing retina from oxygen-induced retinopathy.[76] The fact that nonspecific inhibitors of NOS activity produced quantitatively similar levels of vaso-obliteration compared to eNOS gene disruption also suggests that eNOS may be an isoform involved in blood vessel regulation. Evidence suggests that NO and VEGF are reciprocally regulated such that stimulation of VEGFR-2 activates eNOS leading to NO formation.[76] NO inhibits VEGF production in adjacent cells by a paracrine feedback mechanism involving inhibition of AP-1 binding to the VEGF promoter.[77]

iNOS has consensus sequences in its promoter for the transcription factors hypoxia inducible factor (HIF) and NF-kappa B, both of which are activated under conditions of ischemia.[78] Consequently, iNOS is thought to be induced under conditions of ischemia. Sennlaub *et al.* perfused retinas of wild type and iNOS knockout (iNOS $^{-/-}$) mice exposed to hypoxic conditions. They found that iNOS $^{-/-}$ animals had normal intraretinal vasculature patterning whereas wild type animals had persistent avascular areas.[79] Interestingly, there was a reduction in preretinal neovascularization in iNOS $^{-/-}$ mice indicating a dual role of iNOS in distinct retina layers. They corroborated these observations with pharmacological inhibition of iNOS which increased retinal neovascularization and decreased preretinal neovascularization. They found that pathological intraretinal neovascularization was more severe in iNOS expressing animals.[80] These studies suggest that NO can be an important modulator of angiogenesis in the retina, and that local levels of NO can influence the location and degree of neovascularization. To our knowledge our model is the only one which allows for the simultaneous examination of preretinal and intraretinal neovascularization at the same time in an adult animal. We will use this model to understand the requirement of beneficial intraretinal neovascularization compared to pathological preretinal neovascularization allowing for the dissection of NO and other molecules which affect vascular growth.

CHAPTER 2
GENERAL METHODS AND MATERIALS

The methods detailed below are used extensively in each chapter. Any modifications made to this framework during an experiment are noted in the specific chapter. Methods will be described in this basic outline: (1) the generation of the GFP/BL6 chimera, (2) the induction of the retinal neovascularization, (3) the enucleation of the eye for mounting, (4) examination of neovascularization via confocal microscopy and (5) immunohistochemistry staining of serial sections.

Generating The GFP/BL6 Chimera

The generation of the chimeric GFP/BL6 animal will be described below. This includes the harvesting of bone marrow from the GFP donor animal, the purification and preparation of the marrow for FACS sorting of HSC, the preparation of the C57BL6 rescue marrow and recipient animals, and the HSC transplant and commensurate animal husbandry concerns.

Harvesting Bone Marrow

The generation of the GFP/BL6 chimera animals requires extensive animal use and cell manipulation. The transgenic mouse used as the donor strain was obtained from Andras Nagy at mount Sanai in Toronto Canada.[81] The strain carries green fluorescent protein (GFP) driven by chicken beta-actin promoter and CMV intermediate early enhancer and is ubiquitously expressed. The BL6 females were obtained from Jackson Laboratories (Bar Harbor, Maine) and were at least 5 weeks old at the time of bone marrow transplantation. Recent controversy concerning the events during stem cell

transdifferentiation for repair has led to the possibility that this may not be an inherent ability stem cells, but rather a fusion event occurring between the stem cell and target tissue. The transplantation of male HSC into female recipients directly addresses this issue by allowing for fluorescent in situ hybridization of tissue samples looking for the Y chromosome and determination if a fusion event has occurred. After fully-grown GFP males are euthanized and sacrificed, the long bones in the legs were immediately removed. All muscle, tendon, and ligature was dissected from the bone which was immediately placed in ice-cold PBS. Each bone end was then pruned back about 1-2 millimeters to expose the hollow core of the marrow space. The bone marrow was flushed out into a tissue culture treated plate by inserting a 26-gauge needle into one end of the bone and washing 1-2 milliliters of Dulbecco's Modified Eagle's Medium (Gibco) through the hollow bone core. The cells were kept on ice at all times. The liberated marrow was then triturated with a 26-gauge needle to break up the cell clumps and allowed to adhere to a tissue culture treated plate (Gibco) for 120 minutes. This step allows for an initial enrichment of HSC from other adherent progenitor cells such as mesenchymal stem cells (MSC) since hematopoietic progenitor and stromal cells adhere to the tissue culture treated plastic, while HSC will remain suspended in the media. The complete volume of media containing the nonadherent HSC was then gently drawn up, washed in >10mL volume of cold media, and pelleted by centrifugation at 1000 x g performed at 4 degrees Celsius. The cells were resuspended and stained as outlined by the protocol of the Milteny MACS system in the following section.

Initial Purification of HSC by MACS

Initial HSC purification was done through sorting of the cells by magnetic beads using the Milteny Magnetic Activated Cell Sorting (MACS) system. Briefly, cells were

stained with an antibody conjugated to a magnetic bead. The antibody, and subsequently the bead, is bound to the cell. When these cells are then run over a column in the presence of a magnetic field, those cells which have the specific surface antigens, and thus the antibody-bead bound to them, will adhere to the column (termed positive fraction). Cells which do not present that surface marker (negative fraction) will pass directly through the magnetic field and be removed from the positive fraction of cells. The magnetic field can then be removed and the positive fraction collected from the column.

To begin the MACS enrichment, cell number and viability were determined from the total marrow flushed from the long bones to ensure that the correct amount of antibody, beads, and staining volume will be used. To determine the cell number, I resuspended the washed cells in trypan blue and counted bright cells using a hemacytometer under a phase-contrast microscope. The enumerated cells were then washed in >10mL cold PBS and stained with Sca-1 microbeads (Milteny) in appropriate volume. The cells were run over 2 separate columns to insure enrichment, and the flow-through was discarded and the positive fraction retained. At this time a >90% Sca-1 positive purity typically has been achieved. After enrichment, cells were immediately pelleted and placed back on ice for fluorescent antibody staining for FACS sorting.

Final Purification of HSC by FACS

Again all antibody concentrations and incubation times were followed according to the parameters described by the manufacturer guidelines. For HSC purification I used three different fluorochromes: C-KIT conjugated to APC, biotynylated Sca-1 (with Streptavidin-PharRed secondary antibody), and the lineage markers B220, CD3, CD4, CD8, CD11B, GR-1, and TER-119 all directly conjugated to PE (Pharmingen). The

FACSvantage SE is able to isolate single cells based on the surface antigen bound by antibodies and hence the spectrum of absorbance and fluorescence emitted by that cell. Two rounds of purification are needed to ensure complete removal of all non-HSC cells. See Figure 2-1 for of an example of the gates used to enrich and isolate single HSC.

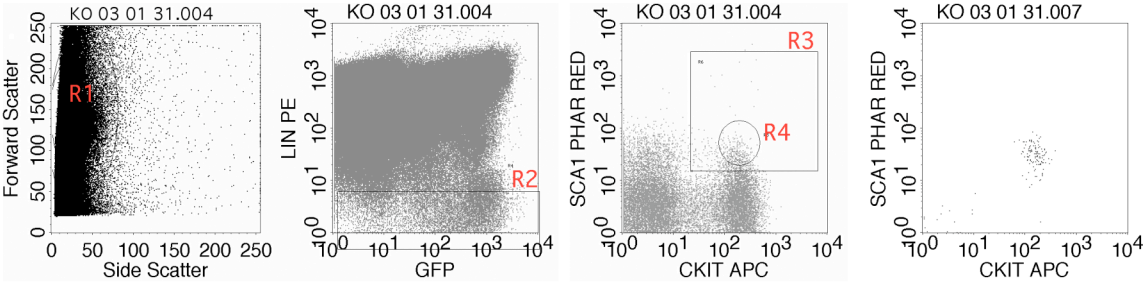

Figure 2-1. Fluorescence activated cell sorting gates for isolating HSC. HSC were removed from bone marrow, enriched by MACS, and stained for SKL surface expression. First panel: Forward and Side Scatter of MACS enriched cells with gate R1 drawn. Second panel: Cells are enriched for GFP and Lineage positive cells (B220, CD3, CD4, CD11b, Gr-1, Ter-119) are depleted excluding gate R2. Third panel: Sca-1 and c-kit positive cells from gate R1 and R2 are enriched in gate R3. Cells are then further enriched by gate R4 based on the same parameters. Panel 4: Reanalysis of cells based on Sca-1 and c-kit expression. These doubly sorted enriched cells were used for transplantation.

The flow rate is set at 10,000 events per second with no greater than a 10% abort proportion. The cells were then collected in media immediately after completion of the sort, isolated, and injected into the recipient animals following "rescue" marrow isolation and recipient preparation kept on ice at all times.

Harvesting of BL6 Rescue Marrow with HSC Depletion, and Irradiation of Recipient Animals.

The harvesting of non-GFP female BL6 marrow was performed in the same manner as the HSC, except these cells were not given time to adhere to the tissue culture treated plate. Once the marrow was flushed, washed, and counted, a Sca-1 depletion was done to remove any HSC from the rescue marrow which would compete with the donor

GFP HSC. This rescue dose is administered for twofold reasons. The immune system of the irradiated animal will experience an interruption and often the animal will become anemic. Until the HSC can engraft and repopulate hematopoiesis, these short term rescue progenitors will help the animal mount an immune response and provide the necessary blood products as needed. Again cells were stained as described in the MACS magnetic bead section, but this time the cells were Sca-1 depleted three times to ensure that the rescue marrow was devoid of HSC. Recipient BL6 mice were finally irradiated with 950 RADS of gamma radiation to prepare the bone marrow for transplantation.

Purified GFP HSC and Depleted Rescue Marrow Transplantation and Ensuing Animal Husbandry Concerns

The HSC depleted rescue marrow was count as above and 1 x 10^6 cells in a 100 microliter volume were aliquoted into a fresh Eppendorf tube. The highly enriched HSC were then singly isolated in the following manner. A volume of the sorted sample was placed on a glass drop slide and examined under a phase-contrast microscope. The cells were diluted to a concentration where single cells can be visualized, isolated, and captured one at a time with a micropipette. Under the scope a single, round, bright, viable cell was isolated and drawn up into a pulled glass micropipette by mouth pipetting with a suction tube. The needle was examined to visualize the cell to ensure that only one cell was drawn. The cell was then place into the 100 microliter aliquot containing the HSC depleted rescue dose. The rescue/single HSC mixture was drawn into a fresh insulin needle and syringe to ensure no contamination of other samples. Finally, an anaesthetized, irradiated BL6 animal was injected in the retro-orbital sinus cavity. The animals were monitored until they overcome the effects of the anesthetic and then be

placed on a regime of antibiotics for the next month until multilineage engraftment had been verified.

Verification of Multilineage Reconstitution

The recipient animals were given one month for the HSC to home to the bone marrow niche and begin to divide to produce progenitor cells which will contribute to the various hematopoietic cell lineages. Determination of engraftment was resolved by peripheral blood sampling and FACS analysis to determine whether the marrow was repopulated or if the animal's native marrow recovered. Each animal had a peripheral blood sample drawn through a tail vein bleed and the blood was collect in a tube containing PBS and 5mM EDTA to act as an anticoagulant. The erythrocytes were removed with a FICOLL PLAQUE (Amersham Biosciences) purification. Briefly, the blood/PBS sample was layered on top of two times greater volume of FICOLL. The emulsion was centrifuged and the "buffy" layer containing the nucleated cells at the interface was removed. The lymphocyte layer containing the nucleated cells was washed in 5X volumes of PBS and stained with the various lineage marker antibodies conjugated to PE. Samples were analyzed by FACS caliber, and animals exhibiting GFP positive cells of the various lineages were scored positive for engraftment. The positive animals were then monitored an additional three months where multi-lineage reconstitution is reconfirmed to demonstrate long-term engraftment by HSC. Exogenous growth factor was then administered as described below.

Induction of Retinal Ischemia

The next step involves administration of an endogenous growth factor and vessel damage in order to promote blood vessel growth in the retina. Fully and robustly engrafted animals were selected and anaesthetized. VEGF was administered directly into

the vitreous using a 36-gauge needle and Hamilton syringe. Either purified (40ug/kg) VEGF protein (Sigma) or (2 x 10^8 particles) AAV-VEGF (VectorCore, UF), where CMV promoter drives expression of VEGF in an Adeno Associated Vector, was used. VEGF is an endothelial cell-specific mitogen which is transcriptionally regulated by the cytomegalovirus promoter/enhancer when packaged in AAV. AAV mediates long-term expression in nondividing cells, which allows for stable expression and constant amounts of VEGF to reach the area of ischemia to promote neovascularization.[30]

The study of clinical diseases such as Diabetic Retinopathy and Retinopathy of Prematurity has led to an understanding of the pathology which occurs in these diseases. In these conditions the eye "detects" a lack of oxygen, either due to the diabetic condition leading to leaky vessels, or the removal of a prematurely born baby from an incubator's oxygen-rich environment. The model takes advantage of this neovascularization by creating a local region of ischemia in the eye through cauterizing of large blood vessels with a laser. As a result, the cells signal new blood vessel growth in the region in an attempt to relieve the ischemic pressure.

Peak expression of VEGF by AAV has been determined to be at 3-6 weeks, therefore the physical disruption of the blood vessels is done during this time (unpublished data). First, mice were anaesthetized normally with a general anesthetic, and concurrently a 10% sodium fluorescein (Akorn) solution was administered intraperitineally. This dye labels blood vessels facilitating visualization during photocoagulation. The eyes were dilated with 1% atropine (Akorn) for 5 minutes, washed with PBS (Gibco), and subsequently dilated with 2.5% phenylephrin (Akorn) for 5 minutes. Immediately after the two 5 minute treatments the mice underwent laser

treatment. An Argon Green laser system (HGM Corporation) was used for retinal vessel photocoagulation with the aid of a 78-diopter lens. The blue-green argon laser (wavelength 488-514 nm) was applied to various venous sites juxtaposed the optic nerve. The venous occlusion were accomplished with >60 burns of 1-sec duration, 50 millimeter spot size, and 50-100 milliwatt intensity. Again the animals were allowed to recover for 30 days while the transplanted HSC, directed by the ischemia and induced by the VEGF, contributed to the neovascularization in order to relieve the hypoxia produced by the cauterizing of the existing vessels.

Eye Removal

One month after ischemic injury the eyes were ready to be enucleated and neovascularization imaged by confocal microscopy. Mice were first anesthetized and then perfused while sedated. Peripheral blood and bone marrow was collected to confirm donor contribution analysis by FACS with lineage specific antibodies conjugated to PE (BD BioSciences) similarly to the procedure outlined above. First, the chest cavity was opened and the ribs cut away to expose the heart completely. The left atria was punctured with a 26-gauge needle and injected with >3 mL of 50 mg/mL tetramethyl rhodamine isothiocyanate (TRITC)-conjugated dextran (160,000 avg. MW, Sigma Chemical) in phosphate-buffered formaldehyde, pH 7.4. The perfusion was performed slowly into the left ventricle and is integral for the functional assay. Immediately afterwards the eyes were removed by sliding a curved forceps underneath the eyeball and pulling the globe out. The eye was punctured with a 26-gauge needle to allow complete perfusion. The eye was placed in fresh 4% PFA and shaken at room temperature for 30 minutes. The globe was then transferred to 1X PBS and washed by shaking at room temperature for 30 minutes to overnight. After washing with PBS the eyes were

dissected. To do this I placed the eye under a surgical microscope and made an initial incision in the cornea. The opening was enlarged until it could accommodate the lens of the eye. The lens was gently pushed forward until it exited through the hole cut in the cornea. The remaining cornea was then trimmed to where the sclera and cornea meet. The retina was dissected away from the retina pigment epithelial (RPE). To do this I gently pushed down on the posterior portion of the RPE and rolled the forceps forward. The retina then detached and was readily mounted. The thickness of the retina (>200um) prevents adequate perfusion of antibody, therefore the retina was placed on a glass slide and 5-6 cuts were made around the periphery so that the retina lies flat when mounted. The tissue was placed in Vectashield mounting medium (Vector Laboratories) to inhibit photo-bleaching. The retinas were immediately imaged. I used an Olympus IX-70, with inverted stage, attached to the Bio-Rad Confocal 1024 ES system for fluorescence microscopy. A Krypton-Argon laser with emission detector wavelengths of 598nm and 522nm differentiated the red and green fluorescence. The lenses used in our system were the (Olympus) 10X/0.4 Uplan Apo, 20X/0.4 LC Plan Apo, 40X/0.85 Uplan Apo, 60X/1.40 oil Plan Apo and 100X/1.35 oil Uplan Apo. The software was OS/2 Laser Sharp.

CHAPTER 3
THE HEMATOPOIETIC STEM CELL HAS HEMANGIOBLAST ACTIVITY

During development there are several types of stem cells broadly classified based on their ability for form specific tissue types. After fertilization during the first few days of division, the embryonic cells are described as totipotent. They have the capacity to produce all the cells, tissues and organs that make up the body along with all of the extraembryonic tissue of the trophectoderm. After the first four to five cell divisions, the embryo forms a hollow sphere called the blostocyst. The blastocyst contains a population of cells located in the inner wall which are capable of producing each of the over two hundred different cell types of an organism. These differ from the totipotent cells in that no one of them can produce an entire organism, nor can they produce the cells of the trophectoderm. Finally, after birth and into adulthood, several types of tissues have cells residing within them which are able to produce the tissue type where they reside. This can occur constantly, such as the hematopoietic stem cell producing all of the blood cells, or only in times of stress or injury such as the oval cells producing hepatocytes. These stem cells are called multipotent, and in most cases under "normal" conditions these cells are thought to produce only one cell type.

In the adult, stem cells are believed to define unspecialized cells that can self-renew (or proliferate) for extended periods of time without differentiating. This process is not well understood, but is believed to involve asymmetric cell division where a copy of itself is produced along with a further differentiated daughter cell. These stem cells exhibit a stable, normal chromosome complement and cannot perform any specialized

functions. However, they do have the potential to give rise to cells with specialized functions-- a process known as differentiation. It is suggested that some of these cells may be able to differentiate into multiple non-related cell types, a characteristic called plasticity.

Adult Hematopoietic Stem Cells

Adult hematopoietic stem cells are defined by their ability to both self renew and provide all of the hematopoietic cells necessary to replace those lost each day. The bone marrow produces an estimated 2-3 million cells per second or over 200 billion per day. The tremendous proliferative potential of these cells would quickly be exhausted throughout a lifetime if there were not some self-renewing parent call to maintain hematopoietic and lymph system progenitor cells. This proliferative and self-renewing capacity make HSC excellent clinical tools for the treatment of hematological malignancies such as leukemias and lymphomas. In these conditions, the bone marrow population, most notably the HSC, is replaced by cells which are non malignant and healthy to reconstitute normal hematopoiesis of an individual. In research, our ability to enrich for HSC coupled with their easy transplantability opens up large realms of exploration. Similarly to other multipotent stem cells, HSC and believed to retain a significant ability to transdifferentiate. These two characteristics make the HSC ideal for identifying the potential of HSC to regenerate or contribute to non-hematopoietic tissues following injury or stress. This data has yielded a large amount of initial excitement, however there has since been a cooling in the enthusiasm due to the increased, though warranted, scrutiny. In order for cell-based therapy to have clinical applications, basic criteria and standard must be established to determine if the phenomenon researchers are characterizing is true HSC plasticity and cannot be attributed to artifact. As a result

several stringent criteria have been outlined which must be fulfilled in order to demonstrate true plasticity.

The criteria demonstrating HSC plasticity is three-fold. First, the cell must be capable of self-renewing and homing to the bone marrow thereby reconstituting hematopoiesis for the lifetime of the organism. This is necessary so that short term progenitors are not used as therapy which may slowly die off as progenitors differentiate and are not replaced. Long-term repopulating self-renewing cells must be transplanted so that the therapy would not fail and the disease or pathologic condition reemerge. Secondly, the bone marrow contains a myriad of cell types ranging from those along any point of hematopoietic development to the supporting cells of the stroma. During a bone marrow transplant, a number of these cells could be transplanted with the bolus containing the enriched HSC no matter stringent the purification parameters. These "contaminating" cells could contribute to the tissue type where the donor-derived tagged cells are found confounding results. In order to conclusively demonstrate the plasticity of the HSC, clonal studies must be done. Through clonal transplants, a single cell must be shown to be able to produce the blood along with the non-hematopoietic tissue. These experiments exclude the possibility of several different cells accomplishing different roles, and tissue which arises from the donor must necessarily be from the single cell. Finally, for these cell based therapies to be practical it must be demonstrated that the plasticity measured is robust and functional transdifferentiation into the non-hematopoietic tissue. Many cells, especially those of the immune system, are capable of assuming the general morphology or even surface marker expression of cells they are nearby either due to stimulation or macrophage engulfment. It must be demonstrated that

the cells are physiologically performing the role of the tissue they are replacing, i.e. cells that are residing in the pancreas having the morphology and characteristics of beta cells must actually produce insulin to be therapeutic. In addition, a few isolated cells capable of producing insulin will not rescue a person from diabetes, therefore the transdifferentiation or plasticity must be robust producing a physiologically relevant amount of tissue. Only when these three stringent criteria have been met can the cell be classified as plastic. To date there has been relatively few examples fulfilling all three, although those that have present some exciting potential.

One of the initial studies have shown that after long term stable hematopoietic reconstitution by a single bone marrow HSC, donor-derived cells could be found in multiple tissues including the brain, skeletal and cardiac muscle, liver, and endothelial cells.[82] This elegant work used a homing assay to isolate HSCs which presented stem cell specific surface markers and then were able to successfully home to the bone marrow niche. These homed cells were then isolated and single cells were transplanted into lethally irradiated recipients. While this work was of note, there was as significantly low level of contribution to the various tissues and there was no functional assay of the donor-derived cells. It does, however, suggest the exciting possibility of regeneration of various damaged tissues by HSC-derived progenitors. Two notable studies also demonstrated the plasticity of the HSC in liver to replace hepatocytes injured chemically.[83, 84] Excitingly, these cells were able to restore liver function, however, clonal assays were not done in these transplant studies. In addition, Orlic *et al.* demonstrated the functional recovery of cardiac muscle through HSC transplantation.[50] After these initial pioneering papers a flood of work was embarked upon, however since then the tide was stemmed due to the

difficulty of meeting all three criteria.[85] Grant *et al.* has developed a model mimicking diabetic retinopathy, and using this model we have been able to expand the understanding of HSC while fulfilling the three plasticity criteria.[30]

Diabetic Retinopathy

Diabetic retinopathy is the leading source of legal blindness among working-age Americans. It is caused by damage to the small blood vessels in the retina as a result of diabetes mellitus. It is estimated that over fourteen million people in the United States have diabetes with approximately half of these individuals not yet diagnosed and unaware of the condition. Ninety percent of patients with diabetes have noninsulin-dependent diabetes mellitus (NIDDM) and control their blood sugar with oral medications or diet alone. The other ten percent have insulin-dependent diabetes mellitus (IDDM), and must use insulin injections daily to regulate their blood sugar levels. Although diabetic retinopathy is frequently seen in both types of diabetes, patients with IDDM are at greater risk for Diabetic Retinopathy complications. The risk increases over time for all patients with diabetes. After five years, approximately one-quarter of patients with IDDM have retinopathy and by fifteen years, nearly everyone with IDDM experiences retinal damage. Diabetics as a group have twenty-five times the usual risk of blindness.

The entire vasculature of a diabetic individual experiences the pathologic changes including plaque formation and swelling of the endothelial cells. These vessels have a diminished capacity to carry blood, and consequently all downstream tissue becomes ischemic. This ischemia causes changes in existing vasculature by stimulating compensatory growth. This pathologic growth is unstable and the vessels are fragile. As a result their rupture can cause leakage of blood into the vitreous and consequently vision loss.

Once pathologic retinopathy has developed, laser photocoagulation is currently the mainstay of treatment. Laser surgery has been used in the treatment of diabetic retinopathy for more than twenty years and its benefit has been clearly established. The abnormal neovascular vessels of proliferative diabetic retinopathy are treated with panretinal laser photocoagulation (PRP). This type of laser involves treatment to the peripheral retina which is not receiving adequate blood flow due to the vessel pathology. By photocoagulating the ischemic regions the stimulus that drives the neovascular process may be halted. This type of laser treatment is frequently successful in stopping the growth of the abnormal vessels, but in some cases they may regress. It is not without side effects as some loss of peripheral and color vision is normal following this type of treatment. Ironically it is the existing PRP laser treatment in humans from which we developed our mouse neovascularization model described in chapter 2 and used throughout this body of work.

Angiogenesis vs. Neovascularization

Our diabetic model is an example of neovascularization. During neovascularization, *de novo* blood vessels are formed which are not derived from preexisting vasculature. The cells which contribute to neovascularization are derived from a distant source, namely the HSC residing in the bone marrow. Contrastingly, angiogenesis is the process of endothelial cell sprouting from pre-existing vasculature.[15] Local endothelial cells, even with their diminished capacity to divide, are able to produce enough daughter cells to supply blood vessel lining, i.e. normal endothelial cells turnover is replaced by neighboring cells. Under conditions of severe injury or in some pathologic condition such as diabetic retinopathy, these vessels are derived from the EPC. *In vitro* studies have shown that EPC are capable of producing tube-like structures under culture

conditions and can be derived from bone marrow cells.[18, 86, 87] Pro-angiogenic factors such as VEGF and GM-CSF increase the number of circulating EPC in the adult and have been shown to promote blood vessel growth.[88, 89] In addition, hydroxymethlyglutaryl-CoA reductase inhibitors are efficient stimulators of EPC transdifferentiation and formation of endothelial cells involving the Akt protein kinase pathway.[90] *In vivo*, several groups have shown that EPC contribute to blood vessels in adult organisms to relieve cardiac ischemia, however these models used short-term progenitor cells in an acute injury model.[29, 91, 92] While clearly the EPC can functionally provide therapy for ischemic injury, these studies did not demonstrate whether these EPC were derived from the HSC or from some other cell such as the mesenchymal stem cell.

During development, the pluripotent progenitors which contribute to the formation of both blood and blood vessels are the hemangioblasts.[93-96] The hemangioblast phenotype can also be derived *in vitro* from embryonic stem cells when cultured with VEGF.[93] The presence of an adult hemangioblast *in vivo* and the role bone marrow derived cells play in neovascularization, however, is incomplete. The work described here will elucidate the role HSC derived cells have in promoting or contributing to neovascularization and describe the plastic nature of these cells in ischemic tissue.

Results

The methods used to obtain the following results are described in detain in chapter two. Any alterations or additions of the model described will be noted.

The C57BL6.*GFP* Chimera

As described above, there are three stringent criteria for the demonstration of HSC plasticity. Briefly, the criteria are 1) the cell must be self renewing and able to provide all of the blood and blood products for the entire life or the organism, 2) the cell must be

able to do so clonally, and 3) the cell must product functional non-hematopoietic tissue in a robust manner. The C57BL6.*GFP* chimera studies will directly address these three criteria. To address question one, HSC were isolated from a donor GFP animal as described. Figure 3-1 is an example of the enriched HSC. The row of panels was obtained from a whole bone marrow preparation purified with a FICOLL gradient. A vast majority of cells are lineage positive (>80%) and Sca-1 negative (>93%) indicating that the bulk of the cellular mass in the marrow is progenitor cells. Once the cells have been enriched for HSC with MACS and FACS, a high proportion of cells have the expected surface marker phenotype of the HSC (>98% Sca-1 positive and >99% lineage negative).

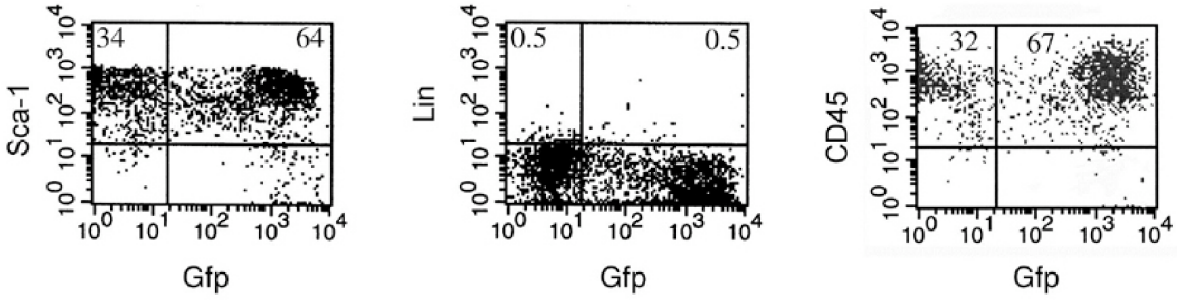

Figure 3-1. Reanalysis of HSC post-enrichment used for transplantation. HSC were flushed from the bone marrow, enriched by MACS, stained for the SKL surface markers, and enriched by FACS. Panel 1: Sca-1 expression of enriched HSC achieving 98% purity. Panel 2: Cells expressing any of the lineage markers were depleted to a 99% purity. Panel 3: 99% of the enriched cells express the pan-hematopoietic marker CD45.

These cells were then transplanted into a lethally irradiated recipient and allowed to long term engraft for three months. Once long term multilineage engraftment was demonstrated in the peripheral blood of the primary recipient, the animal was sacrificed and the GFP HSC isolated from the marrow. These cells were once again transplanted into secondary lethally irradiated recipients and allowed to engraft for four months. This

combined total represents much longer than any short-term progenitor would be able to provide hematopoiesis. Figure 3-2 depicts a representative FACS analysis of the peripheral blood of a serially transplanted mouse with donor GFP HSC. Significant proportions of the T-cell (CD4), B-cell (B220) and mylomonocytic (CD11b) lineages are donor derived (see methods chapter for description of GFP standardization). This contribution could only be from a long term repopulating, and thus self-renewing HSC.

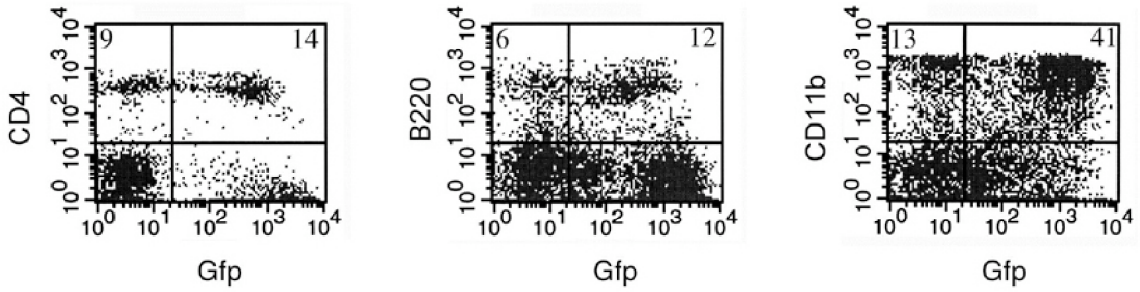

Figure 3-2. HSC can engraft multiple lineages long-term and self-renew. Enriched HSC were transplanted into a primary recipient and hematopoietic reconstitution was demonstrated long-term. HSC were then isolated from the primary recipients and transplanted into lethally irradiated secondary recipients. Peripheral blood was isolated from secondary recipients and stained for various hematopoietic lineages. Panel 1: CD4 (T-cell) lineages were donor-derived. Panel 2: B220 (B-cell) lineages were donor-derived. Panel 3: CD11b (Mylomonocytic) lineages were donor-derived.

The second criteria addresses the clonality of the HSC in its ability to produce all the blood lineages from once single cell. These experiments will also be crucial to demonstrate the ability of the HSC to produce an alternative non-hematopoietic tissue type. In these experiments, HSC were purified as above, except that during the final transplanting into the lethally irradiated recipients, one single cell was isolated and transplanted along with non-GFP rescue progenitor cells. Figure 3-3 is the peripheral blood mononuclear cells stained with the same lineage markers, T-cell (CD4), B-cell (B220) and mylomonocyte (CD11b). This figure demonstrates the clonal ability of the

HSC in hematopoiesis, or the capability of a single cell to provide all of the blood lineages. Each of these cohorts was then placed into the neovascularization model.

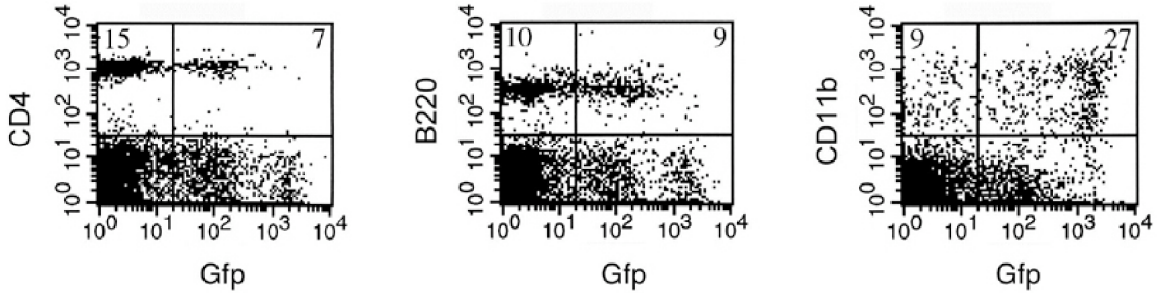

Figure 3-3. HSC can produce all hematopoietic lineages clonally. Single enriched HSC were transplanted into lethally irradiated recipients. Peripheral blood was isolated and stained for various hematopoietic lineages. Panel 1: CD4 (T-cell) lineages were donor-derived. Panel 2: B220 (B-cell) lineages were donor-derived. Panel 3: CD11b (Mylomonocytic) lineages were donor-derived.

Assessment of GFP Retinal Blood Vessel Endothelial Cells

Once long term multilineage engraftment has been demonstrated in these animals, exogenous growth factor (VEGF) was administered to prime the system for blood vessel growth. As noted, VEGF is a potent stimulator of endothelial recruitment and blood vessel formation. The VEGF is packaged into AAV which infects the cells of the retina and causes overexpression and accumulation of the protein. Indeed, the vitreous of the eye is almost completely lacking proteases, so there is ample signal for the endothelial cell formation of blood vessels. After one month to allow for peak VEGF expression, the major blood vessels of the eye are photocoagulated with a laser. This ischemic injury, combined with the VEGF, elicits a dramatic neovascular response in the retina. One month after photocoagulation the animals were sacrificed to measure the amount of HSC contribution to the new vasculature. The mice were perfused with Hoechst stain to mark the nuclei of cells and delineate vessel lumens. Eyes were removed for sectioning and immunohistochemical analysis of the donor cells for both blood and endothelial cell

surface phenotypes. This was done to determine whether the cells were truly transdifferentiated into endothelial cells, or if they were invading leukocytes or macrophages. Eyes were sectioned along both sides of the optic nerve, and more than 30 sections were obtained from each eye. The sections were stained with hematoxylin, Factor VIII, platelet endothelial cell adhesion molecule, or mouse endothelial cell antigen-32.

Figure 3-4 shows the GFP cells which surround the lumen of the newly formed vessels. These same sections when counterstained with the endothelial specific markers demonstrate that the cells lining the lumen of the vessels are endothelial in nature. Each row is of a different capillary tuft, and each is stained with a different endothelial marker. The top row is stained with Factor VIII conjugated to PE. Panel C shows that the vessel lumen is endothelial, as expected, and the GFP cells seen in panel B colocalized with Factor VIII which merge yellow in D. Panel F shows another vessel with donor derived cells (GFP in Panel F) which costain with PECAM in panel G. Another vessel has the same donor derived endothelial phenotype expressing MECA-32 (Panel J and K). Finally, in these vessels when stained with CD45, a hematopoietic specific marker, the GFP cells did not express CD45 and had entirely adopted the endothelial phenotype.

This result was also readily observed on a whole mounted retina. Figure 3-5 shows an entire retina from an animal perfused with the red fluorescent dye as described in the methods chapter. Under low power (Panel A), areas of donor derived GFP cells are visible contributing to the vasculature in the treated eyes. The contralateral, untreated eye has no such endothelial contribution, although areas where the blood was not

removed by the perfusion can be seen containing the GFP hematopoietic cells (Panel B). Under higher power magnification, GFP cells can be seen wrapping around the vessels containing the red dye in various stages of blood vessel formation (Panels C-F). Of note, this HSC contribution to endothelial cells did not occur where no ischemic injury was induced.

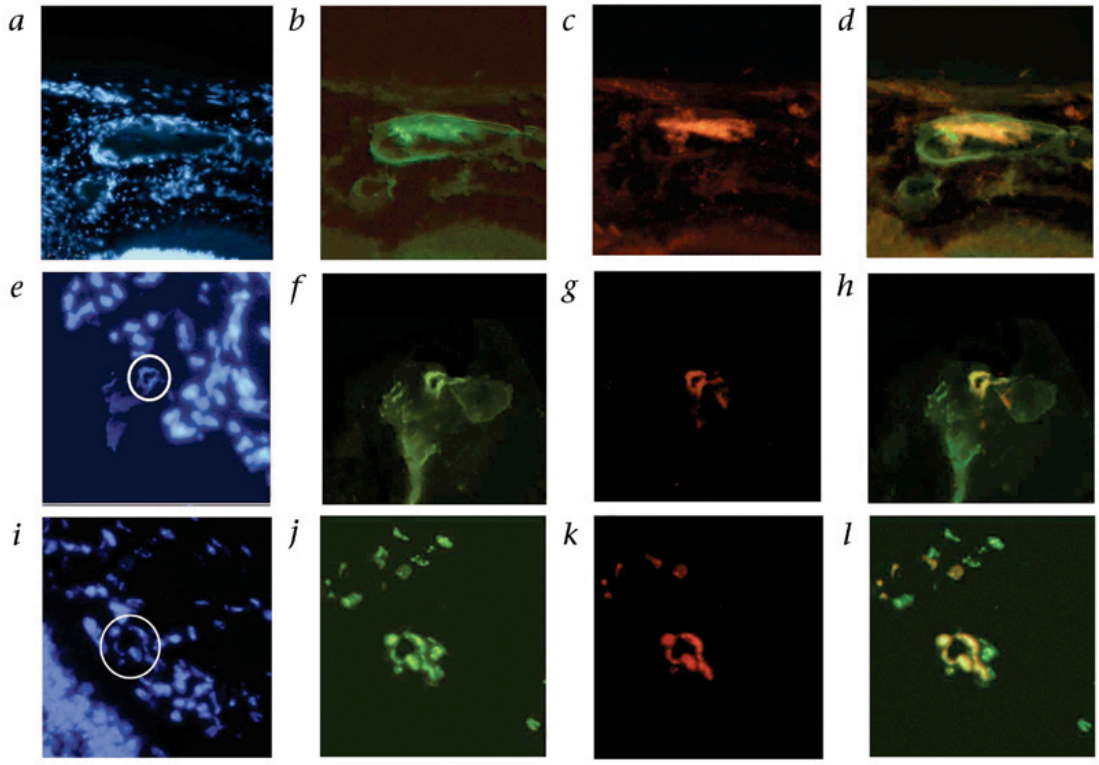

Figure 3-4. Donor-derived HSC contribute to endothelial cells of blood vessels in the eye. Neovascularization was induced in HSC engrafted animals. Retinas were sectioned and stained with endothelial specific markers. Panel A; A treated animal was perfused with a buffer containing Hoescst dye to delineate vessel lumen and a treated control retina was cross-sectioned. Panel B: The same cross-section had GFP donor-derived cells lining the blood vessel lumen. Panel C: The same cross-section was stained with an antibody to Factor VIII conjugated to PE to stain endothelial cells. Panel D: Merged images of B and C demonstrating endothelial cells which were donor-derived. Panels E-H: Another cross-section was stained with Platelet Endothelial Cell Adhesion Molecule-1 and illustrated in the same manner as A-D. Panels I-L: Another cross-section was stained with Mouse Endothelial Cell Adhesion-32 and illustrated in the same manner as A-D. Magnification is x60.

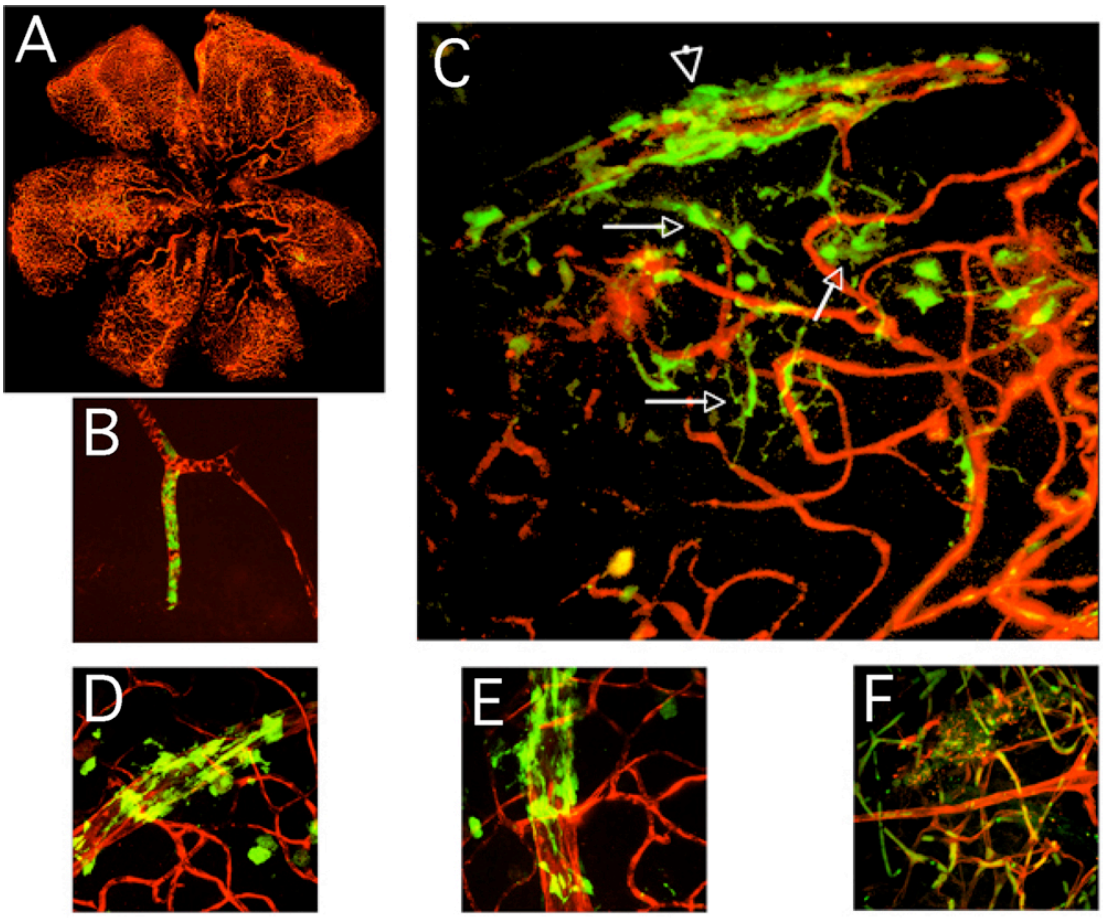

Figure 3-5. Donor-derived HSC produce functional endothelial cells surrounding blood vessel lumens. Mice were long-term hematopoietic engrafted with GFP HSC and placed into the neovascularization model. The animals were perfused with TRITC labeled dextran, sacrificed, and retinas were imaged by confocal microscopy. Panel A: A whole mounted retina is demonstrated under low magnification (x4). The red fluorescence fills the perfused, functional blood vessels. Small capillary tufts of donor-derived GFP cells, which are magnified in C, D, E, & F, can be observed around areas of photocoagulation. Panel B: From a contralateral, untreated eye, circulating donor-derived GFP hematopoietic cells are present in the lumen of a blood vessel. Magnification x40 Panel C: Donor derived-GFP cells are associating with a perfused blood vessel (large arrowhead). Other donor-derived cells have either not directly associated with vessel lumens or have extravasated and not formed endothelial tubes. Magnification x40. Panels D-F: High magnification images show GFP cells surrounding vessel lumens (D&E) and forming early neovascularization (F). Magnification x60.

The HSC has Hemangioblast Function

The recruitment of HSC derived cells to regions of injury agrees with studies done which have found limited contribution to non injured tissues.[85] In these experiments, the donor derived GFP cells were able to contribute to both the blood products and endothelial cells of the vasculature in the same mouse, however the exact cell which could accomplish these feats cannot be established by these experiments.

The classic definition of a HSC is a cell capable of long term hematopoietic reconstitution after irradiation, or self-renewing. We have fulfilled this definition through the series of transplantation studies described above, however the ability of a single HSC to do so clonality, and thus ruling out any contribution by other "contaminating" cells, was necessary to prove HSC plasticity. As described in the methods chapter, a single HSC was enriched and isolated though micromanipulation. The HSC was transplanted along with Sca-1 negative non-GFP bone marrow cells (short term progenitors) and transplanted into a lethally irradiated recipient. Of the 80 mice transplanted, peripheral blood long term multilineage engraftment was demonstrated in 3 animals which were then subjected to the ischemic neovascularization model. Each of the three animals exhibited the capillary tuft growths seen in the previous experiments that were entirely donor derived as demonstrated by GFP expression. In addition these vessels were functional in their ability to hold the red fluorescent dye perfused into the vasculature. Since these animals had both blood and blood vessels which were derived from a single transplanted HSC there can be no contribution from another source and any GFP cells must necessarily be derived from the HSC. As shown in figure 3-6, the HSC demonstrated hemangioblast activity in their ability to produce both blood and blood vessels in a clonal manner. Panels A-C are from a serially transplanted mouse. The red

perfused blood vessel (Panel A) colocalizes with the GFP donor-derived endothelial cells (Panel B) to show donor derived neovascularization (Panel C). These vessels were derived from a self-renewing HSC. Panels D-F are from a single cell transplanted animal. The red perfused blood vessel (Panel D) colocalizes with the GFP donor-derived endothelial cells (Panel E) to show donor derived neovascularization (Panel F). These vessels arose from the HSC in a clonal manner, therefore the HSC can give rise to both blood and blood vessels and function as a hemangioblast.

Discussion

The previous work demonstrates the true plasticity of the HSC and fulfills the three criteria established to prove this capacity. The self-renewing capability was demonstrated through serial transplantations and long term hematopoietic reconstitution. The ability of the HSC to provide hematopoiesis along with non hematopoietic tissue in a clonal manner was shown through single cell transplants. Both experiments demonstrated the ability of the HSC to produce functional vessels in a robust manner. Taken together, these experiments outline an alternative developmental fate of the HSC, namely the EPC, and describe how this outcome can be induced through growth factor administration and ischemic injury. The EPC was shown to be derived from the HSC, and not the MSC as previously posited.[97] This understanding is especially valuable in current treatments where the EPC has been shown to have the ability to contribute to therapeutic neovascularization in several studies of ischemic injury, some in human clinical trials.[51, 88] This work demonstrates that vessel growth is not only carried out by local or circulating endothelial cell angiogenesis, but under conditions of injury the HSC can provide neovascularization. New blood vessels formed were largely derived from the recruitment of undifferentiated precursors cells from the bone marrow.

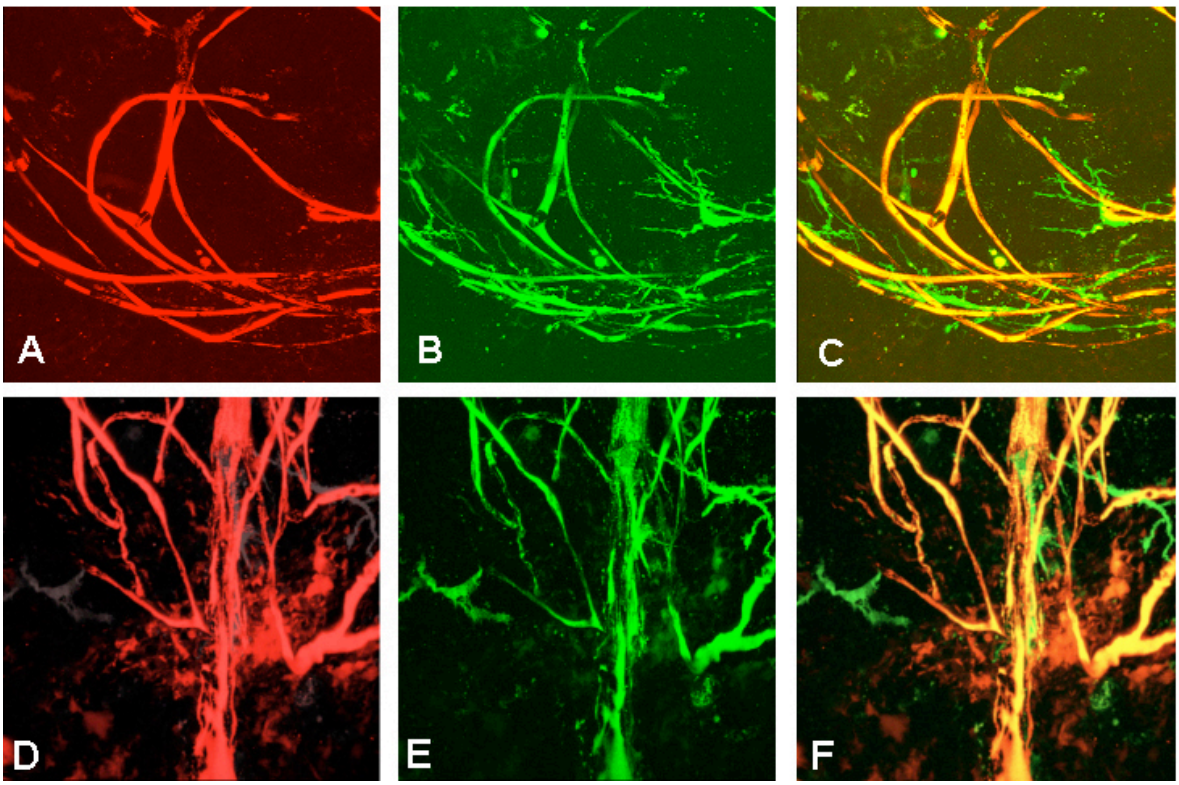

Figure 3-6. The HSC is self-renewing and can clonally form endothelial cells. Both serially transplanted long-term engrafted and single cell transplanted animals were placed in the neovascularization model. Animals were perfused with the TRITC-labeled dextran and retinas were imaged by confocal microscopy. Panel A-C: A long-term engrafted serially transplanted mouse retina was imaged. Panel A shows the red channel only indicating perfused, and therefore functional blood vessels. Panel B is the donor GFP HSC contribution to the neovascularization. A and B were merged in C and yellow areas are donor derived cells colocalizing with the perfused vessel. The HSC is self-renewing and can produce all blood lineages and endothelial cells of the vasculature. Panel D-F: A single-cell transplanted mouse retina was imaged. Panel D shows the red channel only indicating perfused, and therefore functional blood vessels. Panel E is the donor GFP HSC contribution to the neovascularization. D and E were merged in F and yellow areas are donor derived cells colocalizing with the perfused vessel. The HSC can clonally produce all hematopoietic lineages and endothelial cells lining blood vessel walls. Magnification x60.

The next series of experiments will ascertain the potential to modulate hemangioblast function. Understanding the growth factors and biological conditions during ischemia and how they regulate contribution to neovascularization by the HSC

and HSC progenitors may provide methods to manipulate blood vessel formation. Modulation of the HSC/EPC pathway may allow for tailoring of therapies to increase neovascularization in ischemic conditions such as stroke, wound healing, or cardiac muscle damage. Conversely, the ability to decrease pathologic or undesirable neovascularization as seen in tumor neovascularization or diabetic retinopathy could stem from a greater understanding of the HSC to EPC developmental fate.

CHAPTER 4
MODULATORS OF HSC/HEMANGIOBLAST ACTIVITY

Few topics have stirred more recent debate than the promise of hematopoietic stem cells (HSC) exhibiting functional plasticity. Indeed, the candidacy of HSC for therapeutic treatment of disease is contingent upon demonstrating their ability to fulfill stringent plasticity criteria. Initial papers described HSC transdifferentiation into a variety of non-hematopoietic tissues in various organs such as the liver, brain, cardiac muscle, blood vasculature, intestine and pancreas.[30, 50, 82-84, 98] Using various tissue specific markers and phenotypic characteristics these authors have described tissues to which HSC are able to contribute demonstrating plasticity of the HSC in their experimental settings. However, attempts to recapitulate these studies have found limited HSC plasticity.[85, 99] A possible rationale for these dichotic accounts is that these studies employed differing methods to isolate HSC and examine the target organ of potential transdifferentiation. Specifically, a variety of HSC isolation and purification schema have been employed including: elutriation, or separation based on relative density, serial transplantation where only those cells with the capability to home and long term repopulate the bone marrow niche rescue a mouse, and cells isolated through fluorescence activated cell sorting broken down into "side-population" studies of dye exclusion, and single KTLS (c-kit^{+}, Thy-1^{lo}, lin^{-}, Sca-1^{+}) cell transplantations.[30, 82, 100, 101] Each method isolates functional HSC as defined by long-term multilineage hematopoietic reconstitution *in vivo*. However, each technique may isolate "functional" HSC at different developmental stages with respect to plasticity. It may be improper to compare

HSC isolated by physical means, i.e. elutriation and side-population, with those isolated by binding of antibodies to cell surface receptors. The differing populations isolated and the manipulations which the cells undergo may impact their behavior in the experimental settings. These methods must be reconciled before a definitive answer can be reached.

Sharing a mesodermal kinship to the HSC are the endothelial cells (EC) of the vasculature. During embryogenesis hematopoietic and endothelial precursors develop in both spatial and temporal immediacy. In the adult, EC circulate in the peripheral blood which are phenotypically similarity to mature EC.[16] These cells have the ability to contribute to new vessel formation either in place of or in addition to resident EC proliferation. In addition, it was shown that these circulating cells contained a population which were derived from the bone marrow called endothelial progenitor cells (EPC).[17] It is now accepted that these bone marrow EPC exist and contribute significantly to adult blood vessel formation, and that these EPC are HSC derived.[30]

Several experimental systems which damage blood vessels have been able to induce robust HSC transdifferentiation.[30, 102] The preceding chapters described how adult HSC exhibit hemangioblast function by producing both blood and blood vessels in a novel model of retinal neovascularization. The model uses long-term bone marrow chimeric mice that have been stably reconstituted with hematopoietic stem cells from GFP donor mice (fulfilling the first plasticity requirement). These cells are positive for the surface markers Sca-1 and c-kit and demonstrate robust GFP expression in blood products after a four month period. This time is sufficient to eliminate any contaminating progenitor cell which would have since died off and created a deficiency leukemia in mice engrafted with these short-term progenitor cells. The long-term engrafted chimeras

then receive a combination of growth factor administration and laser induced ischemic injury to promote new blood vessel formation in adult murine retinas. Briefly, Adeno Associated Virus (AAV) expressing Vascular Endothelial Growth factor (VEGF) is administered intravitreally and allowed one month to reach peak expression. The retina is then photocoagulated and new vessels attempt to grow into the ischemic region.

Since HSC have the ability to long-term repopulate hematopoiesis, lethally irradiated mice were then transplanted with a single HSC. These animals exhibited significant GFP in peripheral blood and bone marrow all of which was derived from the single transplanted HSC proving clonality in transplanted HSC hematopoiesis. Chimeras derived from both serially transplanted and single GFP+ HSC produced whole GFP vascular beds after acute injury and VEGF induction.[30] The vessels produced were not only robust, but were functional as determined by perfusion after cardiac administration of a fluorescent dye.

The previous chapter has illustrated a new developmental outcome of the HSC: the production of EPC in response to vascular injury. The work demonstrated that both blood and blood vessels can be clonally derived from adult HSC via a combination of growth factor administration and ischemic injury. Thus, adult HSC meet the definition of a plastic stem cell in that they have the ability to act as a hemangioblast *in vivo*. Whether the HSC participates in everyday maintenance vasculogenesis or partakes only in response to chronic vessel injury remained to be determined and was one of the focuses of this work. In addition, it was determined that some form of significant injury is needed for induction of the HSC to EPC pathway, presumably since resident EC are inadequate to seed and proliferate the damaged areas. While the HSC is now known to

produce EPC under injury conditions, the potential role of physiologic mediators which impact vasculogenesis in relation to hemangioblast HSC activity merit examination.

Since their first description in 1989, the nitric oxide synthases (NOS) have been shown to play a role in a myriad of biological functions. The free radical NO, produced from the conversion of L-arginine to citrulline in the presence of oxygen, has been shown to function in distinct processes such as inflammation, host defense, neurotransmission, and smooth muscle contractility. Three distinct isoforms of the enzyme have been characterized including nNOS which is expressed in neuronal tissues, iNOS, expressed in a wide variety of tissues, and eNOS which is predominately expressed in the endothelial cells of the vasculature. The nNOS and eNOS isoforms are constitutively active in their expressing tissues, which the iNOS isoform is induced in response to proinflammatory cytokines or endotoxins from foreign bacteria. This induction of iNOS produces a 100-fold increase in NO as part of an immune response, and NO production is much higher than is seen compared to the basal levels of the constitutively active isoforms.[103] NO produced by iNOS acts as an antimicrobial and antiviral agent by decreasing DNA replication.

Nitric Oxide (NO) also mediates endothelial cell function and hence blood vessel formation. It has been shown to influence neovascularization in several models of angiogenesis.[104-106] The role of NO in promoting angiogenesis has been controversial in part because of the complex regulation of NO generation and inactivation. In addition to vasodilatation, increased local concentrations of NO stimulate proliferation and migration of endothelial cells, both of which are essential for angiogenesis.[107-109] The NO produced by the three separate isoforms are activated under distinct activities and have unique

regulatory controls.[109] Since iNOS is activated under certain pathological conditions, such our injury model, and eNOS is constitutively activated in endothelial tissues, these isoforms may influence the process of neovascularization. The altered amount of NO due to lack of these enzymes in the cell will affect hemangioblast recruitment and formation of blood vessels.

Angiogenesis is initiated by vasodilation in order to open up vessels facilitating introduction of cells in circulation to the site of blood vessel growth. NO is known to have several angiogeneic affects, including increasing matrix metalloprotease expression along with tyrosine phosphorylation of proteins in cells populating the sprouting capillary region.[74] Interestingly, in various neovascularization models NO has been shown to be both proangiogenic and antiangiogenic.[74, 104] The theory is that the two isoforms are activated under differing circumstances and hence are thought to affect blood vessel formation differently. Indeed, *in vivo* this is the case. Blood vessel formation due to HSC contribution under conditions of ischemic injury are influenced by NO as produced by the iNOS and particularly the eNOS isoforms.[110-112]

The endothelial NOS (eNOS) isoform is constitutively expressed at basal levels by endothelial cells and is thought to promote branching, organization, and maturation or endothelial cells during vessel development. eNOS deficient (eNOS-/-) animals exhibit fetal growth restrictions, reduced survival, and an increased rate of limb abnormalities.[113] They also demonstrate marked vascular pathology such as increased cardiomyocyte apoptosis, congenital septal defects, postnatal heart failure, decreased capillary density and vascular permeability.[114] Endothelial cells from eNOS-/- animals demonstrate decreased rates of angiogenesis with reduced branching *in vitro*.[115] These animals also

exhibit an impairment of postnatal angiogenesis in response to growth factors and ischemia.[116] Correspondingly, eNOS has been shown to mediate the mitogenic effect of VEGF on cultured microvascular endothelium.[106] These findings led to the *in vivo* work demonstrating that NO production is essential for angiogenesis in hindlimb ischemia, for wound healing, and coronary collateral growth after myocardial ischemia.[115, 117]

VEGF has been shown to be a potent vascular permeability factor and plays a leading role in angiogenesis, and our model takes advantage of this ability to promote blood vessel synthesis.[73] The angiogenic effect of VEGF under both pathological and physiological conditions has been revealed to be predominantly mediated by eNOS.[118] VEGF promotes NO production from eNOS in EC cells, and inhibition of eNOS by pharmacological agents *in vivo* have decreased angiogenesis and vascular permeability induced by VEGF.[105] This demonstrates that eNOS is both a downstream mediator of VEGF induced angiogenesis and an upstream promoter of VEGF expression. This results in putative positive feedback loop between NO and VEGF which promotes angiogenesis.[119]

The inducible NOS (iNOS) isoform is expressed by endothelial cells in response to external stimuli such as VEGF, proinflammatory cytokines or lipopolysaccharide. iNOS activation results in a 1000-fold greater generation of NO then eNOS activity alone.[120] Its induction is thought to promote tube elongation during vessel development, although evidence supports that it may have a contrasting anti-angiogenic effect.[79] iNOS deficient animals (iNOS-/-) are relatively healthy but do have a slight decrease in NO production and vascular permeability during angiogenesis in collagen gels placed in a mouse cranial window.[106] During normal blood vessel formation the interplay between eNOS and

iNOS activity has been postulated to dictate vessel size and degree of branching. In this chapter I will describe experiments where wild-type GFP+ HSC are transplanted into eNOS-/- and iNOS-/- recipients to assess the effect of NOS dysfunction in tissue on hemangioblast activity.

Results

iNOS and eNOS GFP chimeras demonstrated robust HSC engraftment.

To directly assess the role of NOS activity in the promotion of HSC trans-differentiation into blood vessels, cohorts of wild-type (WT) C57BL6, iNOS-/-, and eNOS-/- animals were generated. Animals were transplanted with 2,500 highly enriched GFP+ HSC. It was necessary to use a highly enriched HSC population rather then a single HSC due to the poor survival of eNOS-/- animals during transplant and the difficulty in producing single cell transplanted animals in general. The enriched HSC populations used were isolated using the same protocol previously employed for single cell transplants in WT animals.[30] Briefly, whole bone marrow was obtained from the bone marrow of GFP animals. Cells were plated on tissue culture treated plates for 2 hours during which time the adherent cell population, which contains progenitor cells such as the mesenchymal stem cell, stick to the plate. Non-adherent cells are collected and stained with Sca-1, c-kit, and the lineage markers. Cells which were sorted by FACS for the stem cell markers of Sca-1 and c-kit and were lineage negative were injected intravenously through the retro orbital sinus. Long-term multilineage hematopoietic engraftment was confirmed >3 months post transplant by flow cytometry analysis of peripheral blood and is shown in figure 4-1. The first column in each cohort represented is peripheral blood stained for B-cells expressing B220, with the second column stained for macrophages expressing CD11b, and the third column T-cells expressing CD4. The

top row (C57BL/6) and the second row (*GFP* donor strain) are supplied for reference controls to facilitate comparison between recipient and donor background fluorescence. The third row is a typical C57BL/6.*GFP* chimeric mouse demonstrating robust hematopoietic engraftment. The forth row (iNOS.*GFP*), and fifth row (eNOS.*GFP*) are representative of engraftment levels of transplanted knockout animal's blood lineage profiles. The bottom row is peripheral blood stained for VEGFR2 demonstrating that GFP EPC are in the circulation of C57BL/6.*GFP,* iNOS.*GFP,* and eNOS.*GFP* animals. Engrafted recipients were subsequently termed C57BL6.GFP, iNOS.GFP, or eNOS.GFP chimeras. Recipients that were robustly reconstituted by donor HSC (>75% donor derived myeloid cells) then underwent our model of ischemic injury to induce adult retinal neovascularization (n > 10 for all cohorts). By waiting >3 months post transplant before inducing retinal ischemia, it is assured that the ability of HSC exclusively to regenerate blood vessels is being assessed. No other cell that can be directly isolated from the marrow has been shown to be capable of long-term reconstitution in a transplant setting. Any contaminating precursor cells would not have had the ability to repopulate the bone marrow for this extended period of time and would have long since disappeared from the circulation. Further proof of the plastic ability of the HSC is demonstrated in previous work where we illustrate how a single adult HSC is capable of making both blood and blood vessels in a transplant recipient eliminating the possibility of any other contaminating cell. Also, this activity is serially transplantable producing functional vessels in a robust manner.[30]

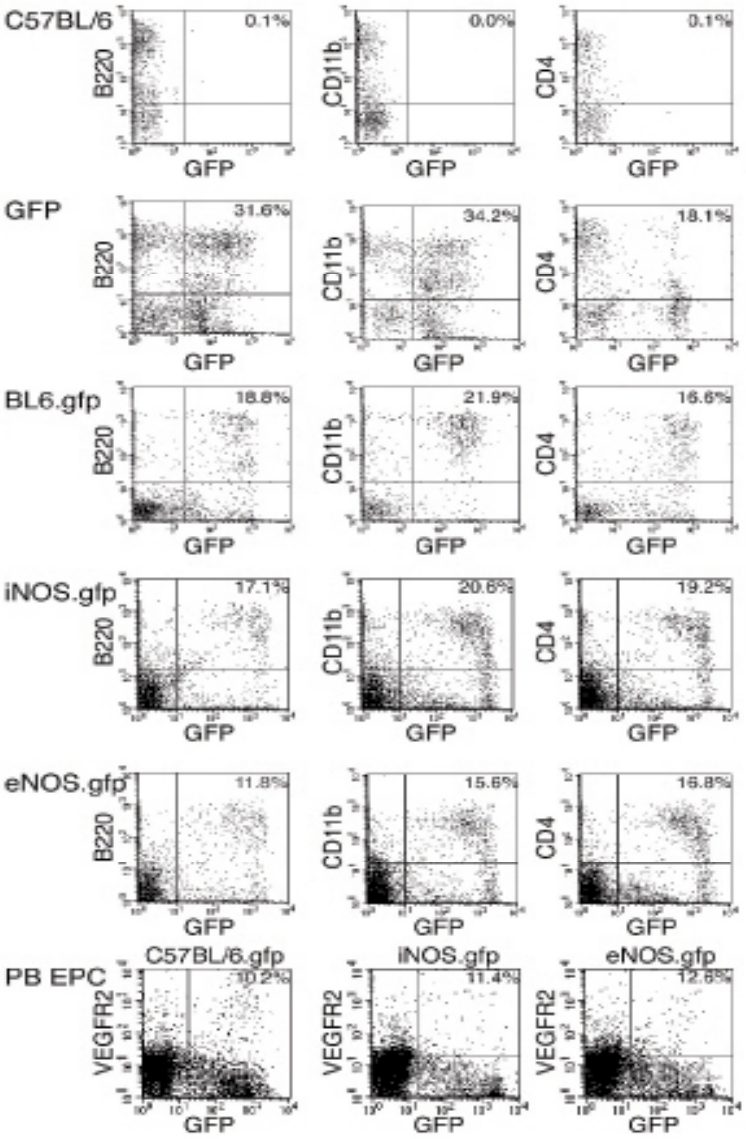

Figure 4-1. NOS knockout animals exhibit long-term, multi-lineage, donor *GFP* peripheral blood engraftment. Peripheral blood mononuclear cells were analyzed by flow cytometry 3 months post-transplant. The first column is B-cells expressing B220, the second column is macrophages expressing CD11b, and the third column is T-cells expressing CD4. The top row (C57BL/6) and the second row (*GFP* donor strain) reference controls show recipient and donor fluorescence. The third row is a representative C57BL/6.*GFP* chimeric mouse demonstrating robust hematopoietic engraftment. The forth row (iNOS.*GFP*), and fifth row (eNOS.*GFP*) are representative of engraftment levels of transplanted knockout animals. The bottom row is peripheral blood stained for VEGFR2. Circulating VEGFR2 positive cells are in C57BL/6.*GFP,* iNOS.*GFP,* and eNOS.*GFP* animals. Numbers in the top right corner are percentages of doubly lineage stained and *GFP* positive cells. iNOS= Inducible Nitric Oxide Synthase, eNOS= Endothelial Nitric Oxide Synthase.

The NO pathway affects blood vessel formation

After induction of retinal ischemia by laser ablation injury, C57BL6.GFP chimeras produced a variety of GFP+ blood vessels at the sites of injury ranging from small capillaries to larger vessels. Size was most likely dictated by the degree of the laser injury as seen in the original hemangioblast characterization (Fig. 3-5 C and Fig. 3-6 C & F). Strikingly, the NOS.GFP chimeras produced a marker phenotype change from the wild-type mice indicating a role for the NOS pathway in hemangioblast function. iNOS.GFP chimeras produced primarily small, highly branched blood vessels that perfused readily (Fig 4-2 E and G) when injured. These vessels were largely donor-derived as shown in the red-green merged images demonstrating the colocalization of the perfused fluorescent dye and the GFP cells. The contralateral eyes had little to no donor contribution as is seen in Figure 4-2 D and F. This indicated that the eNOS isoform, which is still present, is sufficient for maintenance of vascular health, and that iNOS plays a role in blood vessel branching.

In contrast, eNOS-/- mice retinas exhibited a marked phenotype when compared to both control and iNOS -/- animals done in parallel. Strikingly, eNOS.*GFP* chimeras only produced relatively large and unbranched vessels of donor origins regardless of ischemic insult (Fig. 4-3 D-F). These vessels tended to perfuse poorly in spite of their large size. Of note, this phenotype was not due to the inability to visualize the red dye in large vessels due to the fact that the fluorescent perfusant can be easily visualized in B6 control vessels of similar size. In addition, a few small vessels were readily perfused and the animal demonstrated the gross muscle contraction and liver color change indicative of sufficient perfusing. This is consistent with the known vascular defects of $eNOS^{-/-}$

animals. Whether this lack of vessel functionality is due to some vascular blockage of some alternative defect is not known.

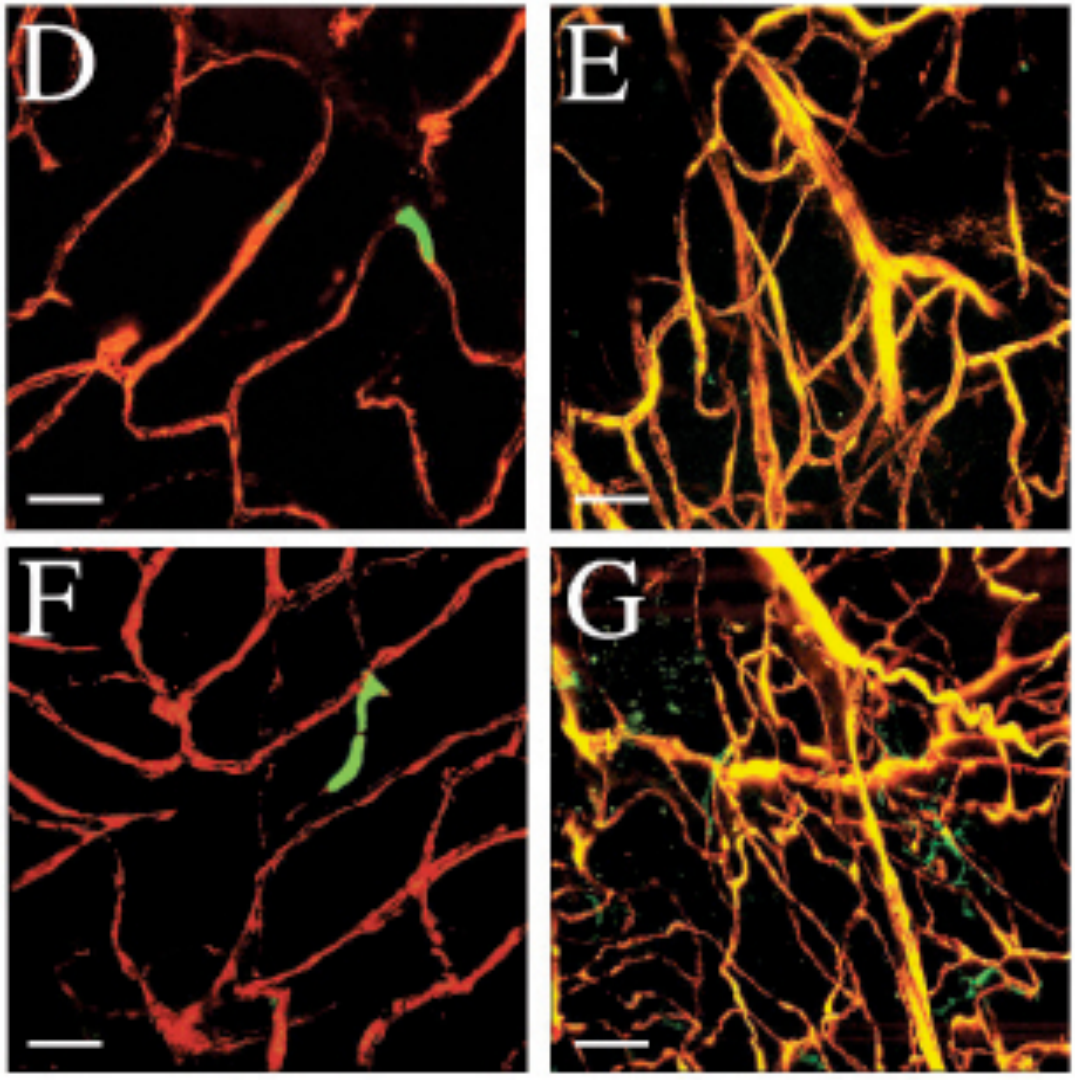

Figure 4-2. The iNOS pathway modulates hemangioblast neovascularization. iNOS.*GFP* chimeric mice underwent the retinal ischemia model followed by perfusion with TRITC-labeled dextran before eye enucleation and confocal imaging of the retinas. All panels are red and green merged confocal images. Panels D and F are retinas from control, untreated eyes. There is little GFP contribution observed (yellow). Panels E and G are from treated eyes where robust GFP contribution can be seen to vasculature. Magnification is 60X and size bar is ~10μM.

Figure 4-3 demonstrates the large and unbranching characteristics of the donor-derived vessels. These pictures are red-green merged confocal images and the lack of red perfuasnt indicates how poorly these vessels function. Panels E and G are from treated eyes where robust GFP contribution can be seen to vasculature forming large, unbranched vessels that do not contain the TRITC-dextran. Panels D and F are retinas from control, untreated eyes. There is significant GFP contribution observed with or without ischemic treatment indicating that the eNOS pathway plays a significant role in endothelial cell maintenance.

The profound contribution of HSC derived GFP+ cells to the untreated retinas of eNOS-/- recipients strongly suggested that deletion of this gene induces chronic vascular injury. While injury was known to be necessary for HSC hemangioblast activity, this work demonstrates that a chronic lack of eNOS can also induce neovascularization. If this postulate is true the transplanted GFP+ HSC should contribute to vascular regeneration throughout the eNOS-/- recipient. The evaluation of neovascularization in contralateral eyes demonstrates and agrees with current studies that determined some type of injury is required for functional plasticity of HSC. In typical physiologic conditions little or no HSC contribution to “normal” tissue occurs, but when acute (ischemic injury) or chronic (eNOS knockout) pathologic conditions arise HSC readily contribute to vascular tissue.

These experiments formally demonstrate that iNOS activity at the site of vascular injury dictates the size and branch characteristics of new vessels formed in adult animals. Furthermore, the new vessels are formed in all, or large part, from circulating endothelial progenitors of HSC origin.

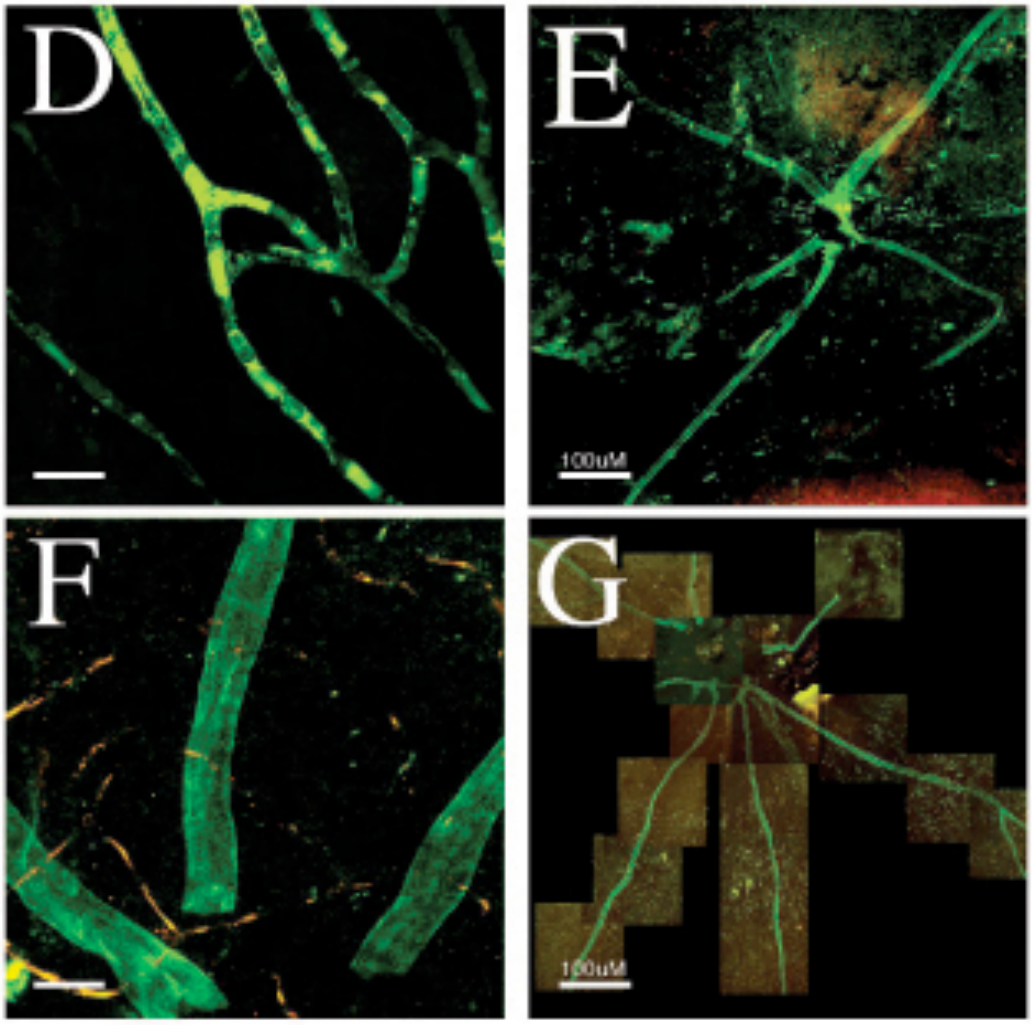

Figure 4-3. The eNOS pathway modulates hemangioblast neovascularization. eNOS.*GFP* chimeric mice underwent the retinal ischemia model followed by perfusion with TRITC-labeled dextran before eye enucleation and confocal imaging of the retinas. All panels are red and green merged confocal images. Panels D and F are retinas from control, untreated eyes. There is significant GFP contribution observed, however the vessels formed do not contain the TRITC-dextran, therefore are poorly functional. Panels E and G are from treated eyes where robust GFP contribution can be seen to vasculature forming large, unbranched vessels which do not contain the TRITC-dextran, therefore are poorly functional. Panel D is 60X. Panel E is 4X magnification. Panel F is 10X magnification. Panel G is a composite of 60X images. Size bar is ~10μM unless noted ~100μM.

The NOS pathway affects blood vessel branching characteristics.

To further examine the role of NOS in neovascularization, retinas from the non-treated contralateral eyes were compared to the injured retinas of WT, iNOS-/-, and eNOS-/- recipients. This was done in order to elucidate whether NOS could drive HSC formation of vasculature without ischemic injury and growth factor administration. iNOS-/- animals responded in a similar fashion to WT animals with production of GFP+ HSC derived vessels in the injured retina (Fig. 4-2 E & G), but little or no contribution could be found in the retinas from the contralateral untreated eye (Fig. 4-2 D & F). Unexpectedly, retinas from eNOS-/- recipients, which as described in other studies have systemic vascular dysfunction, demonstrated robust GFP+ HSC derived contribution to the preexisting vascular endothelium of both test (Fig. 4-3 E & G) and control eyes (Fig. 4-3 D & F).

After induction of retinal ischemia by laser ablation injury, C57BL6.*GFP* chimeras produced a variety of GFP^+ blood vessels at the sites of injury ranging from small capillaries to larger vessels. In C57BL6.*GFP* chimeras, size was most likely dictated by the degree of the laser injury (Fig. 3-5 D-F) and no GFP^+ contribution to vasculature was observed in control eyes (Fig. 3-5 B). iNOS.*GFP* chimeras produced primarily small, highly branched blood vessels that perfused readily in treated eyes (Fig. 4-2 D & F). These animals had limited donor EPC contribution in contralateral untreated eyes (Fig. 4-2 E & G). Strikingly, eNOS.*GFP* chimeras only produced relatively large and unbranched vessels regardless of ischemic insult (Fig. 4-3 D through G). The branching characteristics of the three strains were markedly different suggesting that the NO pathway functions in vessel organization. Total branch points of *GFP* vessels per 60X field of view were counted for each genotype (Figure 4-4). C57BL6 model control

cohorts averaged about 18 branch points per visual field. iNOS$^{-/-}$ recipients had nearly 3-fold more branch points per field, while eNOS$^{-/-}$ recipients averaged 61 times less.

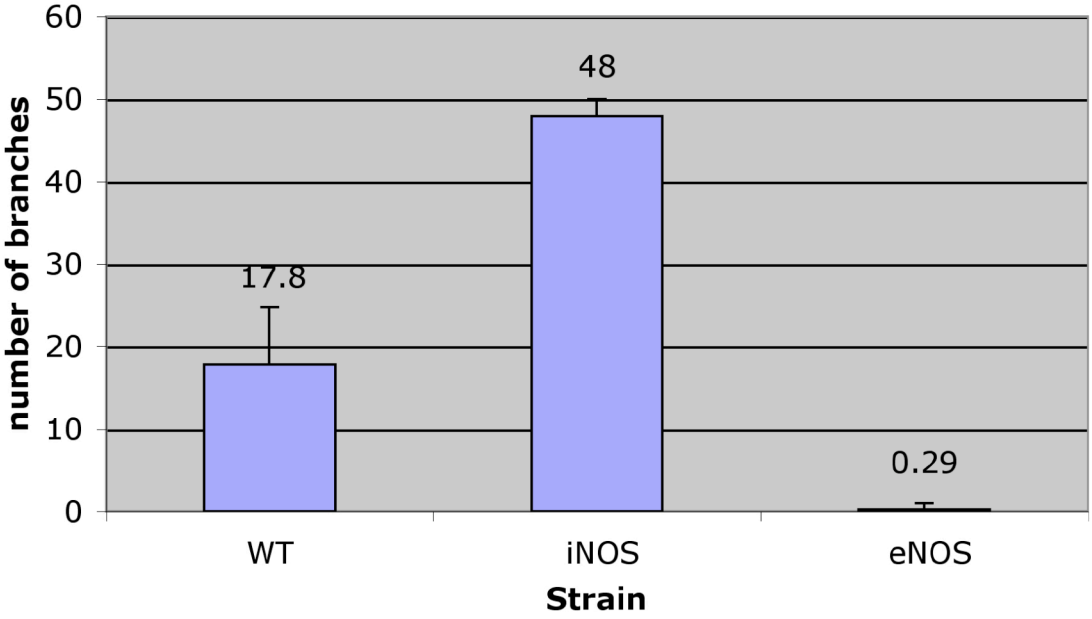

Figure 4-4. The nitric oxide pathway alters hemangioblast blood vessel formed branching characteristics. Confocal Z-series images were compressed and counted "blindly" for number of vessel branch points per image. C57BL/6.*GFP* retinas averaged 17.8 branches per image (n=5). iNOS.*GFP* retinas averaged 48 branch points per image (n=4). eNOS.*GFP* retinas averaged 0.29 branch points per image (n=38). The blood vessels of iNOS$^{-/-}$ retinas were 2.7 times more branched than WT animals ($p< 0.0001$) while eNOS$^{-/-}$ were 61.5 times less branched than WT ($p< 0.0002$).

These experiments formally demonstrate that NOS activity at the site of vascular injury dictates the size and branch characteristics of new vessels formed in adult animals. Furthermore, the new vessels are formed in all, or large part, from circulating endothelial progenitors of HSC origin. In addition, a chronic lack of eNOS activity over time, combined with our ischemic injury model, results in a proliferative retinopathy into the preretinal space, the hallmark of proliferative retinopathy developed in diabetic patients.

NO production affect on vasculature in non-ocular tissue

The finding that HSC have the ability to contribute to vascular tissue in non-treated eyes during a disease state lends to the examination of tissues far removed and unrelated to the eye. To determine the extent of donor GFP+ HSC contribution to the overall vascular system multiple tissues (spleen, thymus, brain, kidney, liver, muscle, skin, and gut) from the C57BL6.GFP, iNOS.GFP and eNOS.GFP chimeras (n=10 per cohort) were harvested. Each of these animals had demonstrated long-term, multilineage hematopoietic engraftment and had undergone the retinal ischemia model. At one month after the induction of retinal ischemia the animals were euthanized and perfused with tetramethyl rhodamine isothiocyanate-conjugated dextran (TRITC, a red fluorescent dye) through the left ventricle. Tissues were harvested and immediately placed in optimum cutting temperature medium and frozen to preserve GFP. 10 micron thick sections were then cut and mounted with DAPI to stain the nuclei. Sections were examined by fluorescent microscopy for GFP+ contributions to the vasculature. Results for the spleen, thymus and brain are shown (Fig. 4-5). In all cases the C57BL6.GFP and iNOS.GFP yielded similar results: limited evidence for GFP+ cells being incorporated into blood vessels in any tissue outside of the treated retina (Fig. 4-5 A-F, and data not shown). This indicates that whole body irradiation alone is not sufficient for induction of HSC contribution to vasculature in tissues which are not treated further. In contrast, eNOS.GFP chimeras exhibited robust GFP+ contributions to the vasculature (as determined by co-localization with the perfused red fluorescent dye) in all tissues examined (Fig. 4-5 G-L, and data not shown). Lack of eNOS creates a pathologic vascular condition where HSC are induced to contribute to vascular repair throughout an organism.

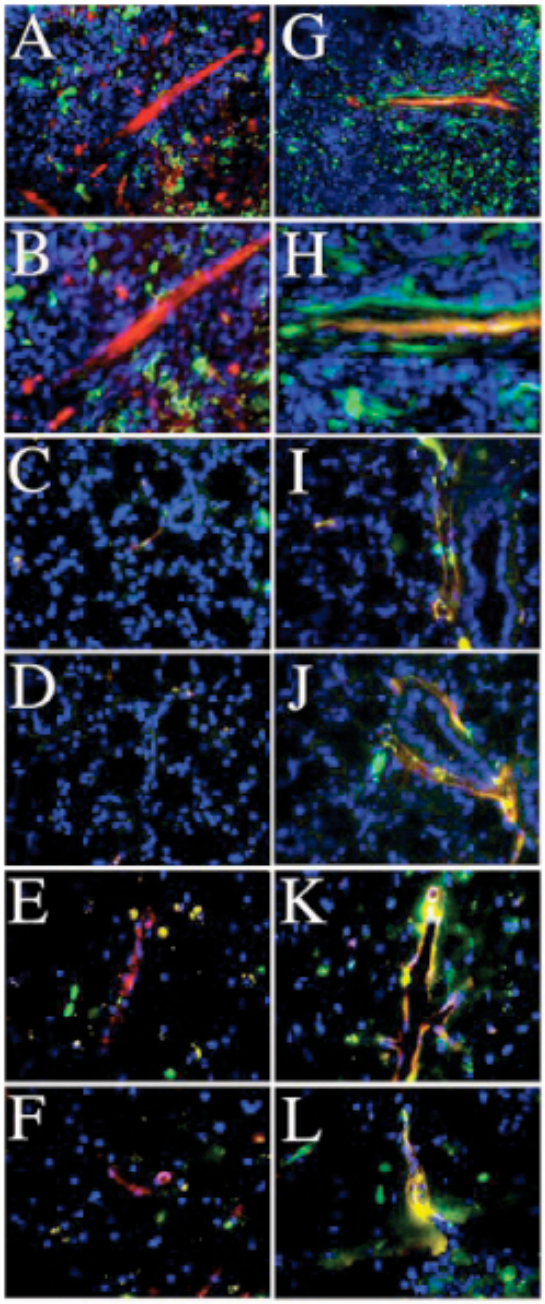

Figure 4-5. Chronic vascular injury in eNOS.*GFP* chimeras induces widespread hemangioblast activity from adult HSC. NOS knockout animals which underwent the neovascularization model, and spleen (A, G, B &H), thymus (C, I, D & J), and brain (E, K, F & L) were harvested from TRITC perfused animals. 10μM cryosections were prepared and mounted with Vectashield plus DAPI. iNOS.*GFP* (A-F) and eNOS.*GFP* (G-L) chimeras were examined by fluorescence microscopy. The donor *GFP* HSC derived cells are green, and the TRITC-labeled dextran perfusant is red. Panels A & G are magnification X40. All remaining panels are all magnification X64.

To ascertain the endothelial cell nature of the GFP+ cells surrounding the vessel lumens tissue sections were stained for the pan endothelial cell marker MECA-32. Ten micron frozen sections were stained with a primary antibody to MECA-32, followed by a Texas Red conjugated secondary antibody and DAPI. Endothelial cells were then scored for the presence of both MECA-32+ and GFP+ cells via fluorescent microscopy. Splenic sections demonstrate the characteristic results observed in all tissues studied (Fig. 4-6). Donor derived hematopoietic cells in the spleen serve as internal negative staining controls for MECA-32 in each section. WT animals showed occasional GFP+, MECA-32+ endothelial cells in the brain (closest organ to the site of VEGF administration) with the majority of tissues such as the spleen (Fig. 4-6 A-D), kidney, liver, and muscle showing no donor derived endothelial cells. iNOS-/- animals, which exhibit minor systemic vascular defects, had occasional GFP+, MECA-32+ endothelial cells in the spleen (Fig. 4-6 E-H) and other tissues.

Overall GFP+ HSC derived contribution to the vasculature of iNOS-/- animals, outside the area of retinal ischemia, was at most 1% in >150 sections examined for MECA-32+ vessels. Robust GFP+ donor derived endothelial cell production was observed in eNOS-/- recipients which have been demonstrated to have chronic and severe vascular pathology. Most vessels were quite large, and most showed extensive GFP+ HSC derived MECA-32+ endothelial cell contributions in the spleen (Fig. 4-6 I-P) and other tissues examined. The HSC contribution to vasculature detected in untreated tissue was analogous to that observed in the treated retinas demonstrating that chronic vascular injury appears to be sufficient to induce the hemangioblast activity of adult HSC.

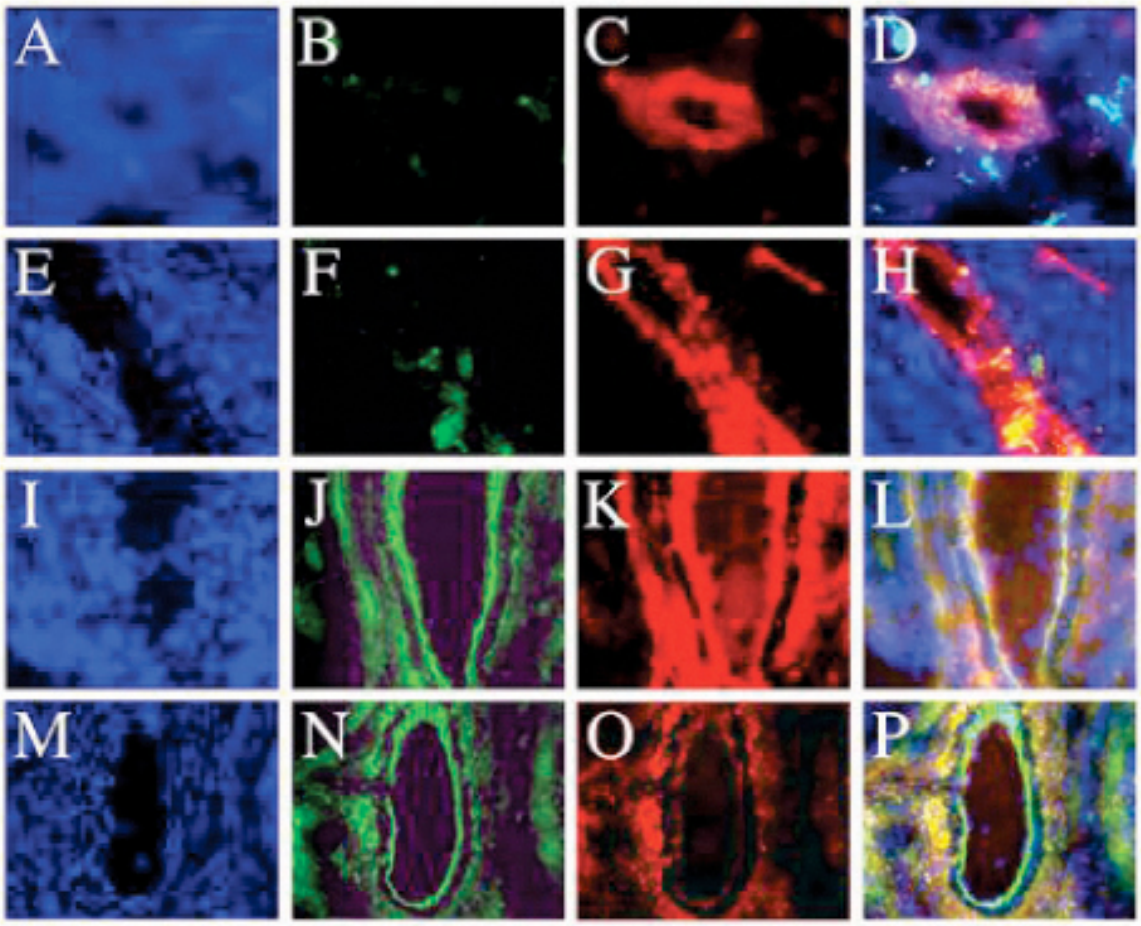

Figure 4-6. Donor-derived cells lining vascular lumens in eNOS.*GFP* animals are MECA-32 positive. Splenic cryosections were prepared from C57BL/6.*GFP* (A-D), iNOS.*GFP* (E-H), and eNOS.*GFP* (I-P) chimeras. Sections were stained with anti-MECA-32 antibody and a Texas Red conjugated secondary antibody to delineate vascular endothelium. Sections were mounted with DAPI (A,E,I,M) to delineate nuclei with blue fluorescence, examined for *GFP* expression (B,F,J,N) via green fluorescence, or MECA-32 staining (C,G,K,O) via red fluorescence. Merged images of the DAPI, *GFP*, and MECA-32 Texas Red stains are shown in D,H,L and P. (A-L) Magnification X64. (M-P) Magnification X32.

Quantitation and location of NOS produced in knockout animals.

To ascertain the influence and determine the expression of NOS in retinas lacking one specific NOS isoform, retinas were dissected and stained with isoform specific antibodies. iNOS-/- (Fig. 4-7 A-C top) and eNOS-/- (Fig. 4-7 A-C bottom) animals were

quantitated for NOS expression in parallel. Animals were sacrificed and the eyes enucleated as described. The dissected retinas were then imaged through confocal microscopy. Figure 4-7 (A top and bottom) demonstrates that in each knockout strain the isoform which is deleted is not expressed *in vivo* at detectable levels. The iNOS -/- has relative amounts of NOS expressed (B and C top), while the eNOS -/- retinas demonstrate an increase in iNOS expression as seen throughout the large, and particularly the smaller vessels (B and C bottom). This confirms that there is an upregulation of iNOS expression in eNOS knockout retinas indicating a dysregulation in amount of NO produced resulting in the pathologic blood vessel formation observed in these animals.

Discussion

Through growth factor administration and ischemic injury to the retina, HSC can be induced to transdifferentiate into vascular endothelium. Furthermore, transdifferentiation can also be observed during a pathologic disease state of chronic vascular injury. The substantial role NO plays in vascular tone, and the presence of a NOS isoform specifically found in endothelial cells hinted at a role of NO in blood vessel formation and remodeling. NOS activity can also dictate the general size and branch characteristics of new blood vessels formed in response to ischemic injury and growth factor administration. Using the neovascular model of inducing HSC hemangioblast activity to promote blood vessel formation in the adult retina, donor WT HSC transplanted into iNOS-/- recipients produced highly branched vessels that are generally smaller in size. These HSC are functioning in an environment where local NO production is similar to what is seen in wild type, non-infection conditions due to the eNOS isoform which is constitutively active in endothelial cells. A wide variety of vessel sizes are formed which are functional as measured by perfusion of marker dye.

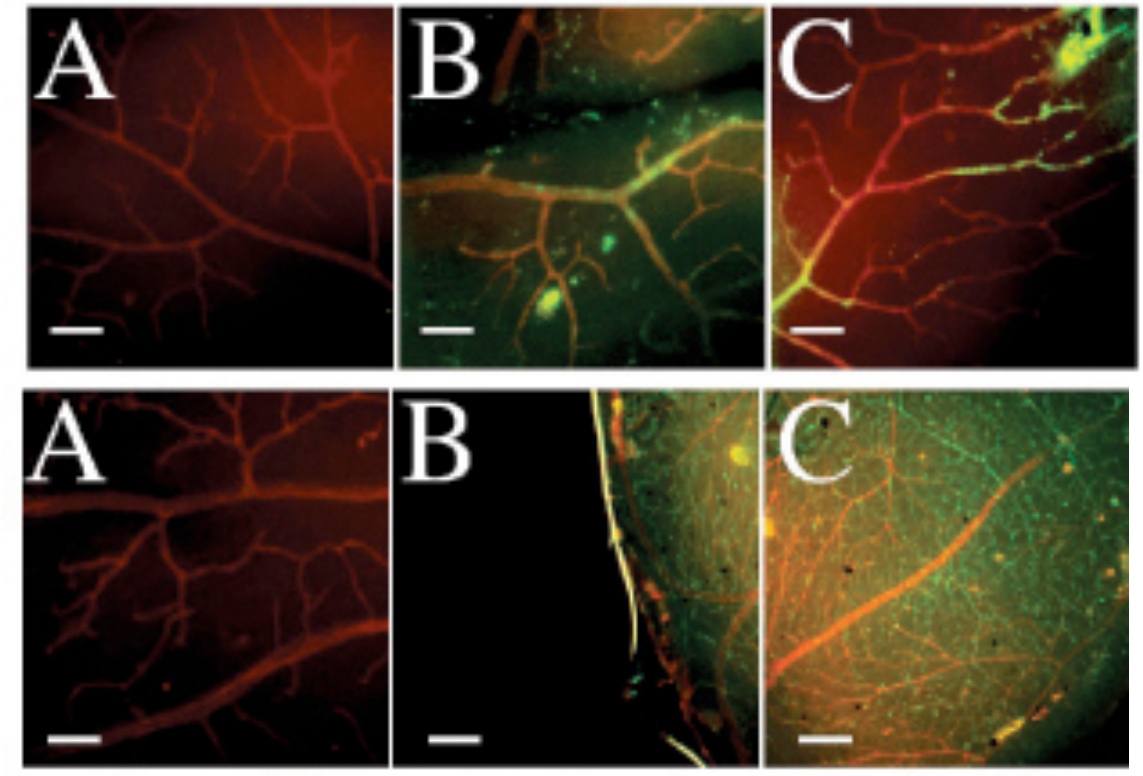

Figure 4-7. Nitric oxide production is dysregulated in eNOS knockout animals. $iNOS^{-/-}$ and $eNOS^{-/-}$ retinas were stained with NOS isoform specific antibodies. Vessels were illuminated by agglutin staining (red) and regions which were positive for the NOS antibody are green. In the top row, panel A depicts an $iNOS^{-/-}$ retina stained with iNOS specific antibody. Panels B and C are $iNOS^{-/-}$ stained with eNOS isoform specific antibody. In the bottom row, Panel A depicts an $eNOS^{-/-}$ retinas stained with eNOS isoform specific antibody. Panel B and C are $eNOS^{-/-}$ stained with iNOS isoform specific antibody.

The branch patterning is similar to normal mouse vasculature, although slightly increased. Contrastingly, eNOS-/- recipients produced primarily unbranched vessels of large size. This indicates that the local NO production due to the eNOS isoform activity is necessary for normal hemangioblast derived blood vessel formation. Modification of NO production via the eNOS isoform, which can now be specifically targeted with pharmacological agents, could provide a means to influence neovascularization and

angiogenesis in pathologic diseases such as Diabetic Retinopathy, and Retinopathy of Prematurity. This altered HSC response was in addition to the widespread vascular remodeling by donor HSC seen throughout the eNOS-/- recipients even in non-injured organs and tissues. The vessels of eNOS-/- recipients were difficult to perfuse indicating their general vascular dysfunction. This further emphasizes the crucial nature of the eNOS isoform's NO production to ensure proper vessel formation and functionality foreshadowing studies occurring outside the realm of the eye on which our model focuses.

This chapter reiterates work demonstrating that an injury state, whether it be acute as seen in the photocoagulation of blood vessels, or chronic as seen in the vascular pathology observed in the eNOS knockout animals, is required for HSC plasticity.[85] Furthermore, local mediators, including VEGF and NO, can greatly influence not only the size and amount of new blood vessels formed but also their functionality. The regulation of NOS activity as a means to influence the remodeling of vascular beds may provide specific treatment regimes. It may prove beneficial to selectively inhibit a particular NOS isoform to correct an imbalance thus altering the development of new blood vessels. Whether a deregulation of NOS or NO activity is a causal effect in human proliferative retinopathy remains to be determined. If this is the case, pharmaceuticals that affect these activities, some already in use for non-related disease treatment, may provide an effective therapy or preventative for human diseases in relation to proliferative or pathological blood vessel formation due to hemangioblast activity.

CHAPTER 5
LIMITATIONS OF STEM CELL RESEARCH AND ETHICAL CONSIDERATIONS

While the promise of stem cells as therapy is considerable, there are limitations to their usage including their biological activity and ethical implications. The biological limitations of stem cell plasticity are related to their ability of to transdifferentiate and their potential to form tissues. The ethical implications are many, and I will address some of them in this chapter.

Biological limitations

In the current research environment, there is currently heated controversy over the reported plasticity of stem cells, particularly the HSC in relation to cardiac muscle, liver, and the nervous system.[121] The limitations of adult stem cells include :their diminished capacity to proliferate when compared to embryonic stem cells. Autologous transplantation of cells back into a patient will still retain genetic abnormalities. Correspondingly, ex vivo epanded or genetically modified cells used for therapy may produce unforeseen consequences. Some tissues arise through a complex developmental fate, such as the pancreas which is derived from the infolding of several tissue layers which will not be easily mimicked in vitro. The resident stem cells within a tissue type are extremely rare. This paucity of cells makes them extremely difficult to identify, isolate, and purify. In addition, it had proven difficult to culture adult stem cells compared to embryonic ste cells in vitro limiting their use as potential therapy. Finally fusion It is believed that cell fusion may be a potential method for introducing new genetic material to correct mutated or malfunctioning genes that cause disease. Cell

fusion occurs when two or more cells combine to form one cell which then contains more genetic material than normal. Fusion has been shown to occur in embryonic along with adult stem cells.[122, 123] In adult mice, fused liver cells may contain 80 chromosomes, double the amount found in a normal mouse liver cells. Other cell types, including megakaryocytes and muscle cells function with an increased ploidy as well. In most cell types, however, aneuploidy would be detrimental often inducing apoptosis or cell transformation. The resulting imbalance in gene dosage could lead to nonfunctional tissue or cancer. It is not clearly understood, nor has it been definitively proven whether fusion is a pathway for stem cell plasticity with research indicating that fusion can result in, but is not necessary for plasticity.[124-126] If the former is the case, then investigation into the effect of producing cells with an increased ploidy at any time must be done. A fusion event during contribution to the regenerated tissue could preclude stem cell based therapy.

The first work characterizing fusion done by Terada *et al.* found that fusion events *in vitro* were extremely rare.[122] Consequently, the robust amount of donor derived contribution is unlikely to arise from such rare events. Recent work from our lab demonstrated that the cells derived are diploid, and any unresolved fusion of the HSC would have resulted in an increase in ploidy shown in figure 5-1.[127] The circulating endothelial precursor cells derived from the donor marrow exhibit normal 2N ploidy when stained with the DNA dye propidium iodide. This does not rule out the possibility of a fusion event which was resolved resulting in normal ploidy, however, and special specific experiments much be done to ascertain if this fusion resolution is occurring.

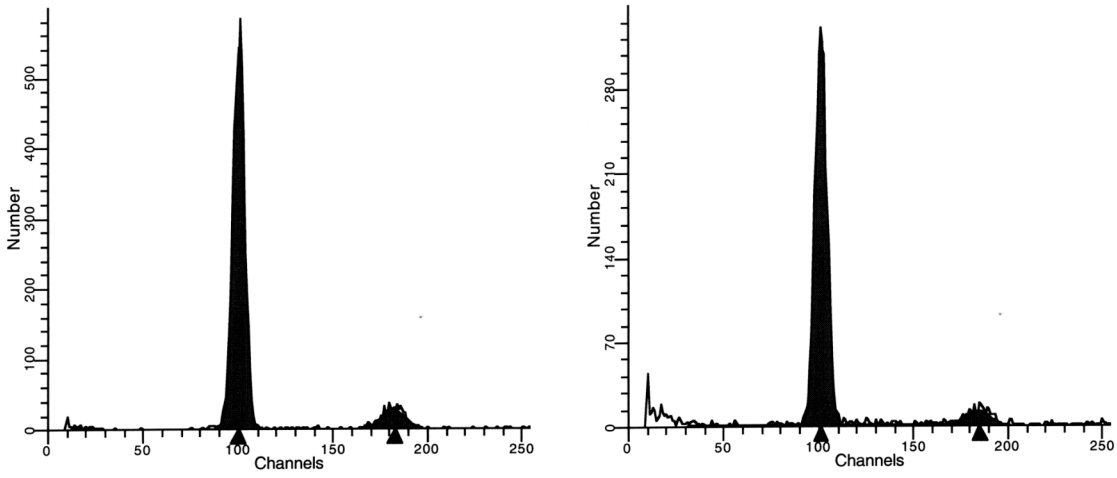

Figure 5-1. Propidium iodide staining of circulating EPC does not indicate abnormal ploidy. Long-term GFP engrafted C57BL/6 recipients underwent the neovascularization model. Animals were bled, and FICOLL enriched peripheral blood was stained and FACS enriched for VEGFR2 expression. Cells were stained with propidium iodide and analyzed by FACS. Left panel depicts EPC from a nontransplanted C57BL/6 animal bled in parallel. Right depicts a test animal. Both exhibit classical diploid staining profiles.

Ethics

Any comprehensive analysis of the stem cell field would be remiss to not include the ethical implications for their use in therapy. The field has proven to be a polarizing issue which influences many religious and political referenda born simultaneously with Dolly, the first cloned mammal. The debate arises from the use of embryonic stem cells which at this time can only be isolated from an embryo resulting in the embryo's destruction. These cells are attractive, however, because of their ability to produce all of the cell types in an adult animal or provide an environment in which DNA can be transferred in nuclear transfer. To date no single adult cell has been shown to have the pluripotency of embryonic cells along with providing the intracellular environmental cues necessary to reprogram transferred DNA. Adult stem cells do not have the same capacity

to produce any tissue or cell types. This inherent extraordinary therapeutic potential resulting from the destruction of an embryo leads to opposition based on the idea of when life begins.

In the United States, many Fundamentalist Christian groups are strongly opposed to embryonic stem (ES) cell research as the destruction of the embryo is considered abortion, or murder. They believe that any and all research using human stem cells is morally unacceptable. Other religions, however, are supportive of embryonic research. Many Jewish groups of differing denominations do not view an early stage embryo as a human being, therefore usage of embryonic tissue is not destruction of a human. Many Humanists, Unitarian Univeraslists, and Muslim clerics have also come out in favor of stem cell research. In addition, proponents point out that stem cell research uses discarded embryos from *in vitro* fertilization, and that fertility clinics routinely destroy thousands of embryos. These unused embryos would normally be discarded or kept frozen indefinitely if not used in research. There is no general consensus among religious groups which gives rise to many concerns over the use of ES cells.

Concerns Over Stem Cell Use

Certainly, stem cells are not the first human discovery to revolutionize scientific knowledge and create waves of ethical debate. Since ancient times society has admonished man for approaching these boundaries as exemplified in the Greek myth of Icarus who did not heed his father's command; he reveled in the "unnatural" sensation of flight and then plummeted to his death after the sun melted his wings. This Greek myth embodies our apprehensions about interfering with nature. Galileo Galilei expanded the frontiers of astronomy and posited that the Earth rotates on its axis and revolves around the Sun. This led to his eventually condemnation for heresy. In Victorian times, society

grappled with the balance between the knowledge gained from performing autopsies for crucial understanding of human anatomy versus the desecration of those who were dead. Even recently, complete consent to produce recombinant DNA for lifesaving medications such as insulin has been granted but only after vehement protestations over genetic engineering. There are shared concerns among all instances of testing medical boundaries, and the concerns of stem cell technology include issues of safety, efficacy, and resource allocation. For decades, patients have undergone adult HSC transplantation in the treatment of immune deficiencies and cancer. Although graft-versus-host disease and posttransplantation infections are major risks of allogeneic bone marrow transplants, investigators have worked to minimize these consequences and many patients accept these risks in the hope of the lifesaving benefit of disease eradication. However, the field of stem cell therapy is still in its infancy and researchers are incrementally improving safety, efficacy, and applicability to a wider spectrum of disease. In all instances of expanding the horizons of our knowledge a societal consciousness was at play, often times encumbering progress and questioning techniques of intervention.

Stem cell therapy differs from previous technologies in how these potential sources of regenerating tissue are tapped. Adult stem cells are typically acquired by harvesting adult tissues. Patients give informed consent and usually undergo little risk at donation. In contrast, human embryonic stem cells (hES) are obtained by culturing cells from the inner cell mass of a blastocyst. This blastocyst is usually acquired from an unused human embryo produced by *in vitro* fertilization or from an aborted fetus. The harvesting process requires dissolving the blastocyst bringing into question the moral and legal status of the human embryo.

Many religious perspectives consider the human fetus an individual human entity. However, there is substantial debate regarding at which specific stage dignity is conferred in development ranging from conception, to primitive streak development, implantation, or birth. Taking into account the many perspectives on the moral status of the human embryo weighed against the scientific promises of a healthier tomorrow through stem cell technology, our society has attempted to define the legal status of the human embryo. In the United States, the first mandate was outlined in 1973 when the US Supreme Court ruled that a fetus is not a person in terms of constitutional protection (Roe v Wade, 410 US 113 [1973]). For a better examination of the decision's effect on research, the National Institutes of Health (NIH) imposed a moratorium on fetal research, and Congress founded the National Commission charging it to put together policy and guidelines on fetal research. The commission published a report encouraging fetal research due to its potential, provided that the research risks to the fetus were minimal and were only those that would be accepted for a term fetus. Thus, despite Roe v Wade, the commission extended protection to a fetus equal to adult patients in research. This included fetuses planned for elective abortion.

The NIH moratorium was lifted in 1975, however during President Ronald W. Reagan's second term Congress enacted legislation that further protected the fetus by ending federal support of fetal research involving any level of risk. In 1996, Congress extended this restriction by banning federal funding for "the creation of a human embryo or embryos for research purposes." This led the NIH to distinguish between deriving and using existing human embryos to support embryonic stem cell research. Under these guidelines researchers using already established hES cell lines derived from private

sector support can receive public sector monies provided that the fertilized embryos would otherwise have been discarded after IVF or were from already aborted fetuses, donors are aware of the research use, and no payment was made to the donors.

President William J. Clinton created The National Bioethics Advisory Commission (NBAC) to thoroughly review moral and legal issues of stem cell re search. This commission largely framed its moral position based on a utilitarianism argument—the good of many outweigh the status of one. In addition, it drew on medicine's aims to heal and prevent disease urging consideration of a long-term benefit-to-harm balance. The NBAC conclusion recommended allowing federal funding for hES research on excess IVF embryos. Reasons supporting this position include the potential of ES cells in regenerative and reproductive medicine and the need for federal support to avoid private sector conflicts of interest which sometimes invokes secrecy, limiting the spread of knowledge, and places shareholders interests ahead of public good. Taking all these guidelines and perspectives into account, President George W. Bush made an executive order on August 9, 2001 to limit federal funding of hES research to cell lines already derived from 64 embryos.

The cost to society of foregoing use of this technology, either by failure to correct genetic abnormalities or by improving the success of lifesaving organ transplantations, may be equal to or greater than the perceived costs to the dignity of life due to destruction of a human embryo. There must be a balance between the perceived costs to the dignity of life held by those with the most sacrosanct concept of the moral status of embryos and those who would directly benefit from stem cell based therapy. The climate in which stem cells are explored can be nurturing or profoundly limiting. As medical scientists,

we must not make judgments or ethical decisions on our own; rather, we must ensure full informed consent of the population as a whole. This approach may limit quick progress and may disqualify avenues of research and therapy, but as responsible researchers we must use the resources of society in a worthy manner to explore fully the tremendous potential of embryonic stem cells.

The use of adult stem cells eliminates any ethical concerns as the cells used for therapy can be obtained with only slight discomfort, and potentially from the individual patient themselves. This is attractive due to the fact that there would be no human leukocyte antigen mismatching, and therefore no need for immune suppressing drugs or the fear of tissue rejection and graft versus host disease. If adult stem cells prove to have the same potentiality as ES cells, either individually or as a collection, their use would end the need for embryonic tissue and their subsequent destruction eliminating any ethical concerns over the ends justifying the means.

CHAPTER 6
GENERAL CONCLUSIONS

The goal of this work was to characterize the hematopoietic stem cell's plastic ability and describe biological pathways which can modulate this ability. In a historic context, this work spans several stages of the he stem cell field as it matured from the beginning flurry of activity into its current stage of careful evaluation. The pioneering work demonstrated the exciting potential of the field as an alternative and powerful tool for use as a cell-based therapy for many diseases ranging from cancer to diabetes, heart attack, and Parkinson's disease. Those founders demonstrated that these cells, specifically the hematopoietic stem cell, was capable of producing many different tissue types. It was found that the HSC is capable of producing not only all of the blood lineages, but also muscle, pancreatic cells, heart, intestinal epithelium, brain, and blood vessel endothelium. These exciting results sparked a revolution in the scientific field with new findings constantly being published in peer reviewed scientific journals along with the front pages of popular newspapers and magazines. This initial impetus soon played out, however, as the heralded plasticity of stem cells came under thorough and critical scrutiny, and justifiably so. It became apparent that the cell types produced were not necessarily functional, nor could there be certainty of the source of the donor cells—several different cell types within the transplant could be individually contributing to the observed tissue, therefore no one cell would be capable of forming several tissue types. This critique was addressed in several studies which began the second stage of stem cell research. Krause *et al.* did a single cell transplant homing assay to prove several tissue

types could arise from one cell.[82] Grant *et al.* also did single cell transplants and demonstrated the functional ability of endothelial cells which clonally arose from the HSC to carry red fluorescent dye perfused into the circulator system.[30] In addition, Lagasse *et al.* rescued liver function in a metabolic liver disease with HSC.[84] Clearly, stem cells, particularly the HSC, are capable of providing functional rescue of disease conditions in a clonal manner. With this understanding, we now embark on the third stage of stem cell plasticity research—defining the genes and biological pathways involved in regulating or controlling stem cell activity.

Many genes have been shown to maintain the "stemness" of stem cells by controlling their self-renewing and proliferating capacity. In addition, we are starting to understand factors, such as the nitric oxide pathway, which play a role in stem cell homing and functional behavior.[128] This work, along with other exciting work done in our lab on chemokines such as stromal-derived factor-1's involvement in homing, highlights the therapeutic benefit of not only stem cell research, but also fortifies the foundation for cell-based therapy.[129] This third age of directed stem cell repair of damaged or non functional tissue has the potential for direct translation into disease therapy along with opening exciting new avenues of original research. This body of work chronicles the relatively new field of stem cell plasticity research from the initial characterizing of HSC plasticity up to describing biological pathways which can orchestrate HSC activity. The founding work and initial application to HSC plasticity described here can lead to many novel stem-cell therapy strategies for debilitating conditions and diseases. Additional effort will focus on direct translation of this knowledge into human disease therapy.

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BIOGRAPHICAL SKETCH

Steve Guthrie was born and raised in Lancaster, Pennsylvania. He attended Albright College in Reading, Pennsylvania where he graduated in 1998 with two majors (biology and philosophy) receiving the Gary Kennis Philosophy Award and Ernest J. Pastorello Biology Prize. He then moved to Gainesville, Florida, where he worked as a lab technician for Dr. Alfred Lewin, and then as a biological scientist for Dr. Edward Scott for 2 years. He joined the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine in 2000 where he began his doctoral study under the guidance of Dr. Edward Scott in the Department of Molecular Cell Biology. He was awarded a Grinter Fellowship, and received first place in his department, and fifth place overall at the 2003 Medical Guild Research Day sponsored by College of Medicine. Steve will be doing post-doctoral research at the University of Alabama at Birmingham beginning Summer of 2005.