REGULATION OF ENDOTHELIAL CELL-DEPENDENT HEMATOPOIETIC STEM CELL REGENERATION

By

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A DISSERTATION

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α-SMA	α-smooth muscle actin
AGM	Aorta-gonado-mesonephros
ALK	Activin like kinase
APC	Allophycocyanin
ARS	Acute radiation syndrome
ATM	Ataxia-telangiectasia mutated
BFU-E	Blast forming unit-erythroid
BMC	Bone marrow cells
BMPR	Bone morphologic protein receptor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BV-421	Brilliant Violet 421
CAFC	Cobblestone area forming cell
CAR cell	CXCL12-abundant reticular cell
CB	Cord blood (umbilical)
CHT	Caudal hematopoietic tissue
CFU-C	Colony forming unit-cell
CFU-S_{12}	Colony forming unit-spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DMSO	Dimethylsulfoxide
DSB	Double strand break
EC	Endothelial cell
EdU	Ethynyldeoxyuridine
EGF	Epidermal growth factor

EGM2	Endothelial growth medium 2
EPO	Erythropoietin
FA	Fanconi Anemia
FDR	False discovery rate
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Flt-3 Lig	FMS-like tyrosine kinase 3 ligand
FOXO	Forkhead box
FPKM	Fragments per kilobase per million reads
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulation factor
GFP	Green fluorescent protein
GSEA	Gene set enrichment analysis
HAEC	Human aortic endothelial cell
HGF	Hepatocyte growth factor
HPAEC	Human pulmonary artery endothelial cell
HSavEC	Human saphenous vein endothelial cell
HUVEC	Human umbilical vein endothelial cell
HDR	Homology directed repair
HPC-1	Hematopoietic progenitor cell 1
HPC-2	Hematopoietic progenitor cell 2
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell
IL-3	Interleukin 3
IL-6	Interleukin 6

IL-7	Interleukin 7
Lepr	Leptin receptor
LSK	Lineage$^{lo/-}$ Sca-1^{+} c-Kit^{+} cell
LTC-IC	Long term-culture initiating cell
LT-HSC	Long-term hematopoietic stem cell
MAPK	Mitogen activated protein kinase
MCAM	Melanoma cell adhesion molecule
MECA-32	Murine endothelial cell antigen 32
MPP	Multipotent progenitor cell
MSC	Mesenchymal stem cells
MSPC	Mesenchymal stromal progenitor cells
Nes-GFP	Nestin-GFP transgene
NHEJ	Non-homologous end joining
Osx	Osterix
PB	Peripheral blood
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PECy7	Phycoerythrin-cyanine 7
PI	Propidium iodide
PRR	Parathyroid hormone and parathyroid related protein receptor
Prx1	Paired related homeobox 1
pSp	Paraaortic splanchnopleura
PTN	Pleiotrophin
PTP	Protein receptor tyrosine phosphatase
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
Runx1	Runt-related transcription factor 1
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel eletrophoresis
SLAM	Signaling lymphocyte activation molecule
ST-HSC	Short-term hematopoietic stem cell
TGF-β	Transforming growth factor-β
TPO	Thrombopoietin
VE-Cadherin	Vascular endothelial cadherin
VEGFR	Vascular Endothelial Growth Factor Receptor
WBI	Whole body irradiation
ZO-1	Zona occludens 1

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Abstract

Hematopoiesis, the life-long process of blood cell production, is maintained through the coordinated division and differentiation of specialized, pluripotent hematopoietic stem cells (HSC). For most of life, HSC reside in bone marrow, where they are maintained in 'niches' by the cells of the hematopoietic microenvironment. These niches are thought to help maintain the HSC pool by tightly regulating stem cell quiescence and supporting self-renewal.

The human hematopoietic system produces about 500 billion cells each day. And amazingly, blood cell production can increase to offset demand after hemorrhage or infection. As a consequence of its high rate of cellular production, the hematopoietic system is very sensitive to cytotoxic therapies. Ionizing radiation and chemotherapy cause direct injury to HSC through DNA damage and cellular oxidative stress. Epidemiologic and mechanistic studies both show that extensive hematopoietic injury from cytotoxic agents is linked to developing bone failure syndromes or hematologic malignancies.

Our knowledge of the niches that maintain HSC has improved substantially in the last 10-15 years. The majority of investigators have shown that HSC reside in perivascular niches. Vascular endothelial cells (EC) are integral niche components that promote HSC retention and survival. Moreover, vascular EC are necessary and sufficient for hematopoietic regeneration after bone marrow injury. Therefore, identifying the factors EC utilize to maintain and regenerate HSC could lead to improved therapies.

The overall purpose of this research is to understand the mechanisms of endothelial-dependent HSC regeneration. Chapters 3 and 4 of this dissertation present the work I have done toward identifying both mechanisms and mediators of vascular EC support for HSC. The studies presented in Chapter 3 introduce a co-culture system we established to measure HSC regeneration by vascular EC. We showed that EC isolated from the aorta can promote the *ex vivo* regeneration and repair of self-renewing HSC with long-term multi-lineage repopulating capacity.

In Chapter 4, I extended these findings to identify growth factor mediators of HSC regeneration. Relative to aorta-derived EC, umbilical vein-derived EC were found to regenerate HSC poorly. Therefore, we used the transcriptomes of these EC subtypes to identify both positive and negative regulators. Transforming growth factor-β1 (TGF-β1), which is a key factor within the quiescent HSC niche and highly elevated in injured bone marrow, completely abolished EC-dependent HSC regeneration and led to a depletion of long-term HSC. These experimental results also uncovered a novel EC-autonomous role for TGF-β1 in suppressing HSC regeneration, indicating that TGF-β1 can participate in an autocrine EC loop. In an attempt to enhance HSC regeneration, several factors were identified as candidates. Hepatocyte growth factor strongly enhanced the regeneration of long-term, multi-lineage repopulating HSC when added to EC-bone marrow cell co-cultures. Mechanistically, HGF activated c-MET on EC, which interfered with TGF-β--Smad2/3 signaling and increased endothelial AKT activation.

Together, these data contribute to the growing knowledge of vascular EC as mediators of HSC regeneration. They indicate that EC-derived TGF-β1 exerts potent suppressive effects on hematopoietic regeneration, part of which is mediated directly through vascular EC. Furthermore, I have generated direct data indicating that growth factors such as HGF can be used to target EC and enhance hematopoietic regeneration. Ideally, these results could be used in the future to inform the implementation of new therapies for HSC transplantation or hematologic malignancies. This work also emphasizes the importance of vascular function in stem cell health which may have multi-system applications for study in stem cell aging, disease, and regeneration.

Chapter 1: "The Problem"
The Need to Repair and Regenerate Hematopoietic Stem Cells

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Part 4: Summary

Part 1: Introduction

Hematopoiesis is the process of blood cell production that occurs through the regulated division and differentiation of somatic, pluripotent stem cells known as hematopoietic stem cells (HSC). Blood cells serve a broad range of vital functions including oxygen delivery, hemostasis, and host defense. Furthermore, disorders of hematopoiesis have widespread effects throughout the body, and can influence a variety of disease states. Therefore, hematopoiesis is of central importance for human health.

Blood cells must continually be replenished throughout life. Adult human hematopoiesis produces 500 billion cells/day at steady state (Fliedner et al., 2002), and hematopoietic demand can rapidly increase due to infection, hemorrhage, or treatment with cytotoxic therapies. Importantly, chemotherapy and exposure to ionizing radiation induce HSC damage and dysfunction, resulting in life-threatening cytopenias and increasing the risk of developing bone marrow failure and hematopoietic malignances. Damage to the hematopoietic system sometimes requires transfusions and cytokine therapy. Severe damage is often curable only with allogeneic HSC transplantation. Despite their importance, the current therapies for hematopoietic injury are limited. Therefore, improving the repair of HSC and regeneration of hematopoiesis is an unmet clinical need. This chapter has three purposes:

- Provide an overview of hematopoiesis and the biology of HSC.
- Describe the pathophysiology of bone marrow injury from ionizing radiation and chemotherapy.
- Discuss standard of care treatments for hematopoietic injury and their current limitations.

Part 2: Origins of hematopoiesis and the biology of the hematopoietic stem cells

Ontogeny of Hematopoiesis

Primitive and definitive hematopoiesis. Two major waves of hematopoiesis occur in mammalian development, both originating from the primitive streak (Figure 1-1a). The first emergence of hematopoietic cells is during the early streak stage, when cells of the posterior streak migrate ventrally to the yolk sac to form the extraembyronic mesoderm and allantois (Dzierzak and Speck, 2008). Blood production from these sites marks the onset of primitive hematopoiesis, in which the extraembryonic blood-forming cells, known as hemangioblasts, are multipotent precursors of hematopoietic, endothelial, and smooth muscle cells. At this stage, hemangioblasts express markers of both mesodermal (Brachyury) and endothelial (vascular endothelial growth factor receptor-2/VEGFR2) cell lineages (Dzierzak and Speck, 2008), and produce erythroid and primitive myeloid cells, but not HSC.

The transition from primitive to definitive hematopoiesis occurs through three additional sub-stages marked by the appearance of new types of blood cells. These extra stages are described as the pro-definitive, meso-definitive, meta-definitive stages, and are punctuated by the appearance of myeloerythroid progenitors, lymphoid progenitors, and $CD34^{+}c\text{-}Kit^{+}$ 'neonatal repopulating' HSC, respectively (Dzierzak and Speck, 2008).

The definitive phase of hematopoiesis begins independently in mesodermal precursors that arise from the mid-late stage primitive streak. These cells migrate ventrally from the primitive streak and contribute to the formation of the axial, paraxial, and lateral mesoderm in the rostral and trunk regions of the embryo (Dzierzak and Speck, 2008). Subsequent development of this mesoderm in the para-aortic splanchnopleura gives rise to the aorta-gonado-mesonephros (AGM) region, which contains the committed precursors to adult HSC.

Cells within the AGM begin to express a variety of endothelial and hematopoietic markers and migrate to the ventral aspect of the developing dorsal aorta, where they integrate with the vascular wall and adopt endothelial cell morphology (Dzierzak and Speck, 2008).

Vascular origins of adult HSC. Most definitive hematopoiesis begins in hemogenic endothelium in the dorsal aorta (Figure 1-1b). HSC were first observed budding from hemogenic endothelium in the dorsal aorta of chick embryos by Florence Sabin in 1917 [republished as (Sabin, 2002)]. Further studies used labeling of aortic endothelium in the chick embryo prior to the onset of HSC appearance to show that circulating hematopoietic cells retain the fluorescent dyes initially injected into vessels (Jaffredo et al., 1998). More recently, improved lineage tracing and live imaging techniques have allowed the direct visualization of emerging HSCs in zebrafish and mouse embryos (Bertrand et al., 2010; Boisset et al., 2010; Kissa and Herbomel, 2010; Zovein et al., 2008). In a process that is dependent on Notch signaling though the master hematopoietic transcription factor runt-related transcription factor 1 (Runx1) (Chen et al., 2009), vascular endothelial cells at the ventral surface of the aorta begin to downregulate endothelial markers such as vascular endothelial growth factor receptor (Vegfr, also Kdrl) and vascular endothelial cadherin protein (VE-Cadherin; Cdh5), and begin to express the pan-hematopoietic marker, CD45 (Bertrand et al., 2010).

In addition to the dorsal aorta, definitive HSC have been observed to arise in hemogenic endothelium in the yolk sac and placenta, as well as the umbilical and vitelline arteries (Orkin and Zon, 2008). Time course studies indicate that these HSC arise autonomously, rather than migrating from the dorsal aorta (Dzierzak and Speck, 2008). Together, circulating HSC derived from these sites begin to populate the fetal liver, where they undergo cycles of expansion along a growing hepatic vasculature (Khan et al., 2016). Following vascularization of the marrow

cavity at E15-17 fetal liver HSC migrate to the bone marrow, the principal site of hematopoiesis during adult life.

Hematopoietic Stem Cell Biology

Functional and phenotypic identification of HSC. Definitive HSC are specialized, pluripotent stem cells possessing the capacity to reconstitute the entirety of hematopoiesis while retaining the ability to self-renew. Pioneering studies by Till & McCulloch led to the concept of a self-renewing HSC that gave rise to a hierarchy of progressively differentiated blood cells (McCulloch and Till, 1960; Till and McCulloch, 1961). When infused with a protective dose of bone marrow cells, lethally irradiated hosts developed discrete nodules on the surface of their spleens after 10-14 days (Till and McCulloch, 1961). These spleen colony forming units (CFU-S_{12}) were shown to be derived from a single founder clone (Becker et al., 1963) and contained donor-derived hematopoietic cell types of mixed myeloerythroid lineages (Wu et al., 1967). Importantly, the clones forming CFU-S_{12} colonies could be isolated and secondarily transplanted to form spleen colonies in irradiated hosts (Siminovitch et al., 1963). The latter finding led to the model that blood cell formation was hierarchical and mediated by a self-renewing, pluripotent pool of stem cells [reviewed by (Eaves, 2015)]. Since this discovery, our knowledge of HSC biology has vastly expanded based on (1) our ability to purify homogenous cell populations on the basis of surface marker expression or biochemical characteristics, and (2) improvements in the specificity of functional assays.

The identification and characterization of HSC has been guided by the use of in vitro and in vivo functional assays to assess the hematopoietic potential within a defined population of cells. As discussed above, the first HSC were found by bone marrow transplantation and subsequent investigation of the CFU-S_{12}. The ability of hematopoietic stem and progenitor cells

(HSPC) to form clonal outgrowth colonies (colony forming unit cells, CFU-C) has led to the development of several types of *in vitro* assays to measure HSPC function. In contrast to CFU-S_{12}, *in vitro* colony formation utilizes semisolid methylcellulose medium supplemented with growth factors that support the clonal outgrowth of HSPC (van Os et al., 2008). Notably, the CFU-C is primarily a measure of progenitor cell activity as HSC will differentiate throughout the culture period. Therefore, more sensitive in vitro assays have been developed as in vitro surrogates for HSC activity.

Two long-term culture assays have been utilized in attempt to better identify HSC in vitro (van Os et al., 2008). The cobblestone area forming cell (CAFC) and long term colony-initiating cell (LTC-IC) assays are performed by culturing HSC-containing populations of cells for 5-6 weeks in direct contact with a stromal feeder layer. At the end of the culture period, a CAFC assay will enumerate HSC on the basis of “cobblestones” – clumps of 5-6 non-refractive cells that appear beneath the stromal layer, which are burrowing HSC – and the number of these CAFC correlates with CFU-C, CFU-S_{12}, and hematopoietic repopulation potential *in vivo*. The LTC-IC assay is carried out similarly; cultures are harvested and replated in methylcellulose. However, in this assay the formation colonies is dependent on the functional potential of HSC surviving long-term culture, which subsequently give rise to amplifying progenitor cells in methylcellulose (van Os et al., 2008).

The gold standard for identifying a functional HSC requires showing their long-term, multi-lineage hematopoietic potential in vivo, as well as the capacity to self-renew (Eaves, 2015). To test this, Hematopoietic Stem Cell Transplantation (HSCT) is performed. A variety of transplantation strategies are employed which differ in their characteristics with respect to recipient conditioning, mode of transplantation, and measurement of transplanted cell function. The general HSCT model involves total bone marrow ablation of recipients through lethal irradiation, followed by intravenous infusion of cells containing putative HSC. A small but life-

saving 'carrier' dose of bone marrow is often co-transplanted with the test population to ensure the recipient is able to meet blood production demands while donor HSC engraft. Donor mice that are congenic to recipients (derived from the same inbred strain, but variant for a particular marker gene) are often used for transplantation studies to distinguish donor from host cells through specific CD45 isoform or GFP expression. To determine long-term multi-lineage hematopoietic reconstitution, donor-derived contribution to peripheral leukocytes is measured at regular (usually 4 week) intervals following transplantation. An overall engraftment of ≥1% is typically used to establish donor chimerism (Eaves, 2015). To determine multi-lineage potential, leukocytes are isolated from peripheral blood, labeled with antibodies to detect myeloid, B cell, and T cell subsets, and measured by flow cytometry. Demonstrating self-renewal potential is the second requirement for the functional identification of HSC. This is done through serial transplantation with the demonstration of donor-derived long-term multi-lineage hematopoietic reconstitution in secondary recipients. Therefore, *bona fide* HSC are identified only retrospectively, through their ability to provide ≥16 weeks of multi-lineage hematopoiesis through at least two rounds of transplantation (Eaves 2015).

Based on the outcomes of functional assays described above, cellular surface marker combinations have been developed to identify HSC using flow cytometry or immunocytochemistry. The HSPC fraction can be enriched by selecting cells that do not express the markers of mature hematopoietic lineages ($Lin^{lo/-}$ cells). In the Black-6 mouse strain, a mixture of short-term (ST) and long-term (LT) repopulating HSC can be further enriched within the $Lin^{lo/-}$ population on the basis of stem cell antigen 1 (Sca-1) and CD117 (c-Kit) expression ($Lin^{lo/-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; LSK cells) (Okada et al., 1992). Additional strategies are used to further subdivide LSK cells and enumerate phenotypic LT-HSC. The most common approach utilizes expression patterns of the signaling lymphocyte activation molecule (SLAM) antigens CD150 and CD48 on LSK cells. Single cell transplantation assays have shown that 1 in 2 $CD150^{+}CD48^{-}$

LSK cells are multi-lineage, serially repopulating HSC (Kiel et al., 2005b; Oguro et al., 2013). Notably, $CD150^{-}$LSK cells define a pool of multipotent progenitor cells (MPP), which are able to provide 12 weeks of peripheral blood reconstitution in primary transplant recipients, but are not self-renewing HSC (Kiel and Morrison 2005). Alternatively, LT-HSC are identifiable based on their $CD34^{-}Flt3^{-}$LSK cell phenotype (Osawa et al., 1996) or from biochemical properties such as Hoechst dye efflux ("side population" HSC) (Goodell et al., 1996). Throughout Chapters 3 and 4 of this dissertation the SLAM-HSC designations, $CD150^{+}$LSK or $CD150^{+}CD48^{-}$LSK, are used to define phenotypic HSC. Despite similarities in function, human LT-HSC do not express the same markers as mouse LT-HSC. Human HSC are enriched in $CD34^{+}$ hematopoietic cells, and LT-HSC can be further fractionated from more differentiated $CD34^{+}$ based on their expression of other cell surface markers including CD38 [reviewed by (Doulatov et al., 2012).

Properties of HSC and regulation of HSC activity. Improvements in the phenotypic and functional identification of LT-HSC have led to a better understanding of their properties and regulation. Under normal conditions, LT-HSC comprise approximately 0.005% of total murine bone marrow cells, which equates to a pool of approximately 3,000 LT-HSC at steady state (Eaves, 2015; Oguro et al., 2013). Analysis of the cell cycle status of LT-HSC has revealed that over 90% are quiescent *in vivo* (Oguro et al., 2013; Wilson et al., 2008). Estimates of HSC cycling rates using label retention assays have suggested that the most dormant HSC divide from as frequently as every 57 days (Chesier, Morrison et al. 1999) to as seldom as 5 times in the lifetime of a mouse (Wilson et al., 2008). Importantly, HSC can be induced into cell cycle entry by several stimuli, including stimulatory cytokines and acute loss of blood cells (Baldridge et al., 2010; Cheshier et al., 2007; Wilson et al., 2008). Therefore, although most LT-HSC are dormant at steady state, they possess the capacity to rapidly proliferate and dramatically expand in response to increased hematopoietic demand. This is particularly evident in the case

of human allogeneic HSCT, where <5% of the donor's HSC pool has the capacity to engraft and provide long-term hematopoiesis in some patients for more than 40 years.

As discussed above, the hallmark properties of HSC are their abilities to repopulate the entirety of the hematopoietic system and to self-renew. Although normally quiescent, when they do divide HSC make cell fate choices that result in three types of outcomes: Self-renewing divisions can be symmetric and result in two daughter HSCs, or asymmetric and produce a single daughter HSC and a second daughter cell that will differentiate. Alternatively, non-self-renewing division results in two daughter cells lacking HSC properties (Morrison and Kimble, 2006). Although the mechanisms of HSC self-renewal potential are not completely understood, in general self-renewal is considered to be an intrinsic property of HSC that is modifiable by extrinsic factors. Several classes of intrinsic and extrinsic regulators have been identified that influence HSC self-renewal potential, including growth factors and chemical modulators, cell cycle regulators, transcription factors and chromatin-associated factors [reviewed by (Zon, 2008)].

The differentiation of HSC to hematopoietic progenitor cells and mature blood cells is a highly regulated and complex process. Generally, hematopoietic differentiation occurs through transcriptional and epigenetic modifications in HSC daughter cells that restrict self-renewal potential and promote lineage specification (Figure 1-2a) (Alvarez-Errico et al., 2015). A detailed description of the mechanisms of mediators of hematopoietic differentiation is beyond the scope of this overview. However, a recent paradigm shift in the way we view hematopoietic differentiation has occurred and is worth brief discussion (Mercier and Scadden, 2015; Schultze and Beyer, 2016).

The classic model of HSC differentiation placed LT-HSC and ST-HSC at the apex of a hierarchy of hematopoietic cells (Figure 1-2b). The production of blood cells occurred through

the gradual commitment of progenitor cells to more and more specific compartments within the hematopoietic tree. For example, in this model, ST-HSC division resulted in the production of common myeloid cells (CMP), and common lymphoid cells (CLP), each of which were restricted to their respective branch of the hematopoietic tree, but had the capacity to produce all myeloid or lymphoid cells, respectively. Only when CMPs and CLPs divided did they subspecialize in to more restricted progenitor cells. Contrary to this classical view, in the new model of hematopoietic differentiation (Figure 1-2b), lineage commitment occurs much earlier at the multipotent progenitor stage (Notta et al., 2016; Paul et al., 2015; Perie et al., 2015). This paradigm shift has been suggested through single cell functional and transcriptome studies. Lineage tracing demonstrated that hematopoietic progenitor cells previously thought to be multipotent, such as CMPs, have very restricted potentials when their differentiation is tracked in vivo at single cell resolution (Perie et al., 2015). Likewise, analysis of global gene expression in individual cells within multipotent populations such as CMP demonstrated that subpopulations of cells expressing highly lineage-restricted transcription programs (Paul et al., 2015). Indeed, the concept of 'lineage priming' – in which HSC transiently express transcriptional programs of committed hematopoietic cells that biases their differentiation potential – has been studied over the last two decades (Orkin, 2003; van Galen et al., 2014; Ye et al., 2003). Therefore the new model, in which HSCs and MPPs differentiate into progenitors with very limited hematopoietic potential, suggests that lineage priming may play an important role in the restricted fate of hematopoietic progenitors.

In summary, the study of hematopoiesis and HSC has a rich history built on the study of model organisms and advancements in technology, which have fueled the identification and functional testing of candidate HSC. Hematopoiesis begins early in development and definitive HSC have the incredible task of producing all blood cells for the remainder of life. In the adult, HSC are not homogenous and represent a pool of pluripotent cells with differential lineage

biases and self-renewal potentials that are dependent on both intrinsic and extrinsic regulation. The process of hematopoietic differentiation is tightly regulated and results in massive expansion of hematopoietic cells in the bone marrow, which perform vital and specialized functions throughout the body.

Part 3: Pathophysiology and consequences of hematopoietic injury

Effects of Ionizing Radiation and Chemotherapy on Hematopoiesis

As a consequence of its constant production of new blood cells, the hematopoietic system is sensitive to any insult that targets cell proliferation. The effects of radiation and chemotherapy on hematopoiesis were discovered independently, but both strongly suppress hematopoiesis by depleting progenitor cells and injuring HSC. Interestingly, the effects of chemotherapy on hematopoiesis were observed long before its use as a cancer therapy. French scientists associated mustard gas poisoning in World War I soldiers with rapid declines in leukocytes, and this later led to the idea that β-chloroethyl amines could be used as a therapy for a variety of lymphoid neoplasms (Gilman and Philips, 1946; Goodman et al., 1946). The effects of ionizing radiation on hematopoiesis were uncovered following the discovery of X-Rays by Wilhelm Röntgen at the end of the 19th century. The later harnessing of nuclear power for war and energy has led to the occurrence of the Acute Radiation Syndromes (ARS) affecting bone marrow, gastrointestinal, and cerebrovascular function (Waselenko et al., 2004). Dosimetry studies have defined dose-response relationships for each type of ARS, and the hematopoietic system is most sensitive showing suppressive effects at doses as little as 1.0 Gy. Currently, the most frequent cause of hematopoietic injury from radiation or chemotherapy occurs in patients receiving cancer therapies. However, radiation-induced ARS has also been a very serious consequence of catastrophic nuclear events including the disasters in Chernobyl and Fukushima; and unfortunately continues to be a worldwide terrorism threat. These sections explain in detail the effects of chemotherapy and ionizing radiation on hematopoiesis and HSC.

Cytopenias from progenitor cell depletion. The kinetics of hematologic suppression induced by ionizing radiation and chemotherapy have been studied in animal models and in human

patients. The effects these insults have on hematologic parameters are both dose and lineage-dependent (Fliedner et al., 2002). Within the myeloerythroid lineage, exposure to ionizing radiation is associated with a gradual decline in erythrocytes and a rapid decline in platelets and neutrophils. Committed myeloid progenitors surviving the initial injury will produce a wave of neutrophils that spikes within the first 2-4 days after irradiation. This is followed by a nadir of myeloid cell production lasting 1-3 weeks (Plett et al., 2012). In certain cases, a short burst of myelopoiesis termed “abortive recovery” occurs, which is attributed to the last rounds of injured HSC division and differentiation prior to their entry into senescence (Fliedner et al., 2002). Lymphoid cells are also damaged by cytotoxic therapies. Declines in lymphocyte numbers in the blood occur within days, and absolute lymphocyte counts will remain low for several weeks following the initial exposure. Lymphopenias resulting from ionizing radiation exposure are longer lasting than the associated myeloid cytopenias (Plett et al., 2012). In addition to cytopenias, after radiation exposure atypical cells are also apparent in a blood smear or bone marrow specimens, including binucleated cells, cells with karyomeres, or giant cells formed from defects in cytokinesis (Fliedner et al., 2002). Therefore cytotoxic agents have effects on all arms of the hematopoietic system, inducing cytopenias through progenitor cell depletion and dysfunction.

HSC in stress hematopoiesis. Depletions within the progenitor pool also induce a substantial proportion of HSC into cell cycle. Both progenitor cell depletion through either 5-FU treatment or administration of G-CSF result in a 8-10 fold decline in BrdU label retaining HSC in mouse bone marrow (Wilson et al., 2008). Loss of HSC labeling with BrdU occurred concomitantly with significant reductions in the HSC G0 fraction and increases in the S/G2/M fraction (Wilson & Laurenti, 2008). These effects are similar to stress hematopoiesis induced by a severe infection or hemorrhage (Baldridge et al., 2010; Cheshier et al., 2007). Stress hematopoiesis involves an

orchestrated response in HSC, progenitors and the bone marrow microenvironment after significant bone marrow injury (Zhao and Baltimore, 2015). Evidence exists that prolonged HSC cycling in the setting of persistent stress hematopoiesis can result in a predisposition for the development of hematologic disease (Anderson et al., 2009; Hasselbalch, 2012; Kristinsson et al., 2011). Therefore, the stress hematopoiesis response of HSC after hematopoietic injury may also result in long-term HSC damage and dysfunction.

Mechanisms of HSC injury

In addition to the replicative demand placed on HSC during hematopoietic regeneration, chemotherapy and ionizing radiation also directly injure HSC. The primary biochemical mechanisms by which radiation and chemotherapy cause HSC injury are DNA damage and cellular oxidative stress (Meng et al., 2003a; Wang et al., 2006; Zhang et al., 2013b).

DNA Double Strand Breaks (DSBs). Several types of DNA damage can occur as a result of ionizing radiation (Azzam et al., 2012). These include base modifications by oxidation, depurination or depryimidination, cross-linking, or DNA strand breaks (Shao et al., 2014). Of all the cellular injuries that chemotherapy and radiation can cause to HSC, double stranded DNA breaks (DSB) represent the most catastrophic because they result in chromosomal fragmentation that, if unresolved or improperly repaired can lead to translocations, large deletions, or the induction of apoptosis (Ceccaldi et al., 2016). High-dose irradiation can directly induce strand breakage (Azzam et al., 2012); however, DNA alkylating or cross-linking agents as well as functional group oxidation induce stalled replication forks, which in the absence of efficient repair can ultimately progress to single strand breaks or DSBs. DNA damage is constantly occurring at low frequency in dividing and non-dividing cells; therefore, elaborate

DNA damage sensing and repair mechanisms have evolved and these are highly utilized by HSC (Lindahl and Barnes, 2000). The response to DNA damage can result in variable outcomes for the cell depending on the degree of damage and the ability of cellular repair pathways to be activated. These include activation of DNA repair machinery leading to repair, temporary or permanent inhibition of the cell cycle, and the initiation of apoptosis (Jackson and Bartek, 2009).

HSC depend on highly functional repair systems during both steady state hematopoiesis and hematopoietic regeneration. Dormant HSC have been shown to accumulate DNA damage throughout life, which can be repaired upon entry into the cell cycle (Beerman et al., 2014); however, DNA damage accrual limits HSC self-renewal potential with aging (Rossi et al., 2007). Furthermore, heritable mutations in the Fanconi Anemia genes involved in DNA repair result in compromised hematopoiesis early in life (Moldovan and D'Andrea, 2009). The readiness of HSC to repair DNA damage in response to injury is a unique property amongst hematopoietic cells. Passague's group showed that DNA repair pathways are preferentially activated in HSC relative to hematopoietic progenitors after low dose (3 Gy) ex vivo irradiation. In contrast to progenitor cells, which undergo apoptosis in response to irradiation, HSC downregulated the apoptotic pathway and activated DNA damage repair machinery (Mohrin et al., 2010). Furthermore, these investigators went on to show that quiescent HSC utilize a different type of DSB repair (non-homologous end joining; NHEJ) relative to cycling HSC (homology directed repair; HDR). This is a notable finding because NHEJ is an inherently error-prone DNA repair process (Lieber, 2010) and can result in lasting mutations, whereas the coupling of HDR to the cell cycle allows HSC to utilize a sister chromatid as a template for error-free repair (Jasin and Rothstein, 2013). Mohrin et al. showed that pre-stimulating HSC to enter the cell cycle prior to irradiation increases the utilization of HDR and reduces chromosomal abnormalities in surviving cells (Mohrin et al., 2010). The switch from NHEJ to HDR has also recently been found to

improve HSC survival in Fanconi Anemia (Zhang et al., 2016). Therefore, DNA damage repair is an essential process during normal HSC function and hematopoietic regeneration. The use of HDR in cycling HSC appears to promote the best response to injury by maximizing survival and minimizing the likelihood of long-lasting chromosomal aberrations.

Oxidative stress. A second major type of cellular injury induced by ionizing radiation and chemotherapy is free radical injury. In addition to the increased metabolic demands placed on HSC after progenitor cell ablation, ionizing radiation also creates free radicals through the radiolysis of water (Azzam et al., 2012), which react with cellular elements to produce reactive oxygen and nitrogen species (ROS and RNS, respectively). ROS and RNS damage can cause macromolecular damage including DNA and lipid peroxidation as well as protein cross-linking by aldehyde lipid peroxidation products (Maier et al., 2010). Furthermore, radiation exposure has been shown to increase the expression of the pro-oxidant enzyme, NADPH oxidase-4 in HSC (Wang et al., 2006). Radiation-induced oxidative stress damages macromolecules and can contribute to long-term DNA damage resulting in the restriction of HSC self-renewal potential (Yahata et al., 2011). In support of this, administration of the antioxidant and sirtuin activator, resveratrol, has been shown to reverse long-term HSC damage and dysfunction after injury (Zhang et al., 2013b).

Regulators of redox balance are important for HSC function. Ito et al. showed that HSC lacking the damage sensor ATM have increased ROS and reduced transplantation potential (Ito et al., 2004). In this setting, increased p38 MAPK activation induces HSC senescence through the upregulation of cell cycle inhibitors $p16^{Ink4a}$ and $p19^{Arf}$, effects that can be reversed by antioxidant (N-acetylcysteine) administration (Ito et al., 2006). The forkhead homeobox type O (FOXO) group of transcription factors also regulate redox balance in HSC. A deficiency of

FOXO3A leads to increased ROS, reduced antioxidant enzyme gene expression, and reductions in HSC quiescence and repopulating potential (Tothova et al., 2007). Other investigators have shown that ROS, which are normally sensed in the cell as second messengers (Urao and Ushio-Fukai, 2013), are responsive to growth factors that induce hematopoietic differentiation (Sattler et al., 1999). Therefore, supraphysiologic levels of ROS induced by ionizing radiation have detrimental effects on HSC function. If not properly handled by the damage sensing and response systems these injuries can result in aberrant HSC cell cycling and differentiation, loss of self-renewal potential, or apoptosis.

Bystander injury. There is some evidence that hematopoietic dysfunction can occur in non-irradiated HSC that enter or are exposed to an irradiated microenvironment. This phenomenon is known as the bystander effect and can occur through proximity interactions between non-irradiated and irradiated cells, or by soluble factors that act over longer distances (Morgan and Sowa, 2007). HSC transplanted into mice receiving 10 Gy ionizing radiation 24h prior to transplantation showed increased levels of apoptosis, reduced c-Kit expression, and increased ROS (Shen et al., 2012). Watson et al. utilized another approach by which both irradiated and non-irradiated HSC were transplanted simultaneously into irradiated hosts. In comparison with pure populations of non-irradiated, transplanted HSC, the mixed transplants showed that non-irradiated HSC acquired chromosomal abnormalities at a frequency of 2-3%, which was not significantly different from irradiated transplanted HSC but was significantly greater than the 0.4% frequency of aberrations in non-irradiated transplanted HSC (Watson et al., 2000). These data provide evidence of a bystander effect in which HSC entering an irradiated microenvironment incur the same types of damage that limit their long term function following direct cytotoxic exposure.

Long term consequences on hematopoiesis and HSC function

The effects of HSC injury from ionizing radiation and chemotherapy are not transient. In fact both chemotherapy and irradiation have been associated with impaired long-term hematopoietic function and a predisposition to hematologic disease.

Loss of HSC self-renewal potential. Long-term HSC damage and dysfunction due to both chemotherapy and irradiation have been documented in rodents and humans (Chua et al., 2012; Meng et al., 2003a; Meng et al., 2003b; Wang et al., 2012; Wang et al., 2010; Wang et al., 2006; Yahata et al., 2011). As discussed extensively in the last section, injured HSC have higher levels of ROS and oxidative DNA damage markers, which results in a reduction in the quiescent HSC pool, and upregulation of cell cycle inhibitors in response to aberrant HSC cycling, and premature HSC senescence. These changes are associated with reduced hematopoietic colony formation and impaired HSC engraftment following transplantation.

Risk for developing hematologic disorders. In addition to limiting their long term function, the damage incurred by HSC after cytotoxic exposures can increase the risk for developing future hematologic disorders (Bhatia, 2013). Epidemiologic studies have demonstrated that survivors from Hiroshima and Nagasaki, as well as workers from Chernobyl, have a significantly elevated incidence of hematologic malignancies (Kesminiene et al., 2008; Tsushima et al., 2012). Cancer patients receiving chemotherapy and radiation are also at approximately 5-fold increased risk for therapy related hematologic disorders (Morton et al., 2013).

Overall, ionizing radiation and chemotherapy are highly detrimental to hematopoiesis and HSC function primarily through the induction of oxidative stress and DNA damage. Although the hematopoietic system is well poised to buffer the effects of naturally occurring stress hematopoiesis, cytotoxic agents can disrupt hematopoiesis to such a degree that prolonged, life-threatening cytopenias and bone marrow failure can occur. The next section of this chapter will focus on the treatments available for hematopoietic injury from radiation/chemotherapy and their limitations.

Cytokine therapy for hematopoietic injury

The vital functions of blood cells make HSC injury and hematopoietic suppression a serious clinical concern. Specifically, patients with suppressed hematopoietic systems are at risk for life-threatening anemia and bacterial infections [reviewed by (Dainiak, 2010)]. Therapies utilizing hematopoietic cytokines can improve blood cell production but are limited in their potency and specificity. This section provides an overview on hematopoietic cytokine therapy, with a focus on granulocyte colony stimulating factor (G-CSF).

Discovery and use of hematopoietic cytokines. The concept of hematopoietic cytokines introduced when patients with anemia were found to have elevated urine levels of a factor that could stimulate erythrocyte production in rats (Plzak et al., 1955). This led to the later discovery and purification of erythropoietin (EPO) (Miyake et al., 1977). The development of *in vitro* CFU assays for hematopoietic progenitors (Bradley and Metcalf, 1966) later allowed Metcalf et al. to purify G-CSF, a hematopoietic cytokine that greatly enhanced the production of neutrophils

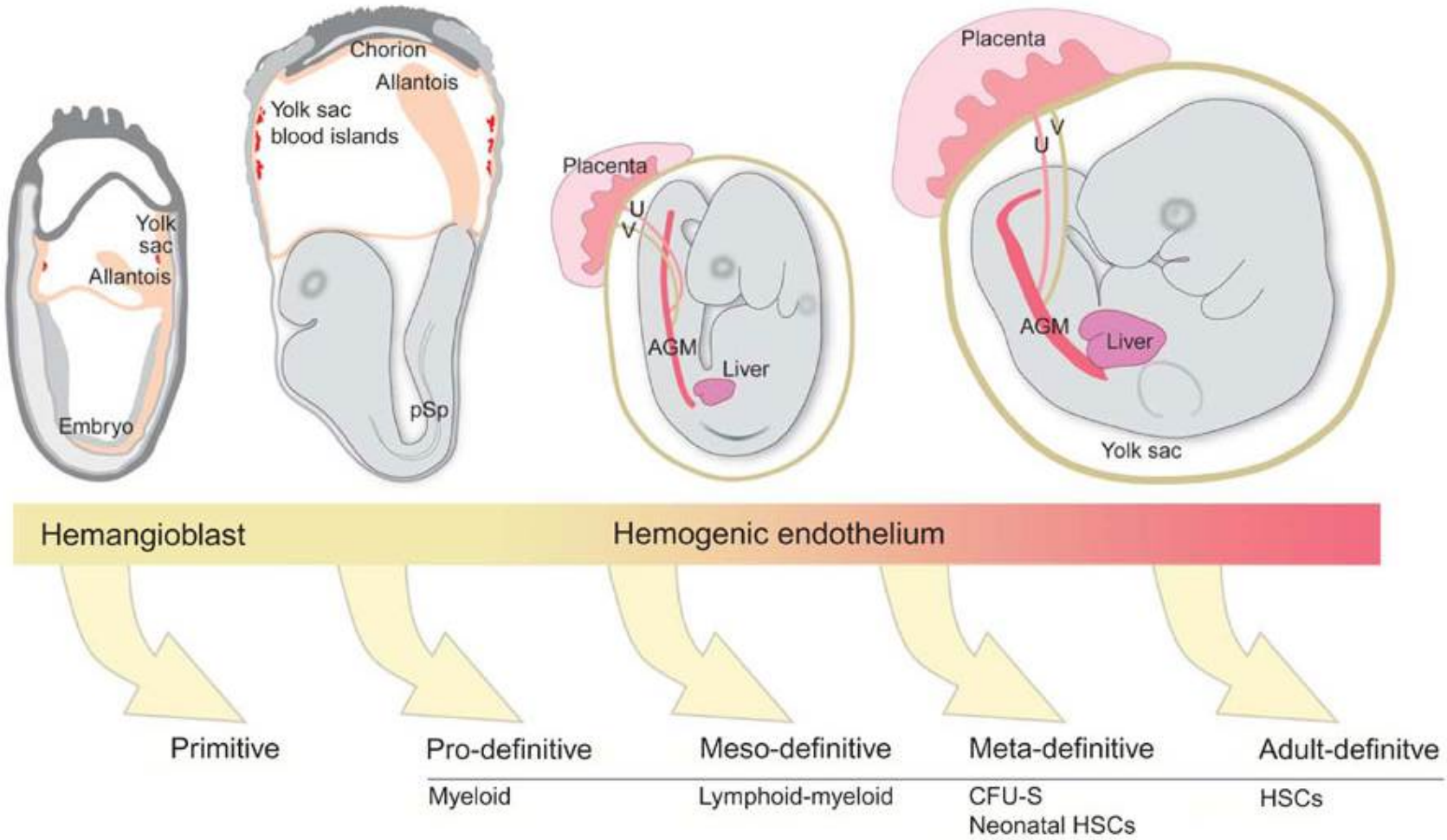
(Metcalf and Nicola, 1983). The discoveries of EPO and G-CSF were significant because they could be used to counteract chemotherapy-associated anemia and neutropenia, respectively, at the HSPC level. Prior to this, therapies to counteract cytopenias were limited to red blood cell transfusions that can cause iron overload, and could not protect patients from life-threatening bacterial infections (Bronchud et al., 1987). Consequently, both EPO and G-CSF have become mainstay therapies for hematopoietic injury from chemotherapy and ionizing radiation [reviewed by (Bendall and Bradstock, 2014; Dainiak, 2010)].

Hematopoietic cytokines other than EPO and G-CSF have been investigated as potential therapies, but have not been as efficacious. Both interleukin 7 (IL-7) and thrombopoietin (TPO) have been administered in attempt to enhance lymphocyte and megakaryocyte differentiation, respectively (Dainiak, 2010; Herodin and Drouet, 2005). Some patients develop paradoxical thrombocytopenia through the production of TPO-neutralizing antibodies (Li et al., 2001). The discovery that irradiation induces apoptosis in HSC led to the use of stem and progenitor cell-targeted cytokine therapy with interleukin 3 (IL-3), stem cell factor (SCF), and FMS-like tyrosine kinase (Flt)-3-Ligand (Dainiak, 2010). These therapies have even been attempted after a 24 h delay, which in theory allows injured HSPC time to react to injury prior to being stimulated (Herodin and Drouet, 2005). In addition, several other non-cytokine factors have been identified that promote hematologic recovery, including certain androgens, angiotensin converting enzyme inhibitors or angiotensin II receptor blockers, and hyaluronic acid (presumed to influence extracellular matrix integrity). Currently however, in the setting of radiation-induced hematopoietic injury only G-CSF, pegylated G-CSF, and granulocyte-macrophage colony stimulatory factor (GM-CSF) are FDA-approved therapies (Dainiak, 2010).

Limitations of cytokine therapy. Although it has become instrumental for treating radiation and chemotherapy induced neutropenia, G-CSF has limitations in its efficacy and specificity. The ability of cytokines including G-CSF to mitigate cytopenias decreases with increasing radiation dose or chemotherapy induced myelosuppression (Herodin and Drouet, 2005). Moreover, prolonged use of cytokines is not feasible because it induces systemic inflammation and in certain cases can lead to myelodysplasia or acute leukemia (Bendall and Bradstock, 2014). Cytokine based therapy is also non-specific. Whereas G-CSF was originally identified as a growth factor for granulocytes *in vitro*, when administered *in vivo* it affects multiple cell types within the hematopoietic and immune systems (Martins et al., 2010). Importantly, G-CSF also induces HSC mobilization from the bone marrow, which could be counterproductive to hematopoietic regeneration. This may be an important consideration with respect to recent findings that G-CSF administration can exacerbate HSC injury from irradiation (Li et al., 2015). Therefore, although hematopoietic cytokines have great promise, better therapies are needed. In the words of Metcalf, "the present situation [of cytokine therapy] is confusing and may encourage a certain level of frustration. However, certain facts stand out clearly. The administration of single agents can have predictable and dramatic effects on hematopoiesis … We need to keep these simple facts in mind … Our future task is now to establish precisely what roles are played by each of these candidate regulators and in what clinical situations the application of these regulators may prove to be of value." (Metcalf, 2008).

Part 4: Summary

Through the use of model organisms and studies of human cells, our understanding of hematopoiesis and HSC biology has expanded tremendously since HSC were identified over half a century ago. Although many pioneering translational advancements in cytokine and cell based therapies have been made, therapies for hematopoietic injury from ionizing radiation and chemotherapy are still quite limited. The lifelong demands of the hematopoietic system require maintenance of HSC self-renewal and differentiation potential, both of which are tightly controlled by intrinsic and extrinsic factors. Restoration of normal hematopoietic function after serious injury or HSCT is not complete, evidenced by prolonged sub-clinical injury to HSC and therapy-related hematologic diseases. Treatments that directly target HSC or the hematopoietic microenvironment have not been well developed, and may result in important advancements for medical hematology. The next chapter will describe our current understanding of how the bone marrow microenvironment maintains HSC function during steady state and regenerative hematopoiesis. Specifically, the critical role of vascular endothelial cells in HSC self-renewal and hematopoietic recovery from injury will be discussed as an important area for identifying new candidates and improving therapies.


Yolk sac
Allantois
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pSp
Placenta
U
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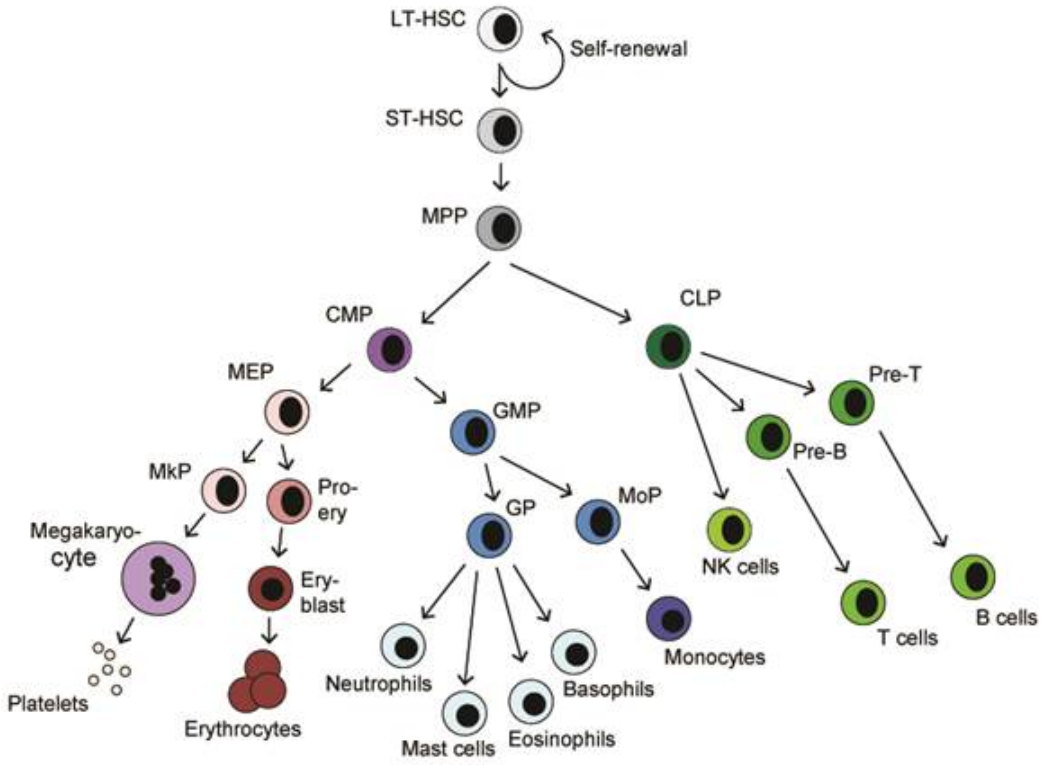
Liver
Placenta
U
V
AGM
Liver
Yolk sac
Hemangioblast
Hemogenic endothelium
Primitive
Pro-definitive
Meso-definitive
Meta-definitive
Adult-definitve
Myeloid
Lymphoid-myeloid
CFU-S
Neonatal HSCs
HSCs

Figure 1-1: The ontogeny of hematopoiesis. Hematopoiesis begins early in development and occurs in two distinct waves. **(a)** The first wave is primitive hematopoiesis, which arises from hemangioblasts that have migrated from the primitive streak to the extraembyronic mesoderm. Hemangioblasts in the yolk sac and allantois (red) have hematopoietic, endothelial, and smooth muscle cell potential, and produce the first erythroid cells and primitive macrophages. As development proceeds, several other subsets of blood cells are produced in the stages of pro-definitive, meso-definitive, and meta-definitive hematopoiesis. **(b)** The onset of definitve hematopoiesis occurs independently from primitive hematopoiesis. In definitive hematopoiesis, hematopoietic stem cells (HSC) are first observed in the aorta-gonado-mesonephros (AGM) region, and eventually reach the circulation via their transition to hemogenic endothelial cells in the dorsal aorta, placenta, umbilical (U) and vitelline (V) arteries. Definitive HSC that arise from hemogenic endothelium migrate to the fetal liver, where the HSC pool expands before migrating to the developing bone marrow cavity. Adapted from Dzirzak & Speck, 2008, with permission. Abbreviations: pSp, paraaortic splanchnopleura; CFU-S, colony-forming unit spleen.

a


LT-HSC
Self-renewal
ST-HSC
MPP
CMP
CLP
MEP
GMP
Pre-T
Pre-B
MkP
Pro-
ery
GP
MoP
Megakaryo-
cyte
Ery-
blast
NK cells
T cells
B cells
Monocytes
Platelets
Erythrocytes
Neutrophils
Basophils
Mast cells
Eosinophils

b

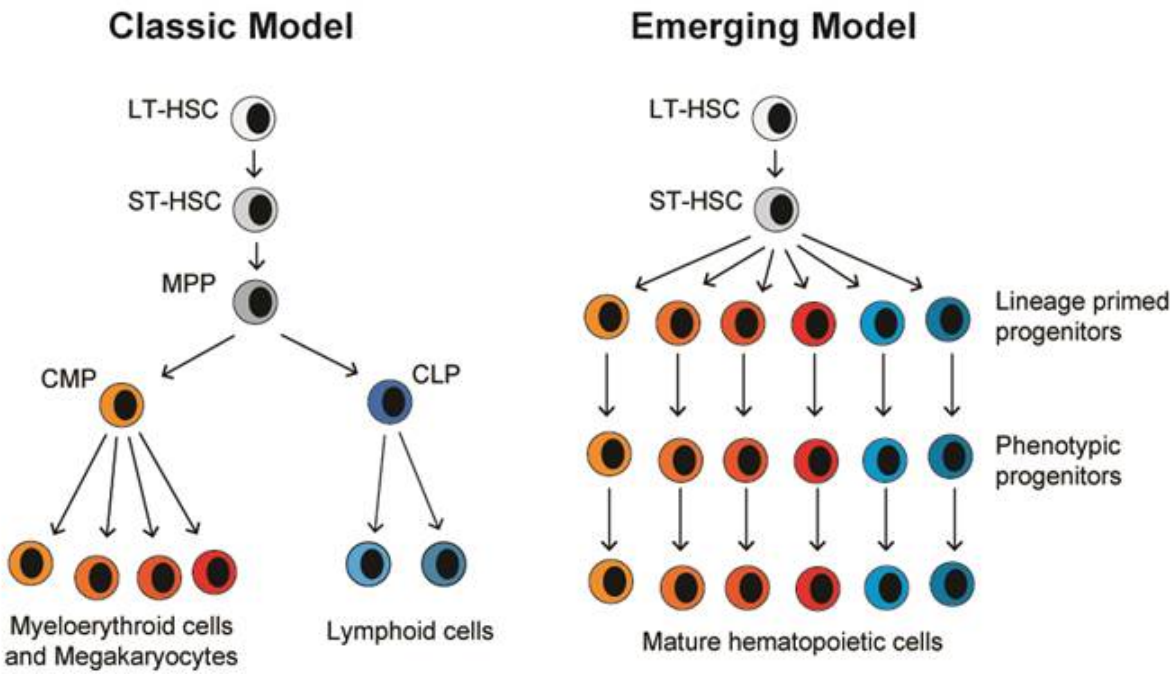

Classic Model
Emerging Model
LT-HSC
ST-HSC
MPP
CMP
CLP
Myeloerythroid cells
and Megakaryocytes
Lymphoid cells
LT-HSC
ST-HSC
Lineage primed
progenitors
Phenotypic
progenitors
Mature hematopoietic cells

Figure 1-2: Hematopoietic differentiation. (a) Hematopoiesis gives rise to all blood cells through the division and differentiation of long-term (LT) self-renewing and short-term (ST)-HSC. Abbreviations: MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; Pre-B, pre-B cell; Pre-T, pre-T cell; MkP, megakaryocyte progenitor; Pro-ery, pro-erythroblast; GP, granulocyte progenitor; MoP, monocyte progenitor; Ery-blast, erythroblast. **(b)** The mechanisms by which HSC and MPP differentiate to mature hematopoietic cells is undergoing a paradigm shift. In the Classic Model, MPP daughter cells formed distinct myeloid and lymphoid compartments, and subsequent divisions within each of these compartments resulted in progressively more lineage-restricted daughter cells. In the Emerging Model, which is based on recent single cell transcriptome and lineage tracing experiments, early progenitor cells are much more restricted to mature cell fates, presumably through the epigenetic phenomenon of 'lineage priming' in HSC and MPPs. Trees adapted from Perie et al. 2015 Cell 163(7), with permission.

Chapter 2: "The Upshot"
Vascular Endothelial Cells Regenerate Hematopoietic Stem Cells

Part 1: Introduction

Part 2: The bone marrow niche for HSC

Early studies of the niche
Discovery that the HSC niche is perivascular
Functional evaluation of the perivascular HSC niche
Heterogeneity of the HSC niche

- Arteriolar niches for HSC
-The role of pericytes in the HSC niche
-Continuing controversy in the niche for HSC

Part 3: The role of vascular endothelial cells in hematopoietic regeneration

Endothelial cells mediate HSC regeneration in vivo
Approaches to define EC factors that regenerate HSC

Part 4: Rationale and specific aims of this research

Part 1: Introduction

Hematopoiesis is a highly dynamic and tightly regulated process. As discussed in Chapter 1, the demand for blood cells can increase with particular exposures or injuries, requiring HSC and progenitor cells to rapidly respond to cues from the bone marrow or systemic circulation. To accomplish this responsiveness and simultaneously maintain HSCs, the bone marrow has evolved "niches"—organized sites composed of several different cell types—that are responsible for regulating HSC and progenitor cell activity. The concept of an HSC niche was proposed by Ray Schofield to explain the discrepancy in serial repopulating potential that occurred between hematopoietic cells harvested from CFU-S_{12} (see Chapter 1) and those transplanted from whole bone marrow (Schofield, 1978). Schofield postulated that the bone marrow contains a particular microenvironment that is essential for maintaining the self-renewal potential of HSC throughout life. The tenets of the niche hypothesis included (1) an anatomically defined location, (2) a site where HSC could be sustained and self-renew, (3) a place where HSC differentiation was inhibited, (4) a space that limited the numbers of total HSC, and (5) a site where reversion of a slightly differentiated cell back to HSC was possible [reviewed by (Papayannopoulou and Scadden, 2008)].

Since Schofield postulated the existence of a niche for HSC, we have learned that even within bone marrow there are likely to be many different anatomic and functional microenvironments that contribute to HSC regulation. The most important of these is the perivascular niche, which is composed of vascular endothelial cells (EC) and associated pericytes derived from mesenchymal stromal progenitor cells (MSPC). The following sections will detail the discovery of the perivascular niche for HSC, as well as the specific role of vascular EC and EC-derived factors in maintaining HSC during steady state and regenerative conditions.

Part 2: The bone marrow niche for HSC

Early studies of the niche

The first attempts to identify the HSC niche compared hematopoietic colony forming activity between marrow sections grossly partitioned into central and marginal regions (Lord and Hendry, 1972; Lord et al., 1975). Hematopoietic potential was found to be higher in marrow cells that were closer to the endosteal surface of bone. In fact, there was a linear relationship between distance from the central axis of bone marrow and CFU-S_{12} potential (Lord and Hendry, 1972). Similarly, CFU-C assays showed a peak of functional hematopoietic potential occurring about 140 μm deep from the endosteal surface (Lord et al., 1975). These initial findings established that the bone marrow cells possessing functional hematopoietic stem and progenitor cell potential were localized away from the central axis and implicated the endosteum as the site of the HSC niche.

Several other lines of evidence initially corroborated the endosteal niche hypothesis, implicating osteoblasts as the primary functional constituents of the HSC niche. For example, HSC express a calcium-sensing receptor (Adams et al., 2006) and home toward the endosteum of bone following transplantation (Lo Celso et al., 2009). Furthermore, genetic models that enhanced osteoblast content in bone marrow, either through bone morphogenetic protein receptor type IA inactivation (Zhang et al., 2003) or parathyroid hormone/parathyroid related peptide receptor activation (Calvi et al., 2003), were found to increase HSC abundance in bone through greater surface contact with osteoblast-expressed N-Cadherin or Jagged-1, respectively.

Although these initial findings supported a principal role for osteoblasts in regulating bone marrow HSC, subsequent investigations have found contradictory results. Whereas selective deletion of Col 2.3^{+} osteoblasts through forced thymidine kinase expression and ganciclovir administration caused a progressive decline in hematopoiesis, this loss in cellularity

was restricted to B lymphocytes and Ter119-expressing erythrocyte progenitors (Visnjic et al., 2004). Neither the frequency of LSK cells nor the ability of bone marrow cells to form myeloerythroid colonies declined during this time period, indicating that osteoblasts only support hematopoietic progenitor cells and not HSC *per se*. Functional studies have shown that osteoblast-specific deletion of a critical HSC maintenance chemokine, CXCL12, has no effect on HSC numbers or repopulating capacity (Ding and Morrison, 2013; Greenbaum et al., 2013). However, consistent with the studies described above by Visnjic et al., loss of CXCL12 from osteoblasts resulted in a decline in B cell progenitors in bone marrow (Ding and Morrison, 2013). Lastly, the findings that suggested HSC utilize N-cadherin to adhere to osteoblasts has been challenged by Morison's group, which has shown through genetic ablation that HSC do not depend on N-Cadherin for their maintenance (Kiel et al., 2009; Kiel et al., 2007). Together, these findings illustrate that while the endosteum is enriched for HSC activity and is an important site for HSC homing, osteoblasts do not maintain HSC in the bone marrow microenvironment.

Discovery that the HSC niche is perivascular

Vascular endothelial cells (EC) are critical regulators of stem and progenitor cell homeostasis in several organs, including lung, brain, liver and testis (Rafii et al., 2016). In these tissues, EC produce tissue-specific membrane-bound and soluble factors that promote progenitor cell maintenance via paracrine signaling. The concept that a vascular niche supports adult hematopoiesis was introduced nearly a century ago by Florence Sabin when she identified HSCs arising from vascular endothelium in the avian dorsal aorta [Sabin 1917; republished as (Sabin, 2002)]. This pivotal finding established a direct link between vascular EC and adult HSC, and provided precedent for a role of vascular EC in hematopoietic homeostasis. Indeed, subsequent investigations have shown that EC are central to HSC biology at each stage of their migration between the dorsal aorta and the bone marrow. Shortly after the endothelial-to-

hematopoietic transition occurs in the dorsal aorta, nascent HSC migrate to the fetal liver where they expand along a growing hepatic vascular system (Khan et al., 2016). During this time, blood vessels also have an essential role in the formation of the bone marrow microenvironment by promoting endochondral ossification. Vascular invasion of a cartilaginous cast provides both cellular and biochemical elements that mediate formation of the bone and the marrow cavity. Around the time of birth, functional changes within the portal vasculature induce egress of HSC from the fetal liver (Khan et al., 2016), which is very soon followed by their population of the bone marrow cavity (Chan et al., 2009). Therefore, vascular EC are critical regulators of HSC biology from the onset of adult hematopoiesis, and a role for EC in the bone marrow HSC niche is well-supported by lessons learned from developmental hematology.

The bone marrow is a highly vascularized tissue, receiving approximately 10% of cardiac output to support the metabolic demands of bone function and hematopoiesis (Marenzana and Arnett, 2013). The positioning of the vasculature at the interface of hematopoiesis and the systemic circulation makes bone marrow vessels an ideal site for the regulation of hematopoiesis. Figure 2-1 provides an overview of the bone marrow blood vascular system. In long bones, afferent arteries penetrate the cortical bone at a primary ossification center located at the end of the diaphysis, branching to form feeder arterioles that course radially along the periphery of the diaphysis. These arterioles transition into a highly branching and hierarchical network of capillaries, the functional divisions and heterogeneity of which have only recently been described in detail with the development of improved bone fixation methods (Kusumbe et al., 2014; Sivaraj and Adams, 2016). This capillary system begins with “transition vessel” loops arising near the endosteal surface and progress to a sinusoidal system of leaky capillaries, which drain blood radially to a central vein that completes the bone marrow micro-circulation.

The discovery that bone marrow vascular EC provide a niche for HSC was made possible through advancements in our abilities to (1) image HSC-EC interactions within a complex bone marrow microenvironment, and (2) evaluate the functional role of EC through

conditional deletion studies. The imaging of HSC within bone marrow sections was first accomplished in 2005 through Morrison and colleagues' development of the SLAM phenotype marks for HSC (Kiel et al., 2005b). These investigators found that HSC with the phenotype $CD150^{+}CD48^{-}CD41^{-}Lin^{-}$ localized to murine endothelial cell antigen (MECA)-32^{+} bone marrow EC at steady state, as well as $MECA32^{+}$ EC in the spleens of mice treated with cyclophosphamide or G-CSF (Figure 2-2). These findings established for the first time a method to visualize long-term HSC in bone marrow sections, and revealed that HSC may localize to sinusoidal capillary EC niches. In the same year, Sipkins et al. used intravital fluorescence microscopy to track the homing and engraftment of fluorescently-labeled HSC in calvarial bone marrow, observing HSC localized to the bone marrow vasculature in as little as 2 h after transplantation (Sipkins et al., 2005). Remarkably, a subset of label-retaining progeny of these HSC remained near vessels up to 70 days post-engraftment, suggesting that a proportion of transplanted HSC remained in a quiescent state near the vasculature (Sipkins et al., 2005). Together, these were the first findings to demonstrate anatomic and functional roles for bone marrow vascular EC in the HSC niche.

Subsequent investigation has extended the findings from Kiel et al. and Sipkins et al. to provide a more detailed description of HSC localization in the niche. Furthermore, these detailed studies have helped to resolve some of the controversial findings that the endosteal surface of bone (and osteoblasts) provide a niche for HSC. Nombela-Arrieta et al. provided a 3-dimensional reconstruction of the bone marrow microenvironment to directly compare the distances of HSC to bone marrow EC versus the endosteal surface (Nombela-Arrieta et al., 2013). This analysis revealed that whereas only 20% of $CD48^{-}CD41^{-}Lin^{-}c\text{-}Kit^{+}$ HSC localized within 10 μm to bone, the vast majority (~80%) of these cells were found <10 μm from a blood vessel, with the majority of those (90%) localized to sinusoidal vessels (Nombela-Arrieta et al., 2013). Importantly, the endosteal region of bone marrow was found to be highly penetrated with the "transition zone" looping capillary network (Figure 2-1). Scanning electron microscopy of

murine bone marrow vessel casts revealed that the average distance separating blood vessels in the metaphysis is 45 μm, whereas micro-CT revealed that the distance separating bony trabeculae in the metaphysis is nearly 200 μm (Ellis et al., 2011). Therefore, these detailed microscopic analyses of HSC localization and bone marrow vascular architecture have revealed that a purely endosteal niche for HSC does not exist *in vivo*, and that the true niche for HSC is most likely to be perivascular.

Functional evaluation of the perivascular HSC niche

Whereas the studies described above revealed a close anatomic relationship between HSC and vascular EC, the functional role of EC *in vivo* has been uncovered through the use of conditional deletion studies. Factors known to be important for HSC survival or retention in the bone marrow have been deleted from vascular EC by using EC-selective promoters to drive Cre recombinase expression, including VE-Cadherin or the Angiopoietin-1 receptor (Tie2). Using this approach, deletion of *Cxcl12* or *Scf* from *Tie2*-expressing vascular EC was shown to cause loss of HSC content in the bone marrow and reduced HSC function in transplantation assays (Ding et al., 2012; Greenbaum et al., 2013). These results indicate that HSC rely on factors derived specifically from vascular EC to maintain hematopoiesis. This conclusion is also supported by studies from Ralf Adams' group, that identified $VEGFR2^{+}$ vessels in the bone metaphysis as a site of HSC proliferation (Wang et al., 2013). Wang et al. termed these microdomains for HSC 'hemospheres' and showed that they support clonal outgrowth of transplanted HSC or confetti-Cre labeled HSC. Moreover, gene targeting of *Vegfr2* disrupted the vascular niche and reduced HSC content in hemospheres (Wang et al., 2013). These functional studies support a necessary role for vascular EC and EC-derived factors for HSC regulation *in vivo*.

Mechanistic insight into the functional role of EC in HSC regulation has also been provided by *in vitro* co-culture studies. Isolated co-culture of EC and HSC has established that EC are sufficient to drive HSC survival, self-renewal, and proliferation in through contact-dependent (Kobayashi et al., 2010) and non-contact dependent (Chute et al., 2005) mechanisms. Furthermore, EC-HSC co-culture systems have become very useful for identifying specific endothelial-derived factors that modulate HSC function (Kobayashi et al., 2010) (Himburg et al., 2010). This strategy has been extensively used in the context of HSC regeneration after injury, which will be discussed in more detail in Part 3 of this Chapter. In summary, our understanding of the niche that maintains HSC during development and in the bone marrow has improved dramatically since the early models proposed by Schofield. Over time, vascular EC have been recognized as critical regulators of stem cell function in several tissue types including lung, liver, brain, and testis (Rafii 2016); and have been shown to be both necessary and sufficient for forming a niche capable of maintaining HSC and promoting their self-renewal *in vivo* and *in vitro*.

Heterogeneity of the HSC niche

Although the importance of the perivascular HSC niche has been well established, our understanding of the interaction between HSC and the bone marrow vascular system is far from complete. Indeed, HSC have been reported to localize to both sinusoidal (Acar et al., 2015; Kiel et al., 2005b) and arteriolar (Kunisaki et al., 2013) vessels, which occupy distinct regions of the bone marrow environment (Figure 2-1). As mentioned above, recent studies are beginning to uncover the heterogeneity of the bone marrow vasculature, and how this impacts HSC biology (Itkin et al., 2016; Kusumbe et al., 2016). Moreover, the majority of bone marrow vessels are associated with pericytes, which have also been shown to release factors that contribute to HSC maintenance [reviewed by ((Boulais and Frenette, 2015)]. The purpose of this section is to

discuss some of the finer details that have more recently been uncovered regarding the perivascular niche for HSC.

Arteriolar niches for HSC

A number of studies have suggested that arteriolar bone marrow vessels, in addition to sinusoidal vessels, may have important functional roles in HSC maintenance (Itkin et al., 2016; Kunisaki et al., 2013). Morrison and colleagues initially localized HSC to the highly branching sinusoidal system that drains radially toward the center of bone (Kiel et al., 2005b). However, sinusoidal vessels occupy a 25-fold greater volume in bone marrow compared to arterioles. To control for this and determine HSC enrichment at either vessel type, Kunisaki et al. compared the mean observed distance of SLAM HSC from either arteriolar or sinusoidal vessels to the distance that would be expected by random HSC placement. Using whole mount imaging and mathematical modeling, they found that HSC proximity to sinusoids was not statistically more probable than chance (14.2 μm predicted and 14.8 μm observed); however, localization to arteriolar vessels was significantly greater than what would be predicted (52 μm versus predicted 78 μm) (Kunisaki et al., 2013). In contrast, there was no enrichment of quiescent HSC near Col2.3-GFP^{+} osteoblasts, suggesting that the arteriolar enrichment of HSC relative to sinusoids and osteoblasts may be more meaningful. In support of this hypothesis, two independent groups have now shown that HSC localized to arterioles are significantly more quiescent than HSC localized away from arterioles (Itkin et al., 2016; Kunisaki et al., 2013). These findings are from pulse-chase experiments with 5-ethynyl-2'-deoxyuridine (EdU) labeling and whole mount staining for Ki-67 (Kunisaki et al., 2013), as well as using metabolic indicator dyes (Itkin et al., 2016) to mark HSC oxidation status. Interestingly, Itkin et al. also showed that HSC localized to sinusoidal vessels are more oxidized relative to quiescent HSC near arterioles, and suggesting that sinusoidal vessels are a site of HSC proliferation and differentiation for

blood-bound progenitor cells (Itkin et al., 2016). Therefore, the perivascular niche for HSC appears to be functionally distinct and divisible based on the type of vessel to which HSC localize.

The role of pericytes in the HSC niche

The majority of bone marrow vessels are in close association with pericytes which are derived from mesenchymal stem and progenitor cells (MSPC) in bone marrow (Zhou et al., 2014) (Boulais and Frenette, 2015). The importance of MSPC-derived pericytes in the HSC niche was first established in 2004 when a non-osteoblastic, non-endothelial cell with reticular morphology was identified as a major source of CXCL12 in the bone marrow. Referred to as CXCL12 abundant reticular (CAR) cells, they were found in close proximity with sinusoidal EC, and were determined to regulate HSC quiescence and bone marrow retention through chemokine associations with HSC expressing CXCR4 (Sugiyama et al., 2006). The role of stromal cell-derived CXCL12 from different stages of MSPC lineage commitment has been further investigated using conditional deletion studies, and indicate that *Cxcl12* expression in cells derived ultimately from early Prx-1^{+} MSPC, but not more mature osterix (Osx)$^{+}$ osteolineage progenitors, are important for HSC maintenance (Greenbaum et al., 2013). These results suggest that subsets of MSPC-derived pericytes play a functional role in the HSC niche, and confirm previous reports of the absence of mature osteolineage cells (e.g. osteoblasts) in the HSC niche.

Another important group of pericytes was discovered on the basis of their expressing GFP under the promoter of the nestin gene. Studies of Nes-GFP expression in bone marrow have revealed that arteriolar and sinusoidal vessels both associate with distinct populations of Nes-GFP^{+} pericytes (Itkin et al., 2016; Kunisaki et al., 2013). Whereas Sca-1^{+} arteriolar vessels associate with Nes-GFP^{bright} pericytes with spindle shaped morphology, (referred to as Nes^{peri});

Sca-1^{-} sinusoidal vessels are enwrapped by reticular-shaped Nes-GFP^{dim} pericytes (Kunisaki et al., 2013). This distinction became important when Kunisaki et al. found that dormant HSC were highly enriched within microdomains that contained Nes^{peri} cells and their associated arteriolar vessels (Kunisaki et al., 2013). Depletion of NG2^{+} pericytes, which labels 30% of Nes^{peri} cells, using a tamoxifen-inducible diphtheria toxin receptor, resulted in significant entry of HSC into cell cycle and a delocalization away from arteriolar vessels (Kunisaki et al., 2013). Together, these data indicate that pericytes have important roles in the HSC niche, and can interact with vascular EC to create functionally distinct domains for HSC.

In addition to their contribution to the perivascular niche for HSC, MSPC play an essential role in the formation and organization of the bone marrow microenvironment in mice and humans. MSPCs enriched by CD146/melanoma cell adhesion molecule (MCAM) in humans, and those expressing the Nestin-GFP (Nes-GFP) transgene in mice, are sufficient to form ectopic sites of hematopoiesis when orthotopically transplanted (Mendez-Ferrer et al., 2010; Sacchetti et al., 2007). Ectopic bone formation involves the recruitment of both a bone marrow vasculature and HSC, indicating that these MSPC play important roles in the formation of the niches that maintain HSC. In addition to pericytes, MSPC also give rise to osteocyte, chondrocyte and adipocyte lineages (Mendez-Ferrer et al., 2010), furthering their importance in the bone marrow microenvironment. Overall our knowledge of the perivascular niche is still limited and quite controversial. Much more work is needed to determine the precise relations between pericytes and vascular EC in forming the HSC perivsacular niche.

Continuing controversy in the niche for HSC

Although our ability to study the HSC niche *in vivo* has improved substantially, several controversies still exist (Kiel and Morrison, 2008). Some of the above mentioned studies are in complete disagreement regarding the relative localization of HSC in the diaphysis area of bone in comparison with the trabecular-rich metaphysis, or whether an enrichment actually exists

(Acar et al., 2015; Guezguez et al., 2013; Itkin et al., 2016; Nombela-Arrieta et al., 2013; Wang et al., 2013). Discordant findings reported between investigators could be due to the several methodologic issues, including choice of markers/functional analysis to identify HSC (Acar et al., 2015; Kiel et al., 2005b), as well as technical differences in tissue preparation or sensitivity of assays (e.g. total number of HSC enumerated). Furthermore, recent reports have added layers of complexity to the perivascular niche by describing heterogeneity in EC and pericyte composition of bone marrow vessels (Itkin et al., 2016; Kusumbe et al., 2016). Some of the markers typically attributed to MSPC-derived pericytes have also been recently shown to overlap with markers of vascular EC (Itkin et al., 2016). These discrepancies could potentially be explained by overlap in the expression of promoters used to activate reporter genes during lineage differentiation (e.g. Nes-GFP) (Ono et al., 2014), or perhaps simply by the continuum that underlies most biologic processes. For example, recent reports have shown that arteriolar EC do not abruptly end in a distinct capillary network (Kusumbe et al., 2016); instead, there is a gradual change in morphology and pericyte association as perfusion pressures decrease. Lastly, the notion that niches for HSC are functional, rather than anatomic, is becoming increasingly important to consider. This has been illustrated through the use of the sulfhydryl reactive dye, pimonidazole, to investigate the relative 'hypoxic' status of HSC and the bone marrow microenvironment (Itkin et al., 2016; Kusumbe et al., 2016; Nombela-Arrieta et al., 2013). Whereas HSC were classically assumed to maintain quiescence by residing at hypoxic sites distant from vessels, more recent studies have shown that HSC do not partition into pimonidazole^{+} regions (Nombela-Arrieta et al., 2013), and instead suggest that the low oxidative status of HSC is maintained functionally through cues from the niche (Itkin et al., 2016). Together, these data highlight the complexity of discerning the true niche for HSC. Although our understanding of how the bone marrow maintains HSC has improved tremendously in the last 10-15 years, it is likely that much more work will be required to fully establish the functional and dynamic characteristics of the niches that regulate HSC.

Part 3: The role of vascular endothelial cells in hematopoietic regeneration

Although several of the essential mediators of HSC homing, retention, and self-renewal potential in bone marrow have been identified through the investigations discussed above, much less is known about the mechanisms and mediators of HSC regeneration after injury. Engraftment studies provided the first clues that the regenerative niche for HSC is vascular, e.g. HSC home to vascular sinusoids and reside in the perivascular space within an irradiated bone marrow environment after transplantation (Sipkins et al., 2005; Wang et al., 2013). The notion that transplanted HSC home to perivascular niches suggests that resident HSC may also utilize the vascular niche for repopulating the hematopoietic compartment after injury. Furthermore, circulating levels of HSC and hematopoietic progenitor cells increase following chemotherapy (Richman et al., 1976; Siena et al., 1989), indicating that a return to the niche is an important characteristic of hematopoietic regeneration and HSC homeostasis.

Endothelial cells mediate HSC regeneration *in vivo*

As discussed in Chapter 1, the regeneration of hematopoietic function following injury is an unmet clinical need. Given that vascular EC are both necessary and sufficient to promote HSC self-renewal, attention has also turned to whether EC or EC-derived factors can be used to treat hematopoietic injury. The direct role of vascular EC in hematopoietic regeneration was first suggested when grafted adult vessels were shown to mitigate hematopoietic failure following exposure to lethal doses of ionizing radiation (Montfort et al., 2002). Later studies confirmed that EC were the vascular component responsible for this effect, as transplantation of purified EC or endothelial progenitor cells (EPC) derived from fetal blood are sufficient to rescue lethally irradiated hosts (Chute et al., 2007; Li et al., 2010; Salter et al., 2009). Peripheral blood analysis demonstrated EC infusion accelerates the recovery of leukocytes and platelets. Furthermore, the transplantation of brain-derived EC accelerates the recovery of BM cellularity and increases the frequency of HSPC in the bone marrow of recovering recipients (Chute et al.,

2007). Long-term hematopoiesis in rescued recipients was derived entirely from the host (Li et al., 2010), ruling out the possibility that transplanted ECs were contaminated with non-irradiated HSC and indicating that EC elaborate factors that are responsible for regeneration of the host hematopoietic compartment.

Interestingly, neither transplantation of EC isolated from fetal brain, nor mesenchymal stromal cells (MSC) were able to recapitulate the mitigating effects of transplanting adult brain EC (Chute et al., 2007). In addition, cytokines (VEGF, IL6, SDF, and PDGF-A) that were produced by brain-derived EC were not sufficient to rescue irradiated mice when administered alone or in combination (Chute et al., 2007). Therefore, although these studies directly illustrate that vascular EC are sufficient to stimulate hematopoietic regeneration, they also show that the requisite EC-derived factor(s) had not yet been identified.

Importantly, it has also been demonstrated that vascular EC are necessary for the reestablishment of hematopoiesis after injury. In addition to effects on hematopoietic cells, chemotherapy and ionizing radiation injure the bone marrow microenvironment (Figure 2-3), resulting in regression of vascular sinusoids (Butler et al., 2010; Hooper et al., 2009; Kopp et al., 2005b) and dysfunction of the other marrow constituents (Cao et al., 2011). With respect to the sinusoids, Rafii's group has shown that the reestablishment of vascular integrity is dependent on VEGFR2 activity and cadherin-dependent homotypic adhesions between sinusoidal endothelial cells. Blockade of sinusoidal regrowth by infusing a combination of anti-VEGFR2 and anti-VE-Cadherin antibodies, or by genetic disruption of VEGFR2, leads to hematopoietic failure after otherwise sub-lethal exposures to ionizing radiation (Butler et al., 2010; Hooper et al., 2009). These studies highlight the importance of stimulating the recovery of bone marrow sinusoids for the ultimately regeneration of hematopoietic function.

Approaches to define EC factors that regenerate HSC

The essential role for vascular EC as a regenerative niche has spurred efforts to identify the mechanisms and mediators of endothelial-dependent HSC regeneration. To do this, platforms have been established in which injured hematopoietic cells are regenerated ex vivo in the presence of primary EC from several mouse or human tissue sources (Kobayashi et al., 2010; Muramoto et al., 2006; Zachman et al., 2013). The ability of EC to stimulate HSC regeneration in co-culture is proof of their powerful role in hematopoietic regeneration. HSC regenerated by EC provide stable multi-lineage repopulation in the peripheral blood of serially transplanted recipients (Chute et al., 2004; Kobayashi et al., 2010), indicating the regeneration of bona fide self-renewing HSC. Mechanistically, EC co-culture results in increased HSPC cell cycling and reduced levels of apoptosis (Chute et al., 2004; Doan et al., 2013b). Both soluble and cell surface factors have been implicated in endothelial-dependent HSC recovery; however, direct comparison has shown that the greatest effects on HSC self-renewal potential have been produced in direct-contact co-culture (Kobayashi et al., 2010; Muramoto et al., 2006). Importantly, ECs also have the capacity to rescue irradiated human hematopoietic $CD34^+$ cells derived from bone marrow or umbilical cord blood (Muramoto et al., 2006). The finding that EC can regenerate human hematopoiesis is an essential underpinning for the translational potential of this field of research.

The capacity of EC to stimulate HSC regeneration in an isolated system has provided the opportunity to much more precisely study the mechanisms and mediators of this process. Different approaches have been developed in attempts to systematically identify the factors that EC utilize to regenerate HSC (Doan et al., 2013c; Himburg et al., 2010; Kobayashi et al., 2010). One common theme is forced activation of survival or growth pathways in EC with subsequent determination of the factors they express that might stimulate hematopoiesis. For example, introducing the adenoviral construct E4ORF into primary human EC leads to elevated AKT activation and an increased capacity of EC to expand self-renewing HSC in co-culture, an effect

that requires direct cell-cell contact and is mediated by endothelial mTOR (Kobayashi et al., 2010). Induced AKT activation in VE-Cadherin expressing EC *in vivo* increases HSC frequency in murine bone marrow 10-fold and improves their engraftment potential (Kobayashi et al., 2010). In these studies, AKT activation in EC enhanced their expression of Notch ligands. A subsequent report from this group has shown that selective Jagged-1 deletion from VE-Cadherin expressing EC impairs hematopoietic recovery from moderate doses of ionizing radiation (Poulos et al., 2013). Thus, AKT activation and Notch ligand expression in vascular EC appear to play an important role in endothelial-dependent HSC regeneration.

Chute and colleagues have also identified factors that are potent mediators of endothelial-dependent HSC regeneration (Doan et al., 2013b; Doan et al., 2013c; Himburg et al., 2010). Initially, the growth factor pleiotrophin (PTN) was identified as a soluble factor that was overexpressed in brain-derived EC capable of rescuing human BM and CB hematopoietic progenitors in non-contact co-culture (Himburg et al., 2010; Muramoto et al., 2006). As its name implies, PTN has widespread effects in HSC through its inhibition of receptor protein tyrosine phosphatase (PTP) activity (Fukada et al., 2006). PTN expands non-irradiated HSC both in vitro and in vivo, and simulates hematopoietic regeneration following sub-lethal exposure ionizing radiation with similar or greater efficacy to G-CSF (Himburg et al., 2010). The effects of PTN have been shown to be sensitive to PI3K, Notch, and Ras inhibition (Himburg et al., 2010; Himburg et al., 2014), suggesting that its inhibition of PTP signaling has multiple effects on the activation status of intracellular signaling pathways. In addition to its role in hematopoietic regeneration, PTN has been shown to regulate HSC retention in bone marrow (Himburg et al., 2012).

A second EC-derived HSC factor identified by Chute's group is epidermal growth factor (EGF). EGF was found to be the most highly overexpressed protein in irradiated bone marrow plasma of mice in which $Tie2^{+}$ EC were protected from radiation-induced apoptosis via deletion of the pro-apoptotic proteins BAX and BAK (Doan et al., 2013c). EGF was subsequently shown

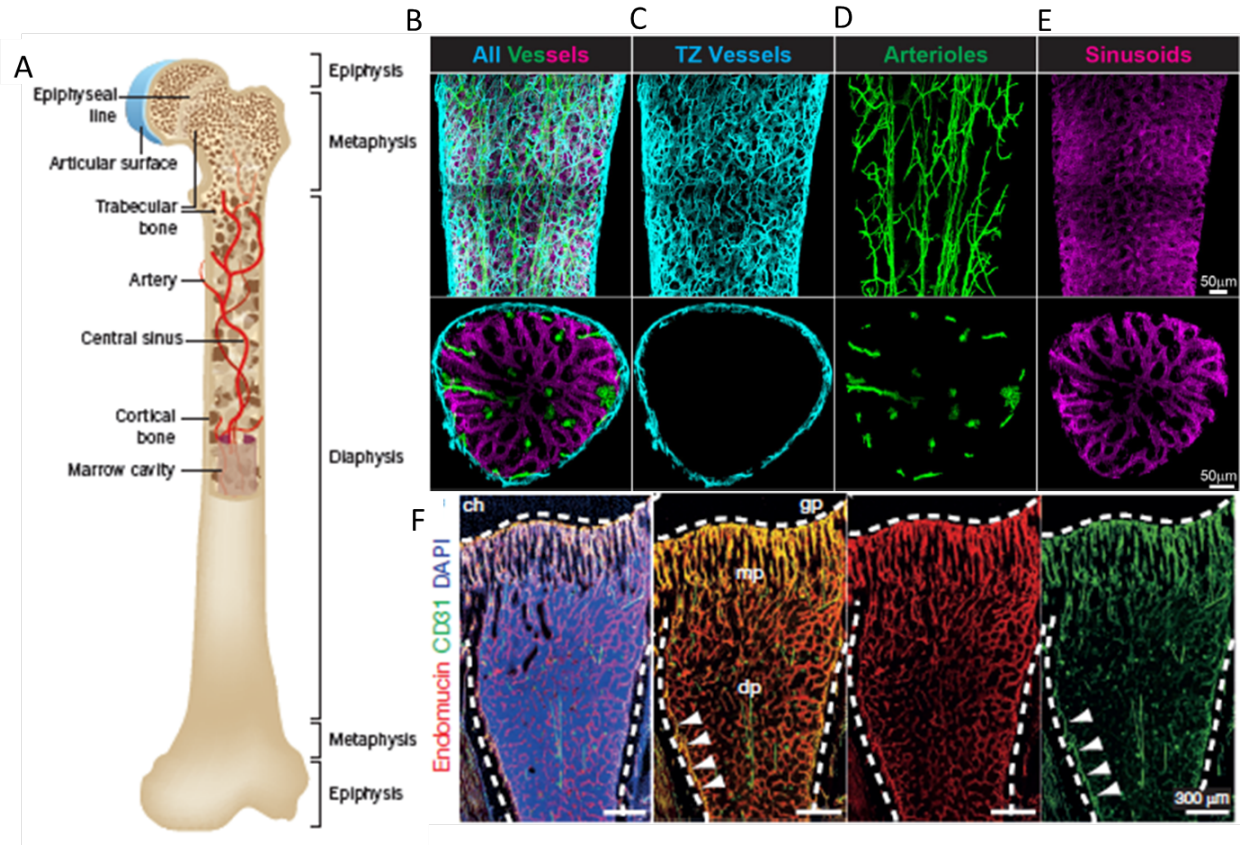
to be a potent HSC regeneration factor *in vitro* and *in vivo* (Doan et al., 2013b). Together, these results show that EC can produce a number of factors that promote the regeneration of HSC after hematopoietic injury (Figure 2-4).

Part 4: Rationale and Specific Aims

The above discussion of the bone marrow niche highlights the importance of extrinsic factors in HSC regulation during steady state hematopoiesis and after injury. Specifically, vascular EC are known to be both necessary and sufficient for HSC regeneration in vitro and possess a remarkable capacity to regenerate self-renewing HSC as measured by serial transplantation. Although the niche factors that regulate HSC during steady state hematopoiesis have been investigated over the last 10-15 years, little is understood about the mechanisms that regulate hematopoietic regeneration. The following chapters will describe my work that addresses this important question. The goals of my studies were to:

Specific Aim 1: Establish a platform to study the mechanisms and mediators of endothelial-dependent HSC regeneration.

Specific Aim 2: Identify specific regulators of endothelial-dependent HSC regeneration.


A
Epiphyseal line
Articular surface
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Central sinus
Cortical bone
Marrow cavity
Epiphysis
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B
All Vessels
C
TZ Vessels
D
Arterioles
E
Sinusoids
50μm
50μm
F
ch
gp
mp
dp
Endomucin CD31 DAPI
300 μm

Figure 2-1: Overview of bone marrow blood vascular system. (A) Image of long bone showing blood vascular supply throughout cortical and trabecular bone of major anatomic divisions. (B-E) Marrow blood vascular architecture (B) depicting distinct regions of transition zone (TZ) vessels near the endosteal surface (C), which are fed by radiating arterioles (D) and drain into a rich sinusoidal system (E) that returns blood centrally to draining veins. (F) Phenotypic heterogeneity of bone marrow blood vasculature highlighting non-uniform expression of CD31 (green) and endomucin (red) by endosteal and central vessels. The bone marrow blood vascular system is still poorly understood and can be characterized on anatomic, phenotypic, or functional properties. Figure adapted with permission from Morrison & Scadden *Nature* 2015; Acar et al. *Nature* 2014; and Kusumbe et al. *Nature* 2014.

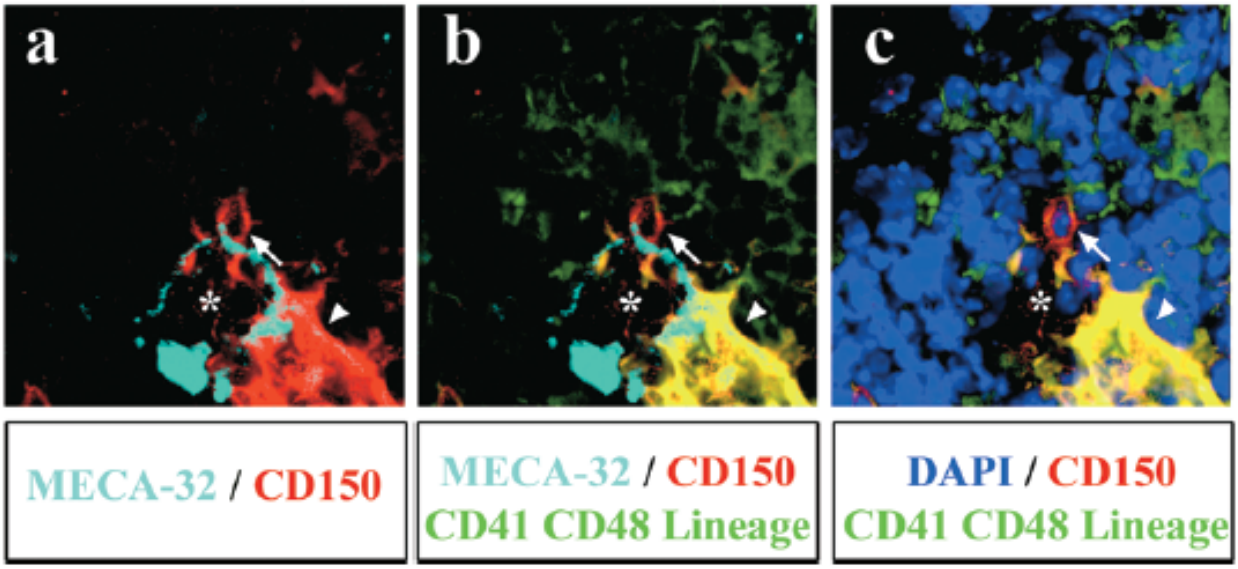
a
b
c
MECA-32 / CD150
MECA-32 / CD150
CD41 CD48 Lineage
DAPI / CD150
CD41 CD48 Lineage

Figure 2-2: Localization of long-term HSC to vascular EC in bone marrow. (a) Bone marrow section showing sinusoid lumen (asterisk) stained with MECA-32 and several nearby cell types staining positive for the SLAM family protein, CD150. (b) Additional staining for CD41, CD48, and hematopoietic lineage markers reveals independently labeled cells localized away from the vascular lumen, as well as $CD150^+$ and $CD41/48/lineage^+$ cells (arrowhead). Full arrow points to HSC labeled with CD150 but not with other antibodies. (c) Composite using DAPI counterstain reveals $CD150^+$ HSC as a rare hematopoietic cell type in bone marrow. Images adapted with permission from Kiel et al., 2005 *Cell.*

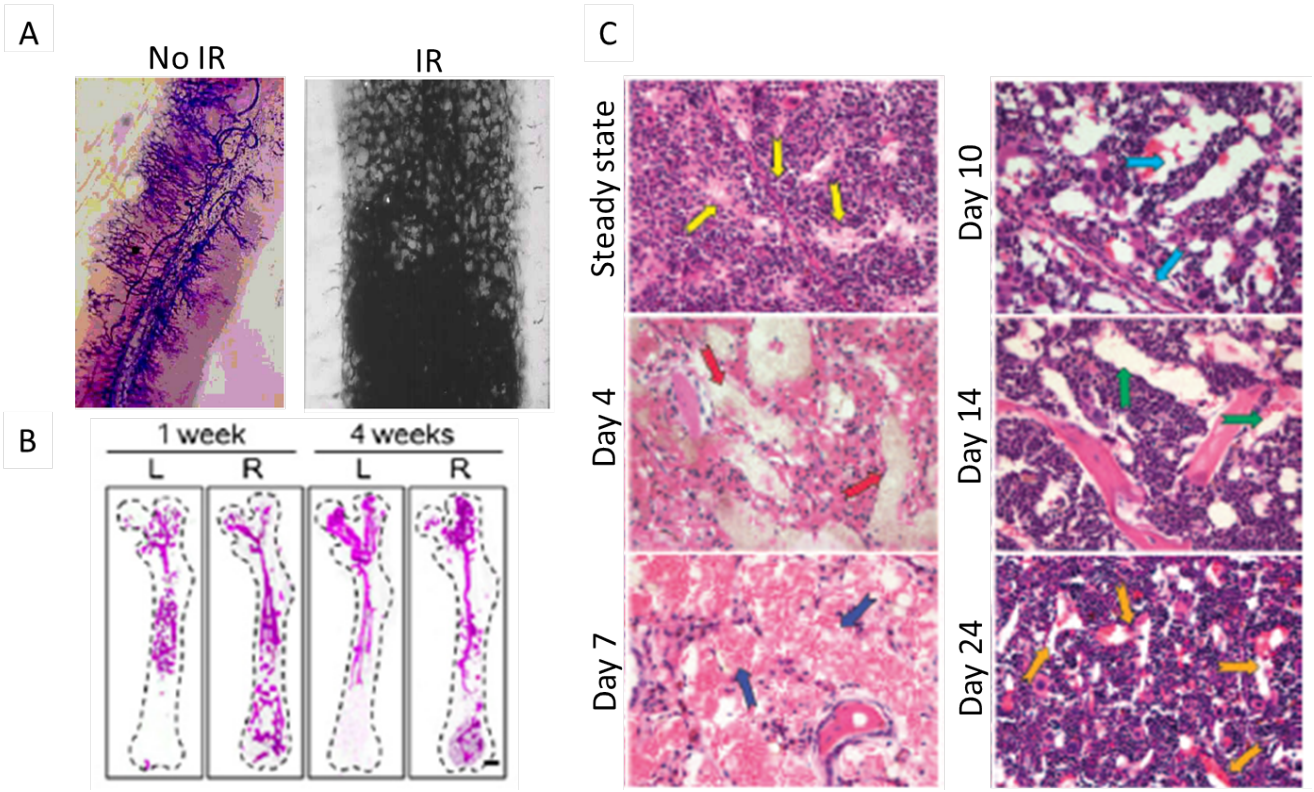
A
No IR
IR
B
1 week
4 weeks
L
R
L
R
C
Steady state
Day 4
Day 7
Day 10
Day 14
Day 24

Figure 2-3: Effects of cytotoxic exposures on bone marrow vasculature. (A) India Ink perfusion of bone marrow vasculature before and after exposure to 650 cGy ionizing radiation showing non-thrombopenic hemorrhage of bone marrow vessels. (B) MicroCT reconstruction of mouse bone marrow vasculature 1 and 4 weeks following local X-ray irradiation to the left (L) distal femur at a dose of 20 Gy distributed equally over a period of 4 days. (C) Regression and regeneration of bone marrow sinusoidal vasculature over the course of 24 days following treatment with 5-fluorouracil. Images adapted with permission from Fliedner et al., 2002; Cao et al., 2011; and Kopp et al., 2005.

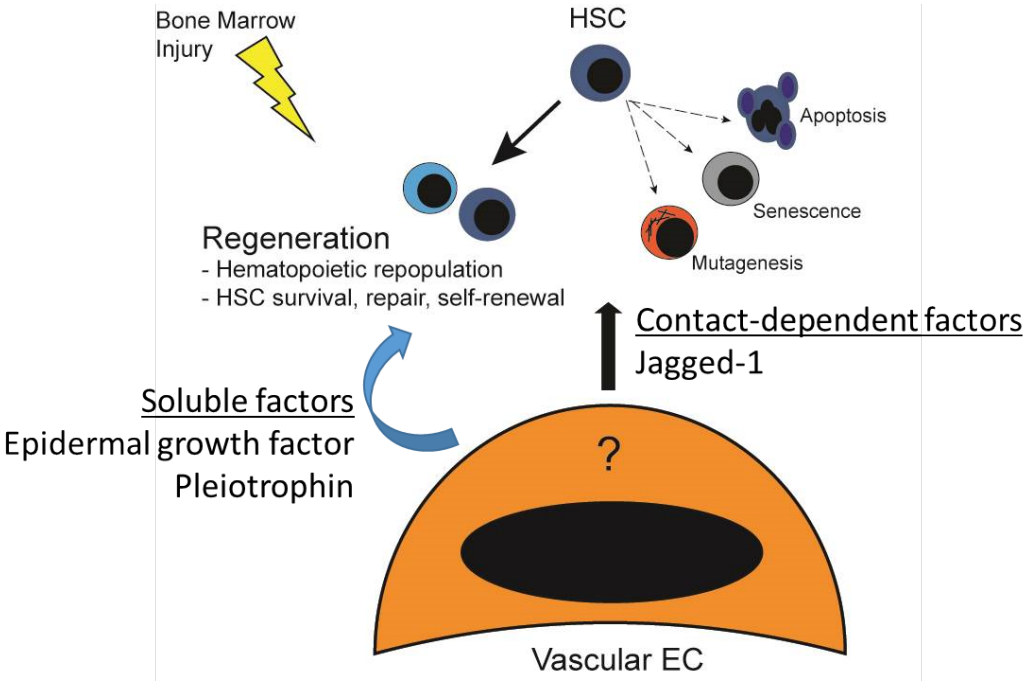
Bone Marrow
Injury
HSC
Apoptosis
Senescence
Mutagenesis
Regeneration
- Hematopoietic repopulation
- HSC survival, repair, self-renewal
Contact-dependent factors
Jagged-1
Soluble factors
Epidermal growth factor
Pleiotrophin
?
Vascular EC

Figure 2-4: Mechanisms of hematopoietic regeneration by vascular EC. Vascular EC have been shown to support HSC regeneration and restore hematopoiesis through the release of soluble factors (epidermal growth factor, EGF; pleiotrophin, PTN) and cell contact-dependent factors (Jagged-1). These factors stimulate HSC regeneration and self-renewal at the expense of apoptosis or senescence induced by radiation-injury. However the mechanisms contributing to endothelial-dependent regeneration are not fully understood.

Chapter 3: Published Manuscript

Endothelial Cells Mitigate DNA Damage and Promote the Regeneration of Hematopoietic Stem Cells after Radiation Injury

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[#]These studies were performed in collaboration with Drs. Ronald P. Leon and Prerna Das, two former post-doctoral fellows. Dr. Das contributed to Figures 1 and 2 by contributing to the establishment of the assay system and performing initial transplantation experiments. Dr. Leon generated the data shown in Figure 3 and contributed the Figure 3-relevant sections of Materials and Methods.

Manuscript Highlights

- Following whole body exposure to ionizing radiation, co-culture with human aortic endothelial cells (HAECs) regenerates hematopoietic stem cells (HSCs) with multilineage reconstituting capability and serial transplant potential.

- Endothelial cell mediated regeneration of HSCs occurs concomitantly with a pronounced reversal of radiation-induced DNA damage in primitive hematopoietic cells.

- Co-culture of irradiated bone marrow with HAECs can be delayed up to 48 hours after radiation exposure and still result in the rescue of functional HSCs.

- *Ex vivo* regeneration of long-term HSCs by co-culture with HAEC is superior to HSC regeneration by culture with G-CSF, the current standard of care therapy for radiation exposure.

Abstract

Endothelial cells (ECs) are an essential component of the hematopoietic microenvironment, which maintains and regulates hematopoietic stem cells (HSCs). Although ECs can support the regeneration of otherwise lethally-irradiated HSCs, the mechanisms are not well understood. To further understand this phenomenon, we studied HSC regeneration from irradiated bone marrow using co-culture with human aortic endothelial cells (HAECs). Co-culture with HAECs induced a 24-fold expansion of long-term HSCs ($CD150^+$, $lineage^{lo}$, $Sca\text{-}1^+$, $c\text{-}Kit^+$; $CD150^+$LSK cells) *in vitro*. These cells gave rise to functional hematopoietic stem and progenitor cells (HSPCs) with colony-forming activity, multilineage reconstitution and serial transplantation potential. Furthermore, HAECs significantly reduced DNA damage in irradiated LSK cells within 24 hours. Remarkably, we were able to delay the exposure of irradiated bone marrow to the regenerative, HAEC-derived signals for up to 48 hours and still rescue functional HSCs. G-CSF is the gold standard for promoting hematopoietic regeneration *in vivo*. However, when compared to HAECs, *in vitro* G-CSF treatment promoted lineage differentiation and regenerated 5-fold fewer $CD150^+$LSK cells. Together, our results show that HAECs are powerful, direct mitigators of HSC injury and DNA damage. Identification of the HAEC-derived factors that rescue HSCs may lead to improved therapies for hematopoietic regeneration after radiation injury.

Keywords

Hematopoietic Stem Cell; Endothelial Cell; Ionizing Radiation; Hematopoietic Regeneration; DNA Damage

Introduction

The hematopoietic system is the most sensitive tissue in the body to the effects of ionizing radiation. Radiation-induced damage to hematopoietic stem cells (HSCs) results in bone marrow failure, which can cause anemia, infection and hemorrhage in irradiated individuals (Mauch et al., 1995); (Chao, 2007). In addition to its acute effects, the induction of oxidative stress and DNA damage in HSCs are thought to underlie the increased risks that irradiated individuals have for developing long term complications, including myelofibrosis, myelodysplasia and acute leukemia (Wang et al., 2010); (Yahata et al., 2011); (Ivanov et al., 2012). Currently, hematopoietic failure following exposure to ionizing radiation is treated with the cytokine granulocyte colony-stimulating factor (G-CSF) (MacVittie et al., 2005); (Dainiak, 2010); however, in the absence of endogenous hematopoietic recovery bone marrow transplantation is the only definitive cure. Thus, discovering the mechanisms responsible for regenerating HSCs and restoring functional hematopoiesis may improve future therapies for hematopoietic radiation injury.

HSCs reside in functional niches within the bone marrow microenvironment, where their asymmetric division and differentiation give rise to all blood cell lineages throughout life (for review, see (Wang and Wagers, 2011)). Coordinate signals from other cellular components of the hematopoietic microenvironment modulate HSC proliferation and differentiation through the elaboration of soluble factors and cell adhesion molecules (Chitteti et al., 2010); (Chen et al., 2013); (Nakamura-Ishizu and Suda, 2013). Endothelial cells (ECs) are microenvironmental components that modulate the proliferation, self-renewal, and differentiation of HSCs at the vascular niche (Kopp et al., 2005a); (Kobayashi et al., 2010). Our group and others have shown that ECs effectively restore hematopoiesis by regenerating irradiated HSCs both *in vitro* and *in vivo* (Chute et al., 2004); (Muramoto et al., 2006); (Hooper et al., 2009); (Li et al., 2010).

However, the mechanisms and practicality of EC-mediated hematopoietic regeneration are still largely unexplored.

In this study, we used a co-culture system to study the regeneration of functional murine HSCs by human aortic endothelial cells (HAECs) following whole body irradiation. We report that HAECs rescue hematopoiesis by reversing DNA damage in primitive hematopoietic cells and expanding long-term HSCs. Furthermore, we demonstrate that HAECs can rescue functional HSCs up to 48 hours following HSC radiation injury, whereas G-CSF cannot. Our results show that HAECs robustly support HSC regeneration following radiation injury, and that *in vitro*, their radiation mitigation is superior to G-CSF.

Materials and Methods

Mice

Congenic male and female 8-12 week old C57Bl/6 mice were used in this study. For transplantation experiments, CD45.2 (Ly5.1) or Ly5.1 $EGFP^+$ (TgN(act-EGFP)OsbY01) mice provided donor bone marrow cells (BMC) and age-matched CD45.1 (Ly5.2) mice were used as transplant recipients. Recipient animals were maintained on acidified water (pH 2.2) for 1 week prior to irradiation and antibiotic-supplemented water for 4 weeks following BMC transplantation. Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Oregon Health & Science University.

Cell Culture Experiments

HAECs (Lonza) were passaged to P3-P5 and then grown to confluence in EGM-2 (Lonza) in $25cm^2$ tissue culture flasks. One day prior to the experiment, confluent HAEC monolayers were irradiated with 1200 cGy using a Shepherd ^{137}cesium irradiator at a rate of 166 cGy/min. On the day of the experiment, BMC were harvested from mouse femurs immediately after 580 cGy whole body irradiation (WBI) and kept on ice. Femurs were flushed with ice-cold 3% serum-modified HBSS, and BMC were washed once before being stained with Turk's solution and counted using a hemocytometer. BMC single cell suspensions ($2x10^6$ BMC/5 mL media) were prepared using EGM-2 supplemented with the following recombinant murine hematopoietic cytokines (purchased from Peprotech): 5 ng/mL IL-3, 5 ng/mL IL-6, 60 ng/mL stem cell factor, 50 ng/mL FMS-like tyrosine kinase 3 (Flt-3) ligand, 2 ng/mL GM-CSF, and 25 ng/mL thrombopoietin. BMC suspensions were cultured on HAEC monolayers (+EC) or plastic (-EC) for 7 days in a humidified 37^oC incubator at 5% CO_2. In delayed rescue experiments,

irradiated BMC were cultured in supplemented EGM-2 for 24 h or 48 h before being seeded on HAEC monolayers or plastic for a subsequent 7 days. For the G-CSF studies, 200 ng/mL human G-CSF (Neupogen) was added to EGM-2 supplemented with murine hematopoietic cytokines. At the end of the 7 day culture period, supernatants were collected, dishes washed once with Ca^{2+}/Mg^{2+}-free PBS, and treated with 0.25% trypsin-EDTA for 1 min at 37^{o}C to dissociate adherent cells. Cells were then collected by washing with ice-cold EGM-2, and BMC were counted as described above. HAECs were excluded from total counts by their large size. For lineage$(Lin)^{lo}$, Sca-1^{+}, c-Kit^{+} (LSK) cultures, equal numbers of FACS-sorted cells (2-3x10^{4}) were seeded into one well of a 24-well plate containing media only and one well containing media and an HAEC monolayer. After 24 h of culture, LSK cells were collected after vigorous pipetting and PBS washes.

Flow Cytometry and FACS

Flow cytometry analysis was performed on a BD LSR-II flow cytometer, and data files were analyzed with FlowJo software (Treestar). For $CD150^{+}$LSK analysis, 1x10^{6} BMC from each treatment group were stained with the following antibodies (purchased from eBiosciences unless otherwise indicated): phycoerythrin (PE)-conjugated CD3, CD4, CD5, CD8 (BD Biosciences), Mac-1/CD11b, Gr-1, B220, and Ter119 (Lin marker cocktail); CD150-Brilliant Violet (BV)-421, Sca-1-PE-Cyanine (Cy)7, and c-Kit-allophycocyanin (APC). All antibodies were used at 1:100 except Mac-1-PE, Gr-1-PE, B220-PE (1:200) and CD150-BV-421 (1:50). Propidium iodide (PI) staining was used to exclude dead cells. Absolute $CD150^{+}$LSK cells were determined by multiplying the $CD150^{+}$LSK cell proportion by the total BMC number in day 7 cultures for each experimental group. Peripheral blood (PB) leukocytes from transplant recipients were stained with anti-Ly5.1-fluorescein isothiocyanate (FITC), anti-Ly5.2-APC, and

Mac-1/Gr-1-, CD3-, or B220-PE to determine the proportion of host- and donor-derived myeloid, T, and B cells, respectively. For secondary transplant experiments, GFP^+ c-Kit-APC^+ cells were FACS purified from primary recipient BMC isolates using a BD Vantage cell sorter. For DNA damage studies, BMC were harvested immediately following radiation exposure and stained with Lin-PE cocktail, Sca-1-PECy7, c-Kit-APC, and PI, and live LSK cells were sorted to purity with a BD Influx cell sorter. Following culture, recovered cells were stained with Sca-1-PECy7, CD45-FITC and PI, and FACS sorted to purity prior to further analysis.

Methylcellulose Assays

Progenitor cell CFU activity was determined as described (Li et al., 2010). Briefly, BMC harvested from day 7 cultures were plated in mouse methylcellulose medium (Stem Cell Technologies, Inc.) at a density of $2x10^4$ BMC/mL in duplicate. Cells were incubated at 37^oC for 7 days and then colonies were scored.

Transplantation Studies

BMC from day 7 cultures were collected as described above and transplanted into recipient mice via retro-orbital injection. $4x10^6$ Ly5.1 or Ly5.1 GFP^+ BMC/recipient mouse were transplanted in 3% serum-modified HBSS into Ly5.2 mice preconditioned with 750 cGy cesium irradiation, and recipients were followed for up to 30 weeks. For PB engraftment analysis, mice were anaesthetized with inhaled isoflurane and PB was collected from the retro-orbital venous plexus. Erythrocytes were sedimented in 3% dextran for 30 minutes, supernatants collected, and erythrocytes further excluded by hypotonic cell lysis using 0.2% NaCl. BMC engraftment

and multilineage reconstitution analysis in transplant recipients were performed as described above.

Comet Assay

To quantify the extent of DNA damage in irradiated LSK cells after 24 h of co-culture, a Comet assay was performed under alkaline conditions as described previously (Olive and Banath, 2006), with the modifications detailed below. The alkaline lysis step was performed overnight at 4^{o}C. A total volume of 1 mL low melting point agarose containing LSK cells was used per slide at a density of 1500-2000 cells/slide. Slides were cooled on ice to facilitate the polymerization of agarose. All solutions were maintained at 4^{o}C with the exception of PI staining solution and washes, and all electrophoresis was also performed at 4^{o}C. Low melting point agarose and alkaline lysis solutions contained 2% DMSO to avoid damage by ion-catalyzed reactive oxygen species. At least 100 LSK cells per condition were scored using Comet IV software and an AVT Marlin Firewire camera. Comet IV software was used to calculate the Olive Tail Moment.

DNA Double Strand Break Analysis

Phosphorylation of the histone variant 2AX at Ser139 (γH2AX) was used to assess the presence of double-strand DNA breaks in LSK cells before and after 24 h of culture with or without HAECs. LSK cells were fixed for 10 minutes in 2% paraformaldehyde, centrifuged, and re-suspended in 150 μl PBS. Fixed LSK cells were placed on Fisher Superfrost Plus glass slides (at a minimum density of 2000 cells/slide), and allowed to settle overnight at 4^{o}C in a humidified chamber. Cells were permeabilized in PBS + 0.15% Triton-X-100 for 5 minutes,

washed once, and blocked for 1 h at room temperature in PBS + 1% BSA. After blocking, a rabbit anti-γH2AX (1:50, Cell Signaling) was applied in PBS + 1% BSA for 1 h at room temperature. Slides were washed twice prior to the addition of secondary antibody (goat anti-Rabbit Cy3) at a dilution of 1:400, and incubated for 1 h at room temperature. After 2 washes, slides were mounted with 100 μL Prolong Gold + DAPI (Invitrogen), and imaged on a Zeiss Axiovert 200 fluorescent microscope. For a negative control, irradiated, sorted LSK cells were fixed at the start of the culture period and stained with secondary antibody only. Images were taken using an Axiocam MRM digital camera, at 100x magnification under oil immersion. Images were processed using Axiovision v4.8 software. For scoring γH2AX, at least 100 cells were counted for each condition using the criteria that < 5 punctate spots was γH2AX negative, and ≥ 5 punctate spots was γH2AX positive.

Statistical Analysis

Data were analyzed using two-tailed Student's t-tests. Statistical significance was considered at $\alpha \leq 0.05$.

Results

HAECs mediate the regeneration of phenotypic HSCs following radiation injury.

To determine whether HAEC monolayers could regenerate phenotypically-identifiable HSCs *in vitro*, bone marrow cells (BMC) were harvested from 580 cGy-irradiated mice and $2x10^6$ input cells cultured in the presence (+EC) or absence (-EC) of HAECs for 7 days (Figure 3-1A). Co-culture with HAECs resulted in a 2-fold increase in total BMC number ($4.0 \pm 0.65 x10^6$ BMC), whereas BMC cultured in the absence of HAECs did not expand relative to input ($1.9 \pm 0.28 x10^6$ BMC; p=0.017 vs. +EC; Figure 3-1B). To determine whether the increase in total BMC included an expansion of the HSC compartment, we analyzed the frequency of $CD150^+$, Lin^{lo}, $Sca\text{-}1^+$, $c\text{-}Kit^+$ ($CD150^+LSK$) cells, which are highly enriched for long-term HSCs (Kiel et al., 2005a); (Chen et al., 2008). Co-culture with HAECs induced an 18-fold increase in the proportion of $CD150^+LSK$ cells after 7 days. The $CD150^+LSK$ cell expansion was a consequence of a 2-fold increase in the frequency of Lin^{lo} cells, a 3-fold increase in the frequency of Lin^{lo} cells expressing CD150, and a 3-fold increase in the frequency of $Sca\text{-}1^+/c\text{-}Kit^+$ cells within the $Lin^{lo}CD150^+$ gate (Figure 3-1C). In total, HAEC co-culture promoted a 24-fold overall increase in the absolute number of $CD150^+LSK$ cells relative to control (3922 ± 705 vs. 160 ± 16 $CD150^+LSK$ cells; p=0.002; Figure 3-1D). These results demonstrate that HAECs can robustly regenerate phenotypically-defined HSCs *in vitro* following radiation injury.

Co-culture with HAECs rescues BMC containing functional hematopoietic stem and progenitor cells.

To query if the BMC regenerated during HAEC co-culture contained functional hematopoietic stem and progenitor cells (HSPCs), we assayed their colony forming activity in methylcellulose and performed serial bone marrow transplantation experiments (Figure 3-2A).

Irradiated BMC cultured in the presence of HAECs had significantly higher colony-forming activity when compared with control-cultured BMC ($27 \pm 4 \times 10^3$ vs. $3.8 \pm 0.7 \times 10^3$ CFUs; $p=0.0002$; Figure 3-2B). Next, we examined HSC functional activity by transplanting BMC into sublethally irradiated congenic recipients. Transplantation of HAEC co-cultured BMC repopulated 20-40% of the peripheral blood (PB) in primary recipients over a 16 week period. In contrast, control cultured BMC contributed only 2.1-4.6% of recipient PB over the same time period ($p<0.05$, Figure 3-2C). Furthermore, analysis of primary recipient PB after 16 weeks of engraftment revealed that HAEC-treated BMC were capable of multilineage reconstitution (Figure 3-2D). To determine whether the functional HSCs regenerated through HAEC co-culture were also self-renewing; donor-derived, c-Kit$^+$ cells were FACS-sorted from primary recipient bone marrow and transplanted into sublethally irradiated secondary recipients. Multilineage hematopoietic reconstitution was detected for up to 16 weeks in these secondary recipients (Figure 3-2E). These results indicate that *ex vivo* co-culture of BMC with HAECs provides an effective means to regenerate *in vivo* functional HSCs following injury by ionizing radiation.

HAECs reverse radiation-induced DNA damage in LSK cells.

Ionizing radiation increases cellular oxidative stress and promotes DNA damage, including double strand breaks (DSBs). Because excessive DNA damage attenuates HSC self-renewal (Yahata et al., 2011), we hypothesized that HAECs may mitigate radiation-induced hematopoietic dysfunction by reducing DNA damage in HSPCs. To test this hypothesis, LSK cells were FACS-sorted from irradiated bone marrow and assayed for DNA damage following culture with or without HAECs (Figure 3-3A). Consistent with the 7 day culture results (Figure 1B), co-culture with HAEC for 24 h resulted in the recovery of 43% more LSK cells relative to controls ($p=3\times10^{-9}$, Figure 3-3B). Phosphorylation of the histone variant H2AX (γH2AX) is a

sensitive, early response marker to DNA-DSBs (Mah et al., 2010); therefore, we assayed DNA-DSBs in irradiated LSK cells by detecting γH2AX immunofluorescence (Figure 3-3C). Nearly all irradiated LSK cells had DNA-DSBs immediately after irradiation (Figure 3D). Although we did not observe a reduction in DNA-DSBs after 3 h of HAEC co-culture (data not shown), extending the culture period to 24 h revealed that HAECs effectively reduce DSBs in LSK cells. Specifically, imaging 24 h after irradiation revealed the persistence of γH2AX foci in 79 ± 2.6% of LSK cells cultured in the absence of HAECs; in contrast, only 26 ± 1.1% of LSK cells cultured with HAECs showed any significant γH2AX signal ($p=2x10^{-6}$; Figure 3-3D). To further validate these results, we also measured DNA strand breaks in single cells using an alkaline Comet assay. HAEC-rescued LSK cells exhibited a 54% reduction in olive tail moment relative to controls (p=0.003; Figure 3-3E), confirming that HAECs reduce DNA damage in HSPCs. Together, these two independent techniques demonstrate that co-culture with HAECs for only 24 h reverses radiation-induced DNA damage in a large proportion of LSK cells. These results suggest that the induction of DNA damage repair is an early event associated with HAEC-mediated mitigation of hematopoietic radiation injury.

HAECs can mitigate HSC loss and rescue functional hematopoiesis up to 48 hours following radiation injury.

Very little is known about how long radiation-damaged HSCs can survive in the absence of environmental factors that promote their regeneration; however, this question has important biologic and therapeutic implications. Therefore, we sought to determine how long after a post-irradiation delay the surviving HSCs remained capable of regeneration by HAECs. To accomplish this, BMC harvested from irradiated mice were cultured under control conditions for 24 h or 48 h before transfer to co-culture with or without HAECs (Figure 3-4A). After a 48 h post-

irradiation delay, HAECs rescued nearly 4-fold more $CD150^{+}$LSK cells than control culture conditions (1026 ± 200 vs. 276 ± 33 $CD150^{+}$LSK cells; p=0.006; Figure 3-4B). We then tested the functional activity of rescued HSPCs with colony-forming assays and bone marrow transplantation. CFU activity was significantly higher in HAEC-treated BMC compared with control BMC after delays of both 24 h (15±4.1 $x10^3$ vs. 2.9 ± 1.1 $x10^3$ CFUs; p=0.019) and 48 h (9.4 ± 1.3 $x10^3$ vs. 2.4 ± 0.7 $x10^3$ CFUs; p=0.0004; Figure 3-4C). Moreover, HAEC co-culture rescued long-term repopulating HSCs that gave rise to 15-32% of total circulating leukocytes and multilineage hematopoietic reconstitution for up to 30 weeks following transplantation into host mice (Figure 3-4D-E). In contrast, after a 48 h post-irradiation delay BMC cultured under control conditions were incapable of engrafting transplant recipients (Figure 3-4D). These results highlight the existence of a 48 h window of opportunity during which endothelial-derived factors can regenerate functional hematopoiesis.

Regeneration of functional HSPCs by HAECs is superior to G-CSF.

The current standard of care therapy for treating unintentional radiation exposure is G-CSF, which improves early hematopoietic recovery in part through HSC mobilization and differentiation (MacVittie et al., 2005); (Dainiak, 2010). To directly compare their potential to regenerate HSPCs, HAECs or G-CSF were co-cultured with irradiated BMC after a 24 h or 48 h delay (Figure 3-5A). Consistent with our previous results (Figure 3-1C), HAECs expanded the proportion of Lin^{lo} cells 2-fold to 14 ± 1.0% of BMC, and this was significantly higher than both control (p=0.009) and G-CSF (p=0.001) treated groups (Figure 3-5B). Further phenotypic analysis showed that HAECs rescue almost 5-fold more $CD150^{+}$LSK cells than G-CSF after a 48 h post-irradiation delay (1107 ± 287 vs. 241 ± 31 $CD150^{+}$LSK cells; p=0.04; Figure 3-5C). The superior ability of HAECs to rescue phenotypic HSPCs in culture correlated with increased

functional activity, as HAEC co-cultured BMC formed 13-fold more colonies than G-CSF co-cultured BMC (17 ± 4.6 $x10^3$ vs. 1.3 ± 0.5 $x10^3$ CFUs; p=0.03) after a 24 h delay prior to HAEC co-culture, and 11-fold more colonies (9.7 ± 1.1 $x10^3$ vs. 0.86 ± 0.17 $x10^3$ CFUs; p=0.004) after a 48 h delay prior to co-culture (Figure 3-5D). Together, these data show that following a post-radiation injury delay of up to two days, co-culture with HAEC is superior to G-CSF for enhancing the recovery of phenotypic and functional HSPCs.

Discussion

We have shown that HAECs mediate the recovery of hematopoietic function following radiation injury by promoting the proliferation of functional HSCs and reducing DNA damage. Relative to control culture conditions, HAEC co-culture regenerated significantly more $CD150^{+}$LSK cells from irradiated bone marrow; furthermore, HAEC-rescued BMC had increased long-term hematopoietic reconstitution potential and contained self-renewing, multilineage-reconstituting HSCs. For phenotypic identification of HSCs we included the SLAM family member CD150, which has been shown to enrich for long-term HSCs within LSK populations (Kiel et al., 2005a); (Chen et al., 2008). HAECs expanded the proportion of $CD150^{+}$LSK cells in culture by 24-fold (Figure 3-1D), and this increase correlated with a log-fold engraftment advantage for HAEC-treated BMC relative to control (Figure 3-2C).

A remarkable finding from our study is the long window of opportunity during which irradiated HSCs can be rescued. Despite the persistence of substantial amounts of DNA damage in LSK cells (Figure 3-3D), a subpopulation of these cells survive for up to 48 h and are responsive to HAEC-derived factors that promote HSC regeneration. In the case of unanticipated exposure to ionizing radiation, the possibility that healthcare intervention may not be immediate is clinically important. Our results show that in the absence of HAEC-derived signals, irradiated HSCs completely lose their ability to repopulate the blood of radiation-conditioned recipients after a 48 h culture delay (Figure 3-4D). Notably, the degree to which irradiated BMC remained capable of producing $CD150^{+}$LSK cells and active progenitors was inversely proportional to the length of the post-irradiation delay. Although we recovered fewer absolute $CD150^{+}$LSK cells from BMC cultures that were delayed 48 h prior to co-culture with HAECs, the percentage of $CD150^{+}$LSK cells in day 7 cultures did not change significantly when compared to BMC cultured immediately on HAECs (data not shown). Thus, our functional studies show that HSCs regenerated by HAECs immediately after irradiation (Figure 3-2C) have

comparable engraftment potential on a per-cell basis as HSCs regenerated by HAECs after a post-irradiation delay of 48 h (Figure 3-4D). These results suggest that HSC death, rather than an intrinsic alteration to the quality and engraftment potential of HSCs, is limiting for the delayed rescue of HSCs through HAEC co-culture.

Currently, G-CSF is the standard of care for the treatment of bone marrow failure from unanticipated radiation exposure or following chemotherapy. The main therapeutic benefit of G-CSF is to enhance neutrophil recovery (Dainiak, 2010). It is less clear whether G-CSF is a direct mitigator of HSPC radiation damage (Drouet and Herodin, 2010). We found that relative to HAECs, treatment of isolated, irradiated BMC with G-CSF after a 48 h delay was less efficacious at promoting the rescue of $CD150^{+}$LSK cells. In fact, when G-CSF was added to control cultures we noted a potentially detrimental effect on the regeneration of HSPCs. Whereas HAECs significantly expanded Lin^{lo} BMC populations relative to both control and G-CSF culture, G-CSF actually induced a shift in BMC populations to the Lin^{hi} phenotype, and this caused a non-significant but pronounced (34%) loss of Lin^{lo} cells in day 7 cultures (Figure 3-5B). Consistent with this loss of Lin^{lo} cells, treatment with G-CSF did not expand active progenitors (Figure 3-5D). This disparate effect of G-CSF treatment *in vitro* may be accounted for by other regulatory signals within the hematopoietic microenvironment that modulate the granulocytic differentiation or asymmetric division of HSCs upon G-CSF stimulation *in vivo*. Indeed, co-culture of irradiated BMC with HAECs + G-CSF yielded intermediate levels of $CD150^{+}$LSK cells and colony-forming activity compared to either treatment alone (data not shown), suggesting that HAECs and G-CSF may induce opposing signaling networks in regenerating HSCs. These data highlight that the continued discovery of endogenous signals that regenerate HSCs may improve our ability to rescue high-fidelity, long-term HSCs with combinatorial pharmacologic treatment.

We have demonstrated that ECs reverse radiation-induced DNA damage in HSPCs, and that this reversal is associated with enhanced bone marrow engraftment and serial transplantation potential (Figure 3-2C-E). We believe these are the first data to suggest that ECs can induce DNA damage repair pathways through paracrine or cell-contact signaling. The identity of the endothelial-derived factor(s) with this activity remains to be determined. Both basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) have been shown to promote DNA damage repair in other cell types (Harfouche et al., 2010); (Bai et al., 2012). Interestingly, systemic delivery of EGF to irradiated mice promotes hematopoietic recovery following radiation injury (Doan et al., 2013a). We did not detect EGF mRNA in HAECs (data not shown), thus ruling out EGF as a candidate HAEC-derived radiation mitigator; however, we cannot rule out the possibility of other ligands signaling through the EGF receptor. Notably, our basal endothelial culture medium contains both FGF-2 and EGF. Thus, the presence of these factors may account for the reduction in the percentage of HSPCs with DSBs (relative to input cells) following culture in the absence of HAEC (Figure 3-3D). Because HAECs promote a much greater degree of DNA repair as well as HSPC expansion in prolonged cultures, we postulate that additional factor(s) from HAECs may be working together with the EGF and/or FGF-2 present in the tissue culture media. To identify these potential candidates, we have undertaken both transcriptome and secretome analyses of HAECs.

In conclusion, our findings add to a growing body of evidence supporting the role of endothelium in HSC maintenance and regeneration following radiation injury (Montfort et al., 2002); (Chute et al., 2004); (Muramoto et al., 2006); (Li et al., 2010). Continued efforts toward identifying endothelial-derived factors that promote the regeneration of HSPCs may lead to improvements in long-term hematopoietic outcomes for individuals exposed to ionizing radiation.

Figures

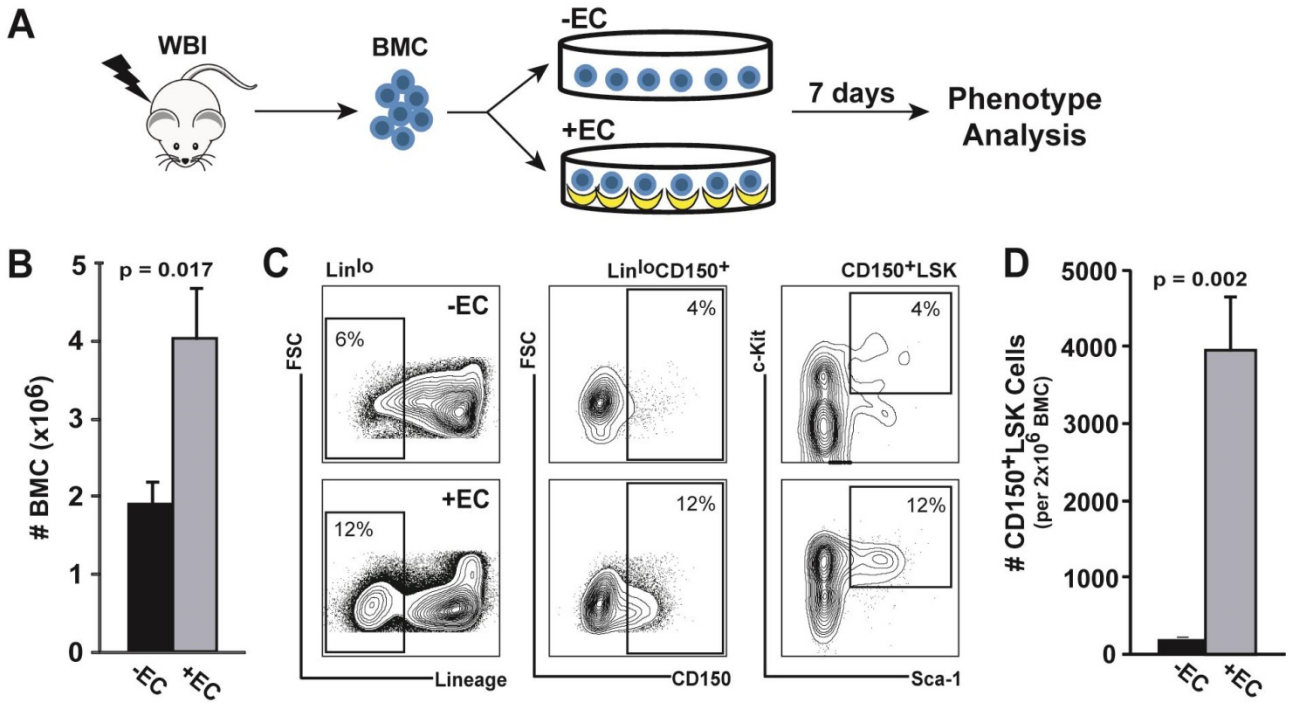
A
WBI
BMC
-EC
+EC
7 days
Phenotype Analysis
B
p = 0.017
# BMC (x10^6)
-EC
+EC
C
Lin^lo
-EC
6%
+EC
12%
FSC
Lineage
Lin^loCD150^+
4%
12%
FSC
CD150
CD150^+LSK
4%
12%
c-Kit
Sca-1
D
p = 0.002
# CD150^+LSK Cells
(per 2x10^6 BMC)
-EC
+EC

Figure 3-1: HAECs promote the regeneration of cells with hematopoietic stem and progenitor phenotypes. (A) Bone marrow cells (BMC) were harvested from the femurs of mice treated with 580 cGy ^{137}Cs whole body irradiation (WBI) and cultured in the absence (-EC, black bars) or presence (+EC, grey bars) of HAEC monolayers (input BMC: $2x10^6$ cells). (B) After 7 days in culture, total BMC were counted and (C) HSCs (Lin^{lo}, $CD150^+$, $Sca\text{-}1^+$, $c\text{-}Kit^+$ ($CD150^+$LSK) cells) were identified by FACS. (D) The absolute number of $CD150^+$LSK cells recovered on day 7 from $2x10^6$ input BMC is shown. Error bars show SEM of 5 independent experiments.

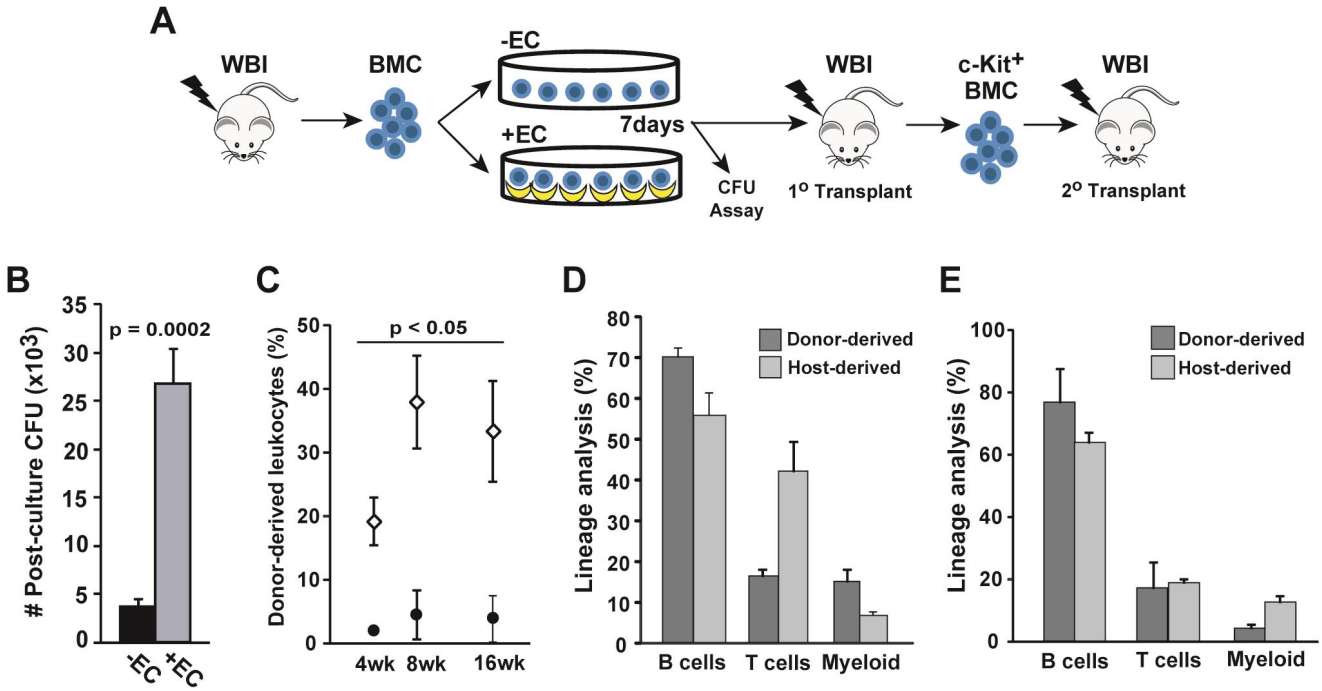
A
WBI
BMC
-EC
+EC
7days
CFU Assay
WBI
1° Transplant
c-Kit+ BMC
WBI
2° Transplant
B
# Post-culture CFU (x10³)
p = 0.0002
-EC
+EC
C
Donor-derived leukocytes (%)
p < 0.05
4wk
8wk
16wk
D
Lineage analysis (%)
Donor-derived
Host-derived
B cells
T cells
Myeloid
E
Lineage analysis (%)
Donor-derived
Host-derived
B cells
T cells
Myeloid

Figure 3-2: HAECs rescue long-term repopulating HSCs. (A) BMC harvested from 580 cGy-irradiated mice were cultured for 7 days in the presence or absence of HAECs and then assayed for CFU activity and *in vivo* hematopoietic potential. (B) BMC were plated in methylcellulose and absolute CFUs per $2x10^6$ input BMC was determined (n=3 independent experiments). (C) Peripheral blood (PB) engraftment of recipients transplanted with BMC cultured in the presence (open diamonds) or absence (closed circles) of HAECs (n=5 recipients/group). (D) Primary transplant recipient donor- and host-derived, multilineage hematopoietic reconstitution of the PB by BMC cultured in the presence of HAECs. Donor-derived, c-Kit^+ cells isolated from the bone marrow of primary recipients were transplanted into irradiated secondary recipients. (E) Multilineage hematopoietic reconstitution in secondary transplant recipients at 16 weeks. Error bars show SEM.

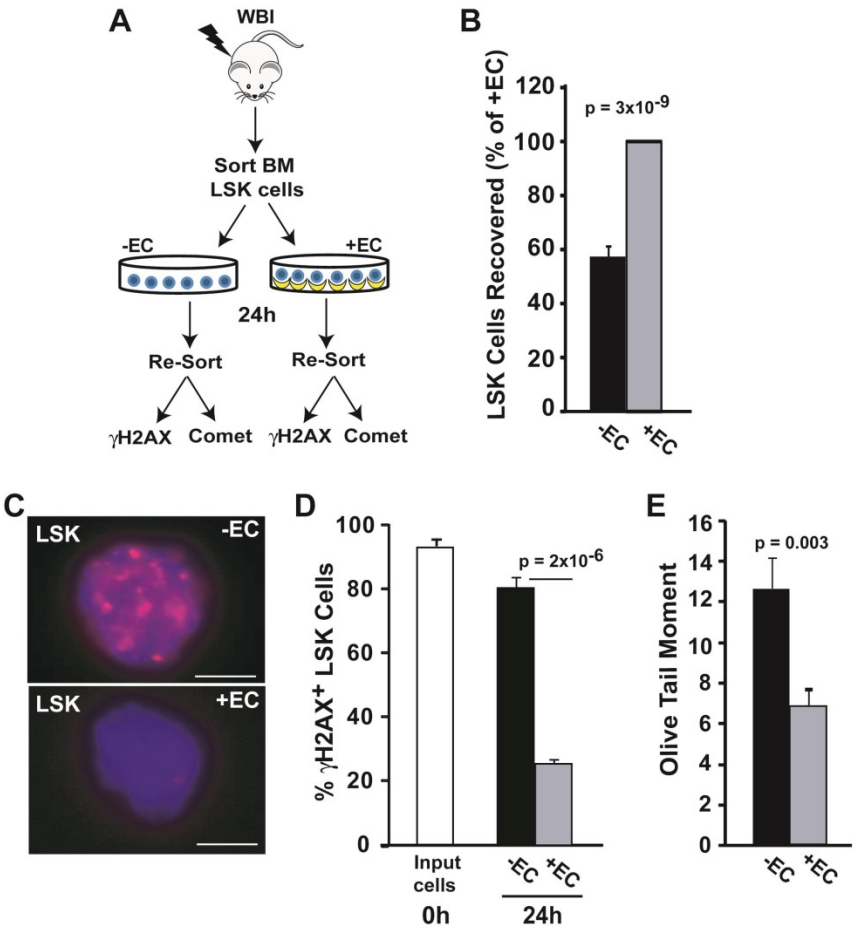
A
WBI
Sort BM
LSK cells
-EC
+EC
24h
Re-Sort
Re-Sort
γH2AX
Comet
γH2AX
Comet
B
LSK Cells Recovered (% of +EC)
p = 3x10-9
120
100
80
60
40
20
0
-EC
+EC
C
LSK
-EC
LSK
+EC
D
% γH2AX+ LSK Cells
p = 2x10-6
100
80
60
40
20
0
Input
cells
-EC
+EC
0h
24h
E
Olive Tail Moment
p = 0.003
16
14
12
10
8
6
4
2
0
-EC
+EC

Figure 3-3: HAECs attenuate DNA damage in LSK cells. (A) LSK cells were FACS-sorted from 580 cGy-irradiated mouse bone marrow and cultured in the presence or absence of HAECs. After 24 h, LSK cells and their progeny were re-sorted based on Sca-1 and CD45 expression, and DNA damage was assessed using γH2AX immunofluorescence and a Comet assay. (B) LSK cells recovered after 24 h, with the +EC group normalized to 100% (n=9 independent experiments). (C) Re-sorted LSK cells were assessed for DNA double strand breaks using γH2AX immunofluorescence. Representative images of irradiated LSK cells cultured in the absence (top) or presence (bottom) of HAECs (scale bar = 5 μm). (D) Quantification of γH2AX-positive cells (≥5 foci/cell) in LSK cells immediately after irradiation (white bar) and after 24 h culture in the presence (grey bar) or absence (black bar) of HAECs. At least 100 cells were scored per group (combined results from 4 independent experiments). (E) DNA damage in re-sorted LSK cells was also determined using a Comet assay. A representative experiment is shown where >100 LSK cells/group were scored for olive tail moment (n=3 independent experiments).

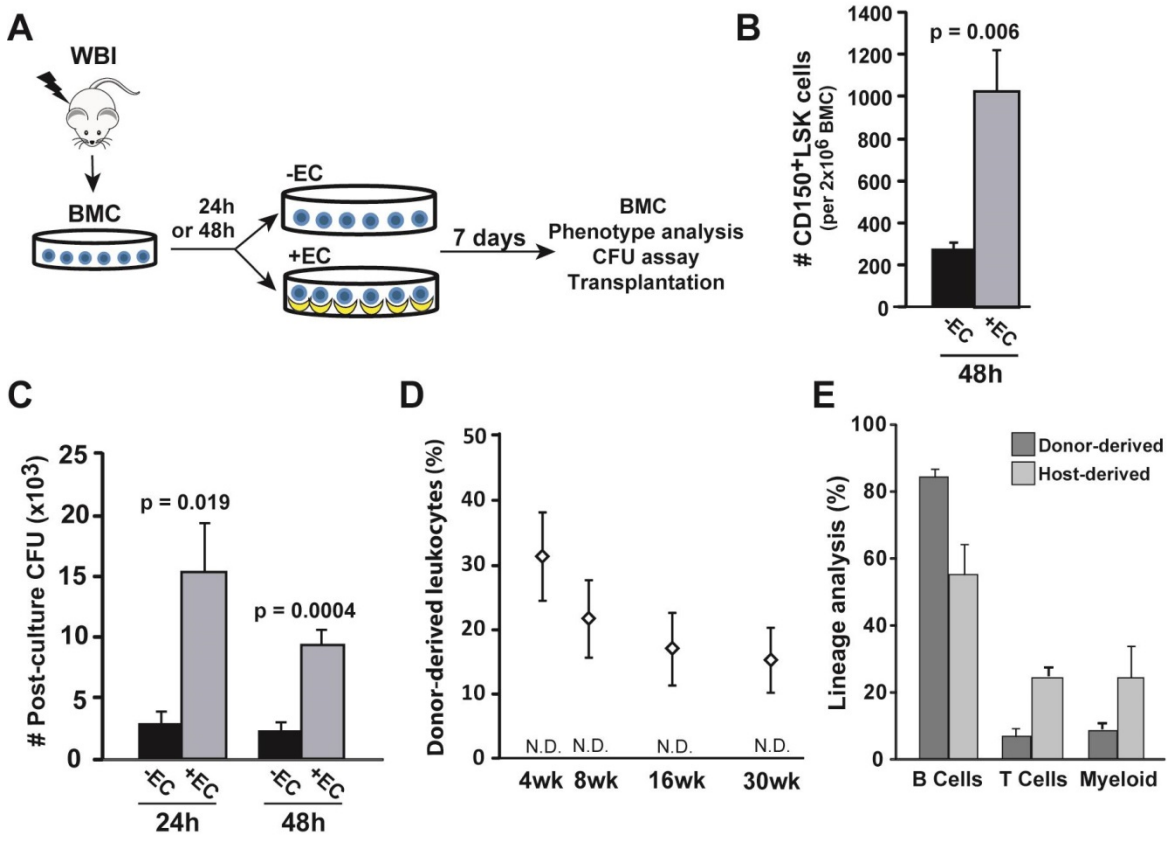
A
WBI
BMC
24h
or 48h
-EC
+EC
7 days
BMC
Phenotype analysis
CFU assay
Transplantation
B
p = 0.006
# CD150+LSK cells
(per 2x10^6 BMC)
1400
1200
1000
800
600
400
200
0
-EC
+EC
48h
C
Post-culture CFU (x10^3)
25
20
15
10
5
0
p = 0.019
p = 0.0004
-EC
+EC
-EC
+EC
24h
48h
D
Donor-derived leukocytes (%)
50
40
30
20
10
0
N.D.
N.D.
N.D.
N.D.
4wk
8wk
16wk
30wk
E
Lineage analysis (%)
100
80
60
40
20
0
Donor-derived
Host-derived
B Cells
T Cells
Myeloid

Figure 3-4: HAECs rescue functional hematopoiesis up to 48 hours following radiation injury. (A) BMC harvested from 580 cGy-irradiated mice were initially cultured for 24 h or 48 h in control conditions and then cultured in either the presence or absence of HAECs for 7 additional days. (B) Absolute $CD150^{+}LSK$ cells (per $2x10^{6}$ input BMC) recovered after a 48 h post-irradiation delay and 7 days of culture (n=5 independent experiments). (C) CFU activity (per $2x10^{6}$ input BMC) after a 24 h or 48 h post-irradiation delay (n=5-7 independent experiments). (D) PB engraftment by 48 h HAEC-rescued BMC for up to 30 weeks following transplantation (n=5 recipients/group). BMC cultured in the absence of ECs provided no measurable level of engraftment (not detectable, N.D., sensitivity = 0.5%). (E) 30 week multilineage hematopoietic reconstitution of the PB by HAEC-rescued BMC after a 48 h delay. Error bars show SEM.

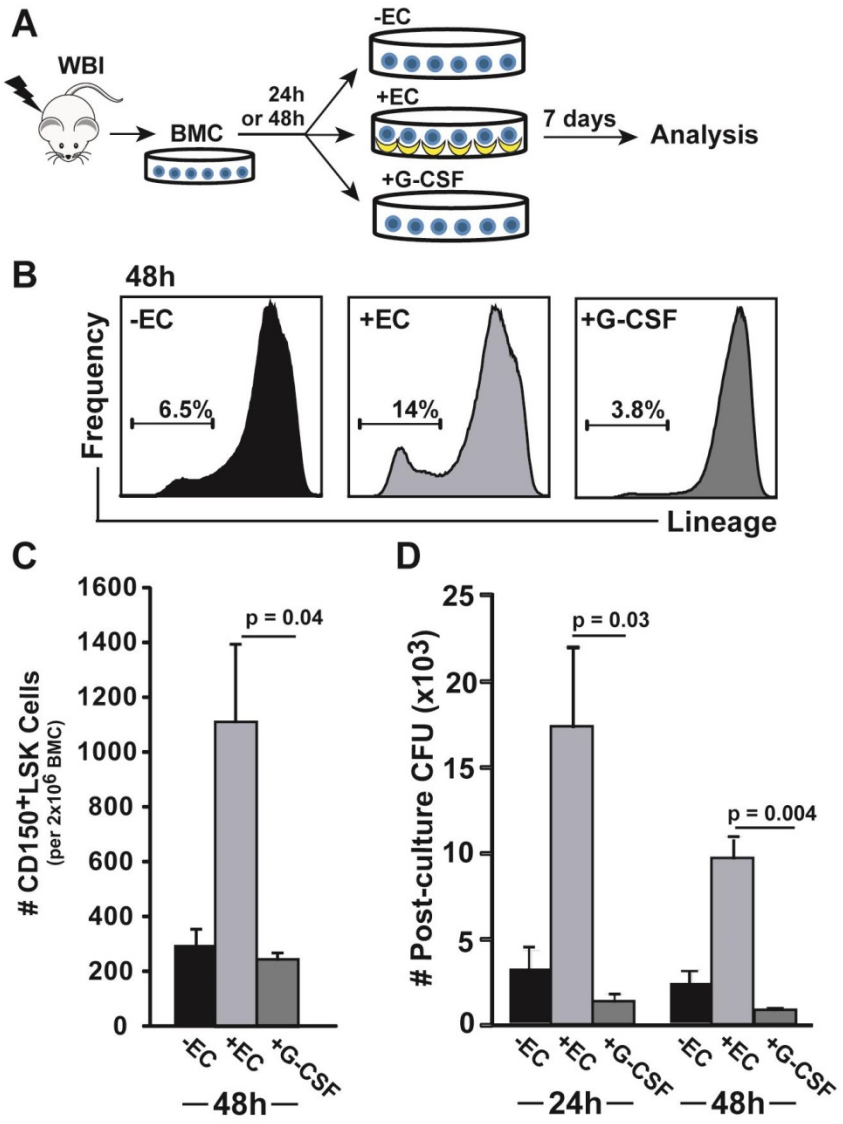
A
WBI
BMC
24h
or 48h
-EC
+EC
+G-CSF
7 days
Analysis
B
48h
-EC
+EC
+G-CSF
Frequency
6.5%
14%
3.8%
Lineage
C
CD150+LSK Cells
(per 2x10^6 BMC)
p = 0.04
-EC
+EC
+G-CSF
48h
D
# Post-culture CFU (x10^3)
p = 0.03
p = 0.004
24h
48h

Figure 3-5: HAECs are superior to G-CSF for promoting HSPC regeneration for up to 48 hours after radiation injury. (A) After a 24 h or 48 h delay, irradiated BMC were cultured in the presence or absence of HAECs, or with G-CSF. (B) Analysis of BMC by FACS after 7 days of culture showed a relative depletion of Lin^{lo} BMC in 48 h delayed, G-CSF-treated cultures (representative flow histograms and a mean of 3 experiments are shown). (C) Quantification of $CD150^{+}$LSK cells following culture in the presence or absence of HAECs, or with G-CSF (n=3 independent experiments). (D) CFU activity of BMC cultured in the presence or absence of EC, or the presence of G-CSF, after the indicated delay period (n=4 independent experiments). Error bars show SEM.

Chapter 4: Manuscript in preparation

TGF-β signaling in vascular endothelial cells inhibits hematopoietic stem cell regeneration[#]

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Manuscript Highlights

- Umbilical vein-derived vascular endothelial cells (HUVEC) poorly support HSC regeneration relative to several other types of primary EC.
- Direct comparison of the transcriptomes of HUVEC and aortic EC (HAEC) can be used to identify candidate regulators of endothelial-dependent HSC regeneration.
- Transforming growth factor (TGF)-β expression and signaling are highly active in HUVEC relative to HAEC and inhibit EC-dependent HSC regeneration through autocrine and paracrine mechanisms.
- Hepatocyte growth factor is overexpressed in HAEC and enhances EC-dependent hematopoietic regeneration.

[#]**Contributions:** Library preparation and RNA Sequencing for these studies was performed in OHSU's Massively Parallel Sequencing Core. Drs. Beth Wilmot and Shannon McWeeney performed differential gene expression analysis and Gene Ontology analysis on RNA-Seq data. Dr. Devorah Goldman performed immunofluorescence microscopy and contributed to editing. Dr. Guha supplied recombinant HGF.

Abstract

Vascular endothelial cells (EC) are integral to the hematopoietic stem cell (HSC) niche and essential for hematopoietic regeneration after injury. However, little is known about the pathways that regulate EC function in this setting. To better understand the mechanisms responsible, we developed a platform to identify soluble factors that enhance or suppress endothelial-dependent HSC regeneration after exposure to ionizing radiation. Transcriptome sequencing of umbilical vein-derived EC (HUVEC), which poorly supported HSC regeneration in direct contact co-culture, revealed highly significant (FDR $<10^{-9}$) overexpression of transforming growth factor-β (TGF-β) ligands relative to aortic EC (HAEC) that robustly support HSC. Functional studies confirmed that TGF-β1 is necessary and sufficient to inhibit endothelial-dependent HSC regeneration in a dose-dependent manner. Increased Smad2/3 phosphorylation and downstream gene set enrichment in isolated HUVEC cultures indicate that TGF-β also participates in an EC autocrine loop. Indeed, pre-treatment of HAEC with recombinant TGF-β1 elevated total TGF-β1 levels during hematopoietic regeneration and led to a 5-fold reduction in HSC transplantation potential. To determine mechanisms that may act on EC to enhance hematopoietic regeneration, we directly compared soluble factor expression between HUVEC and HAEC. HAEC overexpressed nearly two-dozen factors >2-fold with highly significant FDRs. Amongst these candidates, hepatocyte growth factor (HGF) enhanced HSC regeneration by HUVEC >10-fold and significantly improved long-term bone marrow engraftment and multi-lineage hematopoietic reconstitution by regenerated HSC. Treatment of EC with HGF robustly activated endothelial Akt signaling and reduced TGF-β1-dependent Smad2/3 activation. Together, our findings identify an inhibitory role for TGF-β1 on endothelial-dependent HSC regeneration, and demonstrate that EC-targeting growth factors such as HGF have the potential to significantly enhance HSC regeneration after injury.

Introduction

Hematopoietic stem cells (HSC) are tightly regulated to maintain blood cell production throughout life. The precise coordination of HSC self-renewal and differentiation is accomplished by niches within the bone marrow composed of vascular, stromal, neural, and bone elements (Boulais and Frenette, 2015; Morrison and Scadden, 2014). Vascular endothelial cells (EC) are known to be critical components of the HSC niche (Rafii et al., 2016), and mediate hematopoietic stem and progenitor cell (HSPC) trafficking between blood and bone marrow (Itkin et al., 2016; Sipkins et al., 2005). Conditional deletion studies have shown that HSC depend on vascular EC factors for both their bone marrow retention and survival (Ding et al., 2012). Long-term (LT)-HSCs identified using the signaling lymphocyte activation molecule (SLAM) phenotype have localize to both arteriolar (Kunisaki et al., 2013) and sinusoidal (Acar et al., 2015; Ding et al., 2012) vessels. The precise regulation of EC by bone marrow vessel subtypes is actively being investigated (Itkin et al., 2016; Wang et al., 2013).

In addition to their role in regulating HSC during steady state hematopoiesis, EC are also central to regeneration of hematopoiesis after injury (Butler et al., 2010; Hooper et al., 2009; Poulos et al., 2013). Studies from our lab (Li et al., 2010) and others (Chute et al., 2007) have shown that the transplantation of vascular EC rescues hematopoiesis after bone marrow-lethal doses of ionizing radiation by stimulating host hematopoietic recovery. Moreover, in *ex vivo* co-culture primary EC stimulate HSC regeneration by producing soluble and membrane-bound factors that activate HSC self-renewal and improve long-term multi-lineage potential (Butler et al., 2010; Doan et al., 2013b; Himburg et al., 2010; Kobayashi et al., 2010; Zachman et al., 2013). Despite these findings**,** the regulation of endothelial-dependent HSC regeneration is still poorly understood. A better understanding of the mechanisms responsible could lead to improved strategies for HSC transplantation or inform therapies for hematologic malignancies.

The transforming growth factor (TGF)-β superfamily of proteins are powerful modulators of hematopoiesis during development and adulthood (Blank and Karlsson, 2015; Dzierzak and Speck, 2008). Canonical TGF-β1 ligand helps to induce specification of hematopoiesis (Pardanaud and Dieterlen-Lievre, 1999) and have a diverse array effects on hematopoiesis and HSC in bone marrow. In contrast, the regulation of hematopoietic regeneration by TGF-β has not been studied in as great of detail. Neutralization of TGF-β has been shown to prolong HSC cycling and improve hematologic recovery following exposure to 5-fluorouracil, indicating that TGF-β is important for HSC return to quiescence (Brenet et al., 2013). Furthermore, hyperactive TGF-β signaling has been shown to underlie DNA damage accrual and attrition in murine models of Fanconi Anemia (Zhang et al., 2016). Within the HSC niche, TGF-β is highly expressed by type-H $CD31^{hi}Endomucin^{hi}$ ECs and megakaryocytes (Kusumbe et al., 2014; Zhao et al., 2014). Although dysregulation of TGF-β is widely implicated in vascular disorders including Marfan Syndrome (Neptune et al., 2003), Hereditary Hemorrhagic Telangiectasia (McAllister et al., 1994), and pulmonary arterial hypertension (Ogo et al., 2013), the effects of TGF-β on endothelial-dependent HSC regeneration have not been investigated.

In these studies, we uncover a novel inhibitory role for TGF-β in the HSC vascular niche. In a screen of several primary human EC, found that human umbilical vein EC (HUVEC) had a markedly reduced capacity to regenerate long-term (LT)-HSC ($CD150^{+}$LSK cells) compared to adult venous or arterial EC (HAEC). Gene expression analysis showed that TGF-β1 is overexpressed by HUVEC and functional studies confirm it is necessary and sufficient to suppress the ability of EC to support regenerating HSC. Additionally we show that TGF-β1 production and signaling is hyperactive in HUVEC and that TGF-β1 suppresses LT-HSC regeneration through both HSC- and EC-autonomous mechanisms. Lastly, transcriptome analysis to identify EC-specific growth factors that enhance the regeneration of LT-HSC revealed that hepatocyte growth factor (HGF) increased endothelial-dependent HSC

regeneration and multi-lineage hematopoietic potential. Our findings uncover a novel inhibitory role for TGF-β1 in the HSC niche, and demonstrate the utility of targeting vascular EC to enhance hematopoietic regeneration.

Materials and Methods

Mice

Male and female 8-12 week old C57Bl/6 mice were used in this study. For transplantation experiments, Ly5.2 EGFP^{+} (TgN(act-EGFP)OsbY01) mice provided donor bone marrow cells (BM cells) and age-matched CD45.1 (Ly5.2) mice were used as transplant recipients. Recipient animals were maintained on acidified water (pH 2.2) for 1 week prior to irradiation and antibiotic-supplemented water for 4 weeks following BMC transplantation as described (Li et al., 2010). Mice were maintained in accordance with the Institutional Animal Care and Use Committee at Oregon Health & Science University.

Hematopoietic Regeneration Co-culture Assays

Regeneration assays for murine hematopoietic cells were performed as described previously (Zachman et al., 2013). Briefly, human primary endothelial cells from aorta (HAEC), saphenous vein (HSaVEC), pulmonary artery (HPAEC) or umbilical vein (HUVEC) (Lonza) were passaged to P3-P5 and then grown to confluence in EGM-2 (Lonza) in tissue culture flasks. Confluent EC monolayers were irradiated 24 h prior to beginning the regeneration assay with 1200 cGy using a Shepherd ^{137}cesium irradiator at a rate of 166 cGy/min. Following irradiation, EC were fed fresh EGM-2 and in certain experiments supplemented with recombinant human TGF-b1 (Peprotech) at doses ranging from 10-30 ng/mL. The day of the experiment, BM cells were harvested from mouse femurs immediately after 580 cGy total body irradiation and kept on ice. Single cell suspensions of irradiated BM cells ($0.4x10^6$ cells/mL) were prepared in EGM-2 supplemented with the following recombinant murine hematopoietic cytokines (Peprotech): 5 ng/mL IL-3, 5 ng/mL IL-6, 60 ng/mL stem cell factor (SCF), 50 ng/mL FMS-like tyrosine kinase 3 (Flt-3) ligand, 2 ng/mL GM-CSF, and 25 ng/mL thrombopoietin (TPO). BM cell suspensions were cultured in direct contact with EC monolayers or plastic (No EC) for 7 days in a humidified

37°C incubator at 5% CO_2. For certain experiments, recombinant human HGF (20-80 ng/mL) or TGF-b1 (10-30 ng/mL) were added in a vehicle of 0.1% BSA. In other experiments, the small molecule inhibitors SB431542 (1μM) or PF04217903 (10 nM) were added to inhibit signaling by ALK5 and c-Met, respectively (SelleckChem). Both inhibitors were dissolved in DMSO, and an equivalent volume of DMSO vehicle was included in cultures not treated with inhibitor. At the end of culture, supernatants were collected and monolayers thoroughly rinsed to dislodge adherent cells. EC monolayers were inspected under a light microscope for remaining hematopoietic cells and monolayer integrity. Certain regeneration cultures were performed using EC-conditioned media that was collected from HAEC or HUVEC monolayers after 4 days in EGM-2. Conditioned media was centrifuged to pellet any non-adherent cells and added back 1:1 v/v to cultures of irradiated BM cells in the absence of EC. Non-contact co-cultures were performed in 6 well tissue culture plates using Costar transwell inserts (Corning) with a 0.4 μm pore size. Regenerated hematopoietic cells were counted using Turk's stain and a hemocytometer.

Human $CD34^+$ Cord Blood (CB) Cell Regeneration Assays

Human hematopoietic regeneration assays were performed as described by Muramoto et al. (2006) with minor modifications. $CD34^+$ CB progenitors were either purchased from Stem Cell Technologies or purified from fresh CB acquired through the Oregon Health & Science University CB donation program. For the latter, $CD34^+$ cells were purified from unprocessed CB units using a Lymphoprep density gradient followed by a two-step positive selection protocol for $CD34^+$ cells (Cell Signaling Technologies) and FACS. A single cell suspension of $CD34^+$ cells in EGM-2 plus 20 ng/mL TPO, 120 ng/mL SCF, and 50 ng/mL Flt-3 ligand (TSF cytokines) was irradiated with 4 Gy and immediately added to culture for a 10 day regeneration assay. CB cells were regenerated in the absence of EC or in direct contact with HAEC or HUVEC with a 50%

v/v media + cytokines supplement on day 7. At the end of regeneration, total CB cell regeneration was enumerated using a hemocytometer and $CD34^{+}38^{-}$ HSPC frequency quantified using flow cytometry.

Colony Formation Assays

Murine hematopoietic progenitor cell colony forming activity was determined as described (Li et al., 2010). Briefly, hematopoietic cells harvested from day 7 cultures were plated in mouse methylcellulose medium (HSC007, R&D Systems) at a density of $2x10^4$ cells/mL in duplicate. Cells were incubated at 37°C for 7 days and then colonies were scored as an average between the two plates. For human CB cells, $2x10^4$ cells were resuspended in a 10x Cell Resuspension Solution before being diluted to a final plating volume in human methylcellulose medium (HSC003, R&D Systems). Cells were seeded in duplicate and incubated for 14-16 days before scoring colonies.

Flow Cytometry and FACS

Flow cytometry analysis was performed on a BD LSR-II flow cytometer, and data files were analyzed with FlowJo software (Treestar). For analysis of phenotypic hematopoietic stem and progenitor cell regeneration, $2x10^6$ regenerated cells from each treatment group were stained antibodies to mark phenotypic HSPCs and exclude mature lineage-marker expressing hematopoietic cells. Antibodies for FACS were purchased from eBiosciences unless otherwise indicated. The following antibodies were used: phycoerythrin (PE)-conjugated CD3, CD4, CD5, CD8 (BD Biosciences), Mac-1/CD11b, Gr-1, B220, and Ter119 (Lin marker cocktail); CD150-Brilliant Violet (BV)-421 (BioLegend), Sca-1-PE-Cyanine (Cy)7, c-Kit-allophycocyanin (APC), and CD48-fluorescein isothiocyanate (FITC). All antibodies were used at 1:100 except Mac-1-PE, Gr-1-PE, B220-PE (1:200) and CD150-BV-421 (1:50). Propidium iodide (PI) staining was used to exclude dead cells. Absolute $CD150^{+}$LSK cells were determined by multiplying the

$CD150^+$LSK cell proportion by the total BMC number in day 7 cultures for each experimental group. For human CB regeneration experiments, $2x10^5$ CB cells were labeled with CD34-APC ad CD38-PE (both diluted at 1:25). Peripheral blood (PB) leukocytes from transplant recipients were labeled with Mac-1/Gr-1-PE (or CD3-PE in separate tubes) and B220-APC to identify multi-lineage hematopoietic cells. Overall donor engraftment was determined using GFP fluorescence and anti-Ly5.2-PECy7.

Transplantation Studies

Hematopoietic cells from day 7 cultures were collected as described above and transplanted into recipient mice via retro-orbital injection. 3,000 Ly5.2 GFP^+ LSK cells or 4 $x10^6$ total regenerated BM cells/recipient mouse were transplanted in PBS into C57Bl/6 mice preconditioned with 750 cGy cesium irradiation along with 75 x 10^3 non-irradiated carrier cells from C57Bl/6 mice unless otherwise noted. Recipients were followed for up to 30 weeks. For PB engraftment analysis, mice were anaesthetized with inhaled isoflurane and PB was collected from the retro-orbital venous plexus. Erythrocytes were sedimented in 3% dextran for 30 minutes, supernatants collected, and erythrocytes further eliminated by hypotonic cell lysis using 0.2% NaCl. Peripheral blood repopulation and multilineage hematopoietic analysis in transplant recipients were performed as described above. For final BM engraftment analysis in HGF experiments, recipient mice were sacrificed after 30 weeks of engraftment, BM cells isolated, and labeled for HSPC analysis as described above.

RNA Sequencing of Endothelial Cells

For EC transcriptome sequencing experiments, EC monolayers were prepared exactly as described for regeneration assays. Four independent experiments were performed identically with fresh aliquots of cryopreserved EC and separate BM cell donors. Cultures were carried out for 24 h in the absence (EGM-2 media only) or in the presence of irradiated BM cells. Parallel

regeneration assays were carried out for a full 7 days to verify the success of hematopoietic regeneration. In the cultures used for RNA sequencing, BM cells and media were aspirated after 24 h and EC monolayers quickly rinsed twice with ice cold PBS. Following the second rinse, EC were scraped from tissue culture dishes and pelleted by centrifugation. EC pellets were resuspended in Trizol reagent for RNA extraction using an RNEasy Micro Kit (Qiagen). RNA was submitted to OHSU's Massively Parallel Sequencing Core for library preparation and transcriptome sequencing. RNA-seq libraries were prepared using the standard TruSeq (Illumina) RNA-seq protocol. Poly(A)+ RNA was extracted from total RNA using oligo-dT-coated magnetic beads. The recovered poly(A)+ RNA was chemically fragmented. Fragmented RNA was enzymatically converted to double stranded cDNA using random hexamer primers. The resulting cDNAs were treated to remove any single stranded overhanging ends, then a single "A" nucleotide was added to the 3' end of each strand. Illumina adaptors with indices were added to each cDNA, followed by a limited number of cycles of polymerase chain reaction (PCR). The final amplification product was examined on a Bioanalyzer (Agilent) for size, absence of adaptor dimers, and estimated concentration. The concentration of each library was verified by real time PCR on a StepOnePlus (LifeTechnologies). Libraries were mixed and diluted to a target concentration that generated approximately 180 million reads per lane on a single read flow cell. Multiplex sequencing was done on a HiSeq 2000 (Illumina). The base call files from the run were converted to fastq files and separated by sequencing barcode using the CASAVA package (Illumina).

Endothelial transcriptome analysis

Endothelial transcriptome analysis was performed by members of OHSU's Department of Medical Informatics and Clinical Epidemiology. Quality Analysis and Control was performed on EC RNA-Seq data using FastQC v0.9.1 after filtering out low quality reads. Trimmed reads were aligned to the unmasked hg19 genome using bowtie v0.12.7 allowing for two mismatches.

Aligned reads were mapped to known transcripts using Rsamtools package (v1.4.1) from Bioconductor. Tags that had fewer than five counts across the four biologic replicates were excluded from analysis. Data were normalized using the Bioconductor package EdgeR v2.2.6 and the upper quartile method. Sample quality was assessed by clustering count data for raw reads and normalized values. In addition, a distance analysis was performed of the biological coefficient of variation. These analyses resulted in close clustering of independent replicates and EC types, indicating the variability between replicates is low. Differential gene expression was analyzed using both negative binomial and generalized linear models. Tag-wise dispersion estimates increased with decreasing transcript expression by both methods. Tag-wise dispersion in the general linear model was closer to overall common dispersion estimates (0.004) when compared with the negative binomial approach. Batch effects were tested by comparing DE within four biologic replicates. Taking into account batch effects, the common dispersion was reduced to 0.002 and median tag-wise dispersion was 0.003. Differential gene expression was determined using the generalized linear model after adjustments for batch effects. DE genes were determined using an exact test and p-values adjusted for multiple testing using Benjamini and Hochberg's approach for controlling the false discovery rate (FDR) (Benjamani & Hochberg, 1995). An FDR < 0.05 was considered statistically significant.

Gene Set Enrichment Analysis (GSEA)

GSEA software downloaded from the Broad website (http://software.broadinstitute.org/gsea/index.jsp) was used to determine gene set enrichment in EC RNA-Seq datasets. Analyses were performed using curated gene matrices that were either all-inclusive or hand-chosen to represent specific signaling pathways (e.g. gene sets reported to be modifiable through Akt or TGF-β1 signaling). Direct comparisons using all-inclusive gene matrices were made between HAEC-BM cell and HAEC+BM cells, as well as HUVEC-BM cells and HUVEC+BM cells, to determine whether exposure to regenerating hematopoietic cells

alters HAEC and HUVEC enriched gene sets differentially. In addition, specific gene matrices were used to compare HAEC-BM cell and HUVEC-BM cell expression, as well as HAEC+BM cell and HUVEC+BM cell expression, to determine whether Akt or TGF–β1 responsive gene sets were differentially enriched between HAEC and HUVEC at steady state, or in the presence of regenerating hematopoietic cells.

Immunofluorescence Microscopy

Immunofluorescence studies were performed on HAEC and HUVEC monolayers to detect Smad2/3 nuclear localization. Following 48 h of culture, EC monolayers were fixed in 4% PFA and immunostained with anti-Smad2/3 monoclonal antibody (clone D7G7, Cell Signaling Technologies) coupled with goat-anti-rabbit Cy3 secondary antibody. Cells were counterstained with Prolong Gold anti-fade reagent with DAPI (Invitrogen). Immunofluorescence staining was carried out per antibody manufacturer's protocol. Images were acquired using a Zeiss Axiovert 200 fluorescent microscope and an Axiocam MRM digital camera.

Western Blotting

Following growth factor stimulation or at various times during regeneration co-cultures, EC lysates were collected into lysis buffer containing 0.5 M Tris-HCl (pH 6.8), 10% glycerol and 2% sodium dodecyl sulfate. For HGF stimulation experiments, EC were first serum starved overnight. Lysates were passed through an 0.5 mL insulin syringe 10 times to shear genomic DNA and 40 μg aliquots stored at -80^{o}C. Samples were prepared for SDS-PAGE by adding bromophenol blue and 25 mM dithiothreitol. Antibodies were diluted according to manufacturer's instructions in 5% BSA to probe for phospho c-Met (Y1234/1235), phospho-Akt (Ser473), total c-Met (clone H-190), or total Akt. All WB and IHC antibodies were purchased from Cell Signaling Technologies except for total c-Met, which was purchased from SantaCruz. Blots

were developed with SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher), imaged with a BioRad ChemiDoc MP Imaging System, and analyzed using ImageJ.

Statistical Analysis other than bioinformatics

Data were analyzed using ANOVA or Student's t-tests. Statistical significance was considered at $\alpha \leq 0.05$.

Results

Heterogeneity in endothelial cell support of hematopoietic regeneration

To identify mechanisms and mediators of EC-mediated hematopoietic regeneration, we screened primary human EC from different vascular sources in order to uncover functional differences in their regenerative capacity. Bone marrow (BM) cells isolated from g-irradiated (6 Gy) mice were co-cultured in direct contact with aortic (HAEC), pulmonary artery EC (HPAEC), saphenous vein (HSaVEC) or umbilical vein (HUVEC) EC for 7 days (Figure 4-1A). Cell numbers declined within the first 24 h and then remained stable from days 1-3. By day 7, HAEC regenerated the greatest number of total hematopoietic cells (Figure 4-1B-C), $lin^{lo/-}$ cells, and LSK ($lin^{lo/-}$ $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$) cells (Figure 4-1D-F and supplemental Figure 4-8). Similar results were seen with HPAEC and HSaVEC; however, HUVEC supported significantly less hematopoietic regeneration under all conditions, with outcomes similar to No EC (Figure 4-1B-F and supplemental Figure 4-8). Functional studies revealed that HAEC regenerated BM cells contained 4-fold more colony forming activity in methylcelluose assays than HUVEC-regenerated BM (Figure 4-1G).

To directly determine if the hematopoietic regeneration potentials of HAEC and HUVEC were conserved in human hematopoiesis, $CD34^{+}$ umbilical cord blood (CB) cells were irradiated with 4 Gy and cultured in direct contact with HAEC or HUVEC (Figure 4-1H) as described by Chute and colleagues (Muramoto et al., 2006). Whereas total CB cell output was similar (Figure 4-1I), there was a significant increase in the frequency of primitive $CD34^{+}CD38^{-}$ cells regenerated by HAEC (Figure 4-1J). Similarly, HAEC co-cultured CB cells produced significantly more hematopoietic colonies in methylcellulose than HUVEC co-cultured cells, including increased numbers of monocytic and erythrocytic progenitors (Figure 4-1K and supplemental Figure 4-8). Together, these studies highlight the deficiency in HUVEC-mediated human and mouse hematopoietic cell regeneration.

HUVEC fail to regenerate long-term functional HSC

To evaluate the regeneration of long-term HSC, the frequency of $CD150^{+}CD48^{-}$LSK cells was determined in HAEC and HUVEC co-cultures. By day 5 of EC-mediated regeneration, virtually all $CD150^{+}$LSK cells co-expressed CD48 (Figure 4-2A and supplemental Figure 4-9). Quantification of a HAEC regenerated $CD150^{+}$LSK cells revealed a 3.5 fold higher number of these HSC that occurred primarily after 5 days. In contrast, there was survival but no expansion of $CD150^{+}$LSK cells in the presence of HUVEC (Figure 4-2B). To directly test their long-term functional activity, equal numbers of GFP labeled LSK cells sorted from HAEC and HUVEC co-cultures were transplanted into sublethally irradiated (7 Gy) mice (Figure 4-2C). Analysis of the peripheral blood at 4 weeks revealed 4-fold more donor-derived leukocytes in the recipients of HAEC-regenerated LSK cells compared to HUVEC-regenerated LSK cells. Over the next several months, the progeny of the HAEC regenerated HSC gradually increased reaching a mean of 22% of the total leukocytes while the progeny of the HUVEC regenerated HSC fell to 0.4% of circulating cells (Figure 4-2D). Lineage analysis of the donor-derived cells at 20 weeks revealed that 15% of leukocytes were $Mac\text{-}1^{+}/Gr\text{-}1^{+}$ myeloid cells derived from the HAEC-regenerated LSK cells (Figure 4-2E; supplemental Figure 4-9). By contrast, the majority of the recipients of HUVEC co-cultured LSK cells showed no detectable myeloid cells. Together, these results indicate that although HUVEC modestly support the regeneration of hematopoietic progenitor cells relative to cytokines alone, they do not support the maintenance or expansion of HSC with long-term, multi-lineage hematopoietic potential. Based on these findings, we hypothesized that uncovering the molecular basis for the differences between the HSC regeneration activity of HAEC and HUVEC may lead to the discovery of factors that regulate this process.

Marked heterogeneity exists between the HAEC and HUVEC transcriptomes

To determine the identity of endothelial cell-derived factors that regulate hematopoietic regeneration we used RNA-Seq to profile the transcriptomes of HAEC and HUVEC after 24 h of culture. Median gene expression level (FPKM) across all $14{,}000^{+}$ unique transcripts was stable (supplemental Figure 4-10); however, HAEC and HUVEC had markedly heterogeneous gene expression. More than half (7,274) of all genes expressed in both HAEC and HUVEC were differentially expressed at a false discovery rate (FDR) <0.05 (Figure 4-3A). In addition, a subset of 961 genes were expressed only in HAEC or HUVEC. HUVEC were highly enriched in homeobox transcription factor expression relative to HAEC (supplemental Figure 4-11). There was a wide range of genes encoding soluble factors between HAEC and HUVEC. To identify candidates for hematopoietic regulation we queried genes encoding soluble factors that were ≥2-fold overexpressed by either type of EC (Figure 4-3B). These included both known and novel candidate factors for regulating EC-dependent hematopoietic regeneration.

A network analysis of the soluble factors overexpressed by either HAEC or HUVEC showed that their soluble factor production contributed to very divergent cellular responses. Whereas in HAEC G-protein coupled receptor and tyrosine receptor kinases were predicted to mediate signaling pathway activation, factors secreted by HUVEC were more highly linked with extracellular matrix proteins and developmental pathways (Figure 4-3C-D). Specifically, network analysis of HUVEC enriched for the TGF-β isoforms (Figure 4-3D). These results were supported by Gene Set Enrichment Analysis which identified both ECM remodeling pathways and TGF-β1 responsive genes as enriched within the HUVEC transcriptome at an FDRq<0.05 (Table 4-1). Parallel analysis of EC gene set expression after exposure to irradiated bone marrow revealed a substantial enrichment for H3K27Me3-mediated chromatin modifications in

both HAEC and HUVEC (Table 4-2 and 4-3), suggesting that epigenetic modifiers are also key components in the endothelial cell response to hematopoietic injury.

A TGF-β1 driven transcriptional program is highly active in HUVEC

TGF-β is a potent mediator of stem cell quiescence through upregulation of the cell cycle inhibitor p57Kip (Scandura et al., 2004). The functional deficit in regenerating HSC observed in HUVEC suggests that they overexpress inhibitory factors of hematopoietic regeneration. Analysis of the differential gene expression profiles indicates that *TGFB1* and *TGFB2* are significantly overexpressed in HUVEC compared to HAEC (Figure 4-4A). Consistent with this, significantly more TGF-β1 protein was present in HAEC conditioned by ELISA (Figure 4-4B). TGF-β signaling has been implicated in disorders of vascular dysfunction (McAllister et al., 1994; Neptune et al., 2003; Ogo et al., 2013); therefore, we investigated whether TGF-β autocrine signaling was also hyperactive in HUVEC relative to HAEC. Indeed, higher levels of Smad2/3 nuclear localization and phosphorylation at Ser423/5 were found in HUVEC relative to HAEC (Figure 4-4C), and significantly increased TGF-β1-response gene expression was detected in HUVEC by both GSEA and qRT-PCR (Figure 4-4D-E). Based on our findings of increased TGF-β signaling in HUVEC, we hypothesized that it may also have an important role in regulating endothelial-dependent hematopoietic regeneration.

TGF-β1 suppresses endothelial cell-dependent HSC regeneration

To determine the effects of TGF-β1 signaling on hematopoietic regeneration, EC-HSC co-cultures were treated with recombinant human TGF-β1 or the ALK5 small molecule inhibitor, SB431542. Modulation of TGF-β signaling in both HAEC and HUVEC co-cultures had dramatic effects on total hematopoietic cell regeneration and on the regeneration of $CD150^{+}$LSK cells (Figure 4-5). Doses of TGF-β1 ≥10 ng/mL reduced total hematopoietic cell regeneration in

HAEC supported cultures by 60% (Figure 4-5A) and $Lin^{lo/-}$ cells by 50% (supplemental Figure 4-12). TGF-β1 strongly inhibited $CD150^{+}$LSK cell regeneration in a dose-dependent manner, reducing the frequency of these cells by 84% to a level similar to that observed in the absence of EC (Figure 4-5B-C). These findings indicate that TGF-β1 inhibits the regeneration of both total hematopoietic cells and phenotypically defined LT-HSC. To determine whether suppressing TGF-β1 signaling in HUVEC improves HUVEC's ability to support hematopoietic regeneration, SB431542 an inhibitor of ALK5 was added to HUVEC co-cultures. Treatment with SB431542 resulted in dose-dependent increases in the number of total BM cells and $CD150^{+}$LSK cells similar to levels seen in HAEC co-cultures (Figure 4-5D-F). These results reveal that TGF-β1 is a potent, yet reversible suppressor of endothelial-dependent hematopoietic regeneration.

To determine whether TGF-β1 suppresses endothelial-mediated regeneration of hematopoietic cells after injury, we studied the EC-autonomous effects of TGF-β1. EC were pretreated with TGF-β1 for 24 h, followed by washout before the addition of bone marrow cells (Figure 4-5G). Although pre-treatment of EC with TGF-β1 did not cause a decrease in total BM cell regeneration (Figure 4-5H), pre-treatment of EC resulted in greater production of TGF-β1 during co-culture (supplemental Figure 4-12) and a significant reduction $CD150^{+}$LSK cells regeneration (Figure 5I-J). Similar to our findings with continuous exposure to TGF-β1, the frequency of $CD150^{+}$LSK cells was similar to hematopoietic cells cultured in the absence of EC (Figure 4-5 I compared with B&E). To test their multilineage hematopoietic potential, LSK cells were sorted from HAEC or TGF-β1 pretreated HAEC co-cultures and transplanted with competitor bone marrow into lethally irradiated recipients (Figure 4-5K). Strikingly, there was a 10-fold reduction in peripheral blood repopulation by LSK cells regenerated by HAEC pre-treated with TGF-β1 (Figure 4-5L). Multilineage analysis revealed a similar frequency of donor-derived B-cells, T-cells and myelomonocytic cells indicating that the inhibitory effects of TGF-β1

on HAEC-mediated regeneration were not lineage-specific (Figure 4-5M). These results demonstrate the existence of a novel, EC-intrinsic suppressive effect of TGF-β1 on hematopoietic regeneration.

HGF enhances hematopoietic regeneration by the vascular niche

The differential gene expression profiles between HAEC and HUVEC prompted us to evaluate factors overexpressed by HAEC to determine their potential to enhance HSC regeneration. To identify candidate factors, we queried differentially expressed genes that encoded known receptor ligands and were ≥2-fold overexpressed in HAEC relative to HUVEC. This analysis revealed several candidates that were overexpressed in HAEC (Figure 4-3). We focused on hepatocyte growth factor (HGF), a cytokine broadly implicated in angiogenesis and tissue regeneration (Aoki et al., 2000; Taniyama et al., 2001), that was overexpressed by 7.6-fold in HAEC (FDR=$3e^{-5}$). Consistent with this, HGF was readily detected in HAEC conditioned media but not in media from HUVEC cultures (Figure 4-6A). In addition, HGF activated c-Met and Akt phosphorylation in both HAEC and HUVEC (Figure 4-6B), and partially inhibited Smad phosphorylation in response to TGF-β1 stimulation in HUVEC (Figure 4-6C). To directly determine whether HGF could enhance HSC regeneration, co-cultures were treated with HGF. While the addition of HGF to HUVEC co-cultures did not alter total hematopoietic regeneration or $Lin^{lo/-}$ cell regeneration (supplemental Figure 4-13), HGF treatment led to greater regeneration of both LSK and $CD150^{+}$LSK subsets (Figure 4-6D and supplemental Figure 4-13). Notably, there was a marked enrichment of $CD150^{+}$ cells that approached the levels seen in HAEC co-cultures (Figure 4-6E). The expansion of total $CD150^{+}$LSK cells was abolished by treatment with the c-Met inhibitor, PF04217903 (Figure 4-6D-E). Notably, HGF treatment of BM cells cultured in the absence of EC did not enhance HSC regeneration (Figure 4-6D), nor did

adding HGF to HUVEC-conditioned media. (Figure 4-13). Together, these findings indicate that HGF participates in an endothelial cell autocrine loop that enhances HSC regeneration.

To confirm HGF enhanced the regeneration of functional HSC, we transplanted regenerated BM cells from HAEC, HUVEC, or HUVEC+HGF co-cultures and monitored long-term, multi-lineage hematopoietic reconstitution (Figure 4-6F). Consistent with their ability to regenerate 10-fold more phenotypic $CD150^{+}$LSK cells, HGF treated HUVEC co-cultures had significantly higher levels of long-term, multi-lineage repopulating HSC (Figure 4-6G-H). Treatment of HUVEC co-cultures with HGF was also sufficient to maintain myeloid cell reconstitution, which was typically lost by co-culture with HUVEC in the absence of HGF (Figure 4-6E-G and Figure 4-2E). To determine whether HGF increases the engraftment potential of LT-HSC, we evaluated donor-derived cells in the bone marrow of recipient mice >30 weeks following transplantation (Figure 4-6I-J). Strikingly, only recipients transplanted with HSC that were regenerated in HAEC or HUVEC+HGF co-cultures had readily detectable donor-derived cells within recipient bone marrow (Figure 4-6J). In summary, these data show that TGF-b1 directs a transcription program that inhibits HSC regeneration in part by EC-autonomous mechanisms (Figure 4-7). Reversing the effects of TGF-β1 and other HUVEC-derived factors by targeting EC with HGF enhances the regeneration of long-term multi-lineage repopulating HSC. Therefore, specific targeting of endothelial cells during hematopoietic regeneration could potentially reverse bone marrow suppression and improve functional LT-HSC regeneration.

Discussion

Vascular EC are essential components of the hematopoietic microenvironment (Ding et al., 2012; Hooper et al., 2009; Kiel et al., 2005b), and have the capacity to regenerate hematopoiesis and HSC *in vitro* and *in vivo* (Chute et al., 2007; Kobayashi et al., 2010; Li et al., 2010; Montfort et al., 2002; Muramoto et al., 2006; Zachman et al., 2013). However, little is known about the molecular cross-talk underlying these interactions. Here we have used the functional and molecular heterogeneity of EC to identify growth factors that activate or inhibit endothelial-dependent HSC regeneration. Using this approach, we uncover a novel, EC-autonomous, inhibitory effect of TGF-β1 on the vascular niche for HSC (Figure 4-7). Furthermore, our data also indicate that targeting vascular EC in the hematopoietic microenvironment with HGF can inhibit TGF-β1 signaling in EC and enhance their ability to regenerate HSC.

We previously showed that HAEC possess the capacity to regenerate self-renewing multi-lineage HSC in co-culture regeneration assays (Zachman et al., 2013). Consequently we screened a panel of primary EC sources to search for potential functional heterogeneity between arterial and venous endothelium. The capacity of HAEC, HPAEC and HSaVEC to regenerate total hematopoietic cells, LSK cells and colony-forming activity was remarkably similar. By contrast, HUVEC consistently showed significantly diminished regeneration activity although it was clearly superior to the absence of EC. Similar differences were seen when the potential of HAEC and HUVEC to regenerate umbilical cord blood-derived $CD34^+$ hematopoietic progenitor cells was evaluated. These marked differences in functional activity between HAEC and HUVEC provided us with a unique tool to explore the EC-mediated signals that regulate hematopoietic regeneration.

Transcriptome profiling of HAEC and HUVEC revealed that although both EC types share many transcriptome responses following exposure to irradiated BM cells, the hyperactive

TGF-β1 response in HUVEC inhibits their ability to mediate the regeneration of $CD150^{+}$LSK cells. Full inhibition of TGF-β1 signaling in HUVEC-BM cell co-cultures restored hematopoietic regeneration to levels seen with HAEC. Conversely, activing TGF-β1 signaling in HAEC co-cultures completely blocked their ability to regenerate HSC. Taken together these results indicate that TGF-β1 is both necessary and sufficient to inhibit endothelial-dependent HSC regeneration.

These findings are consistent with recent studies which show that the immediate inhibition of TGF-β1 has a protective effect on bone marrow suppression after ionizing radiation (Zhang et al., 2013a) and chemotherapy (Brenet et al., 2013). Furthermore, a recent report showed that blockade of a hyperactive TGF-β1 response in Fanconi Anemia HSPC improves survival after mitomycin C challenge by activating homologous recombination DNA repair (Zhang et al., 2016). In agreement with these studies, TGF-β1 treatment of irradiated BM cell cultures significantly increased HSC regeneration; however, the absolute regeneration of HSC was still much lower than in the presence of EC (data not shown). Notably, TGF-β1 induced a substantial phenotypic shift in the HSPC pool. This included a loss of $CD150^{+}$HSC and an accumulation of LSK cells, which occurred concomitantly with a decrease in total hematopoietic cell regeneration. These results indicate that in the setting of hematopoietic regeneration, TGF-β1 may have dual effect on the hematopoietic compartment. Namely, it depletes LT-HSCs while simultaneously inhibiting the expansion of HSPC to repopulate the hematopoietic compartment.

Importantly, in these studies we have identified an EC-autonomous effect by which TGF-β1 suppresses hematopoiesis. Using a modified regeneration assay in which only EC were treated with TGF-β1, we noted a marked (70%) reduction in LT-HSC regeneration. The loss of CD150 expressing HSC results in 10-fold lower engraftment potential and early hematopoietic reconstitution by LSK cells isolated from co-cultures in which EC were pre-treated with TGF-β1. Notably, total hematopoietic cell regeneration was unaffected in this setting, indicating that

$CD150^+$ LT-HSCs are selectively depleted when EC are inhibited by TGF-β1 signaling. Together, these findings establish a previously unknown suppressive role of TGF-β1 on HSC regeneration by vascular EC.

Our results indicate that EC-derived TGF-β1 can suppress hematopoietic regeneration by both autocrine and paracrine mechanisms. The effects of TGF-β1 on HSC are well described and involve cell cycle regulation through induction of cell cycle inhibitors and preventing lipid raft clustering of hematopoietic cytokine receptors (Chabanon et al., 2008; Keller et al., 1990; Ohta et al., 1987; Scandura et al., 2004; Yamazaki et al., 2009). Importantly however, TGF-β1 effects cannot be generalized to all HSC, as upper and lower side population LT-HSC with different myeloid and lymphoid biases are differentially affected by TGF-β1 (Challen et al., 2010). In contrast, the specific effects of TGF-β1 on bone marrow EC during steady state or regenerative hematopoiesis have not been established. TGF-β1 plays important roles in angiogenesis in some contexts (Carmeliet and Jain, 2011); however, can also block EC proliferation through activation of ALK5 (Goumans et al., 2003). In the bone marrow, type H EC express high levels of *Tgfb1* and *Tgfb3*, have recently been shown to be responsible for repopulating the bone marrow sinusoidal capillary network after injury (Kusumbe et al., 2014). This may support a pro-angiogenic, yet hematopoietic-suppressive role of TGF-β1 in the repopulation of the bone marrow cavity with sinusoids. Alternatively, several studies have shown that TGF-β1 mediated endothelial-to-mesenchymal transition (EndMT) can promote vascular dysfunction and increase organ fibrosis following injury (Krenning et al., 2010; Xu et al., 2015; Zeisberg et al., 2008; Zeisberg et al., 2007). Suprisingly, EndMT has not yet been studied as a potential mechanism of hematopoietic dysfunction or bone marrow fibrosis.

Enhancing the function of the vascular niche has the potential to improve HSC regeneration. Importantly, using HAEC as a model we have also identified a number of factors that may promote EC function after bone marrow injury. HGF was one of approximately 20

soluble factors overexpressed ≥2-fold in HAEC relative to HUVEC and has potent effects on hematopoietic regeneration. Addition of HGF to HUVEC co-cultures enhanced $CD150^{+}$LSK cell regeneration >10-fold and significantly improved long-term peripheral blood repopulation and bone marrow engraftment. In support of this, HGF rapidly activated Akt phosphorylation and prevented Smad2/3 activation in TGF-β1 stimulated EC. Interestingly however, HGF had no effect on HSC regeneration in the absence of EC. HGF is known to promote EC function at sites of revascularization after ischemia (Aoki et al., 2000; Taniyama et al., 2001) and can prevent apoptosis in irradiated EC in vitro (Hu et al., 2009), supporting its role as an EC growth factor during hematopoietic regeneration.

Together, these data establish a novel role for the regulation of EC-dependent HSC regeneration by EC autocrine loops. We show that EC derived from aorta overexpress HGF and other cytokines that have the potential to enhance the regeneration of irradiated HSC in co-culture. In contrast, we have also uncovered a strongly suppressive role of EC-derived TGF-β1 to inhibit HSC regeneration. In addition to its direct role on HSC, TGF-β1 also markedly reduces the ability of EC to support HSC regeneration, which results in LT-HSC differentiation and loss of repopulating potential. These results expand our knowledge of the regulation of bone marrow microenvironment, and suggest that targeting vascular EC is a promising approach for maximizing HSC regeneration after injury.

Figures

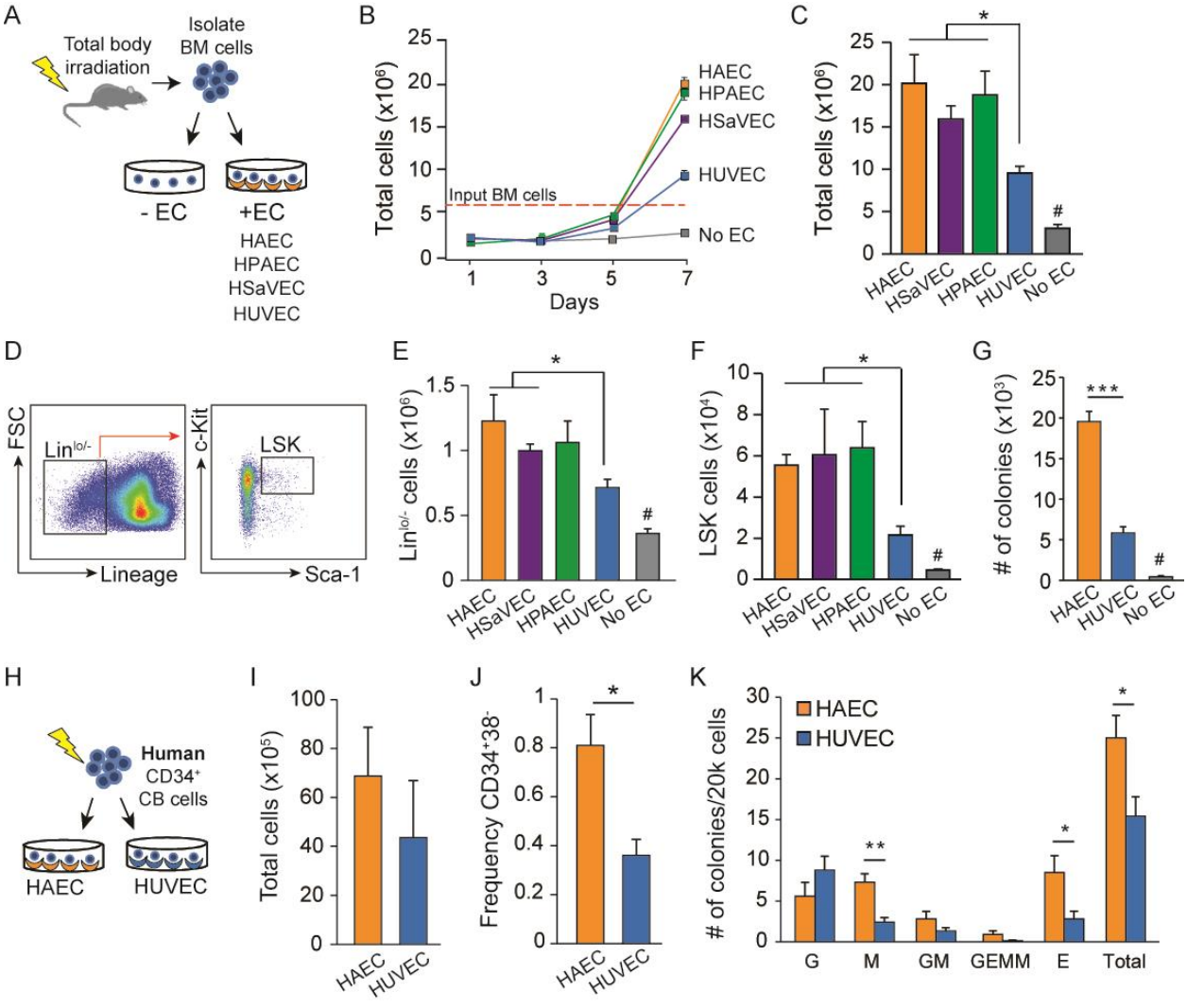

A
Total body irradiation
Isolate BM cells
- EC
+EC
HAEC
HPAEC
HSaVEC
HUVEC
B
Total cells (x10^6)
Input BM cells
HAEC
HPAEC
HSaVEC
HUVEC
No EC
Days
C
Total cells (x10^6)
HAEC
HSaVEC
HPAEC
HUVEC
No EC
D
FSC
Lin lo/-
c-Kit
LSK
Lineage
Sca-1
E
Lin lo/- cells (x10^6)
F
LSK cells (x10^4)
G
# of colonies (x10^3)
H
Human CD34+ CB cells
HAEC
HUVEC
I
Total cells (x10^5)
J
Frequency CD34+38-
K
# of colonies/20k cells
HAEC
HUVEC
G
M
GM
GEMM
E
Total

Figure 4-1: Heterogeneity in endothelial cell support of hematopoietic regeneration. (A) Bone marrow (BM) cells from mice exposed to 6 Gy total body irradiation were cultured in hematopoietic cytokines ± direct contact with primary human EC from different vascular sources, including aorta (HAEC), saphenous vein (HSaVEC), pulmonary artery (HPAEC), and umbilical vein (HUVEC). Hematopoietic regeneration was measured over 7 days. (B) Kinetics of hematopoietic cell regeneration. (C) Total hematopoietic cell regeneration at day 7 was higher in HAEC, HSaVEC, and HPAEC co-cultures relative to HUVEC and No EC. (D) Flow cyotometry analysis of stem and progenitor cell markers on hematopoietic cells after 7 days of co-culture. Using these markers we noted differential regeneration of (E) lineage$^{lo/-}$ hematopoietic cells and (F) stem and progenitor (Lin$^{lo/-}$ c-Kit^{+} Sca-1^{+}; LSK) cells. (G) Hematopoietic progenitor colony forming capacity in methylcellulose. Data are mean ± S.E.M. from 4 replicate experiments. Panels H-K: Heterogeneity in EC support of human hematopoietic regeneration. (H) Purified CD34^{+} cord blood (CB) cells were irradiated ex vivo with 4 Gy and co-cultured with EC for 10 days. HAEC and HUVEC supported the regeneration of similar numbers of total cells (I), but HAEC regenerated more CD34^{+}38^{-} CB cells (J) and functional progenitor cells (K) as assessed by colony-forming assays. Data are mean ± S.E.M. from n=5 biologic replicates. *$p<0.05$, **$p<0.01$, ***$p<0.001$ for comparison indicated, $^{\#}p<0.05$ versus all EC.

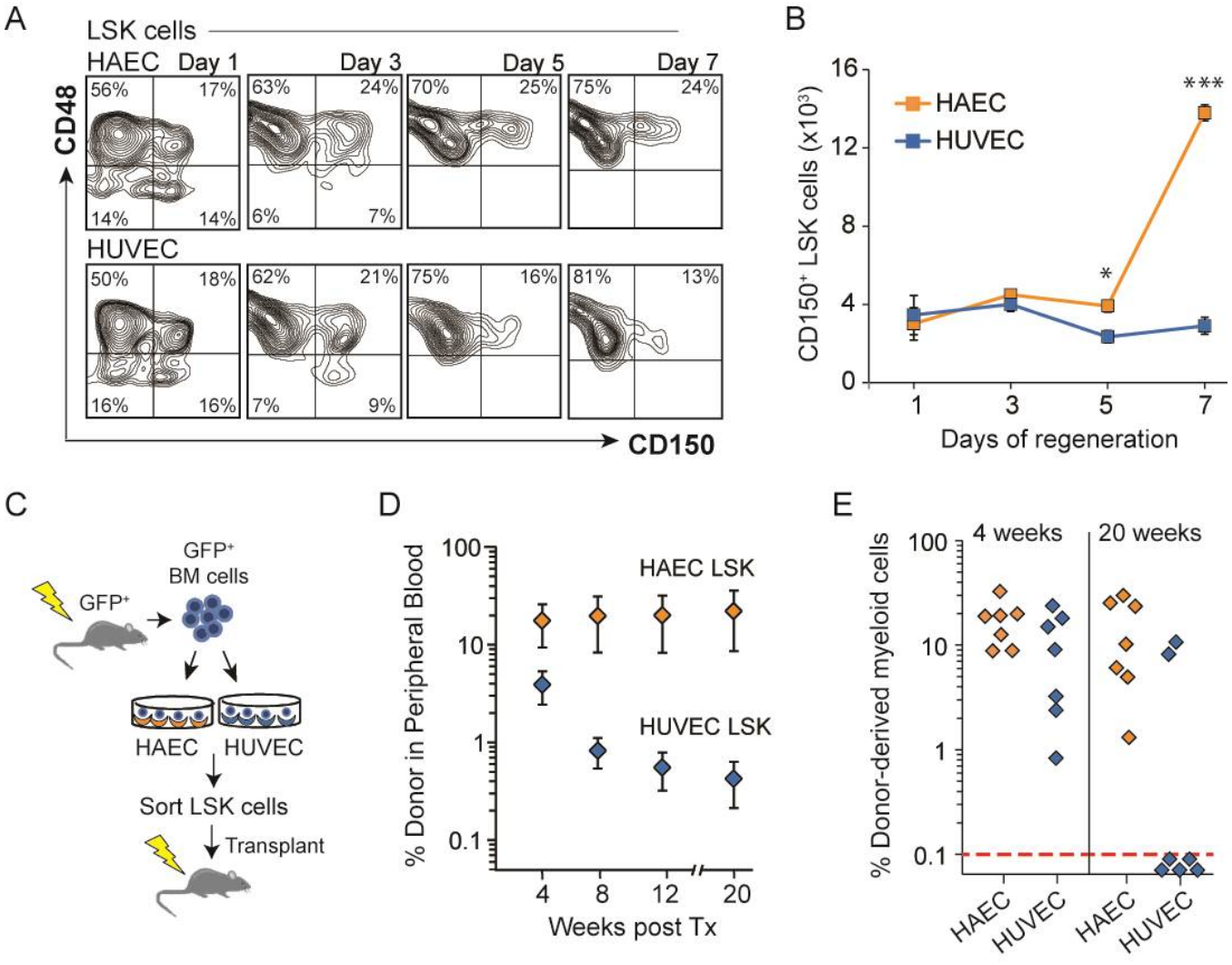

A
LSK cells
HAEC
Day 1
Day 3
Day 5
Day 7
CD48
56%
17%
14%
14%
63%
24%
6%
7%
70%
25%
75%
24%
HUVEC
50%
18%
16%
16%
62%
21%
7%
9%
75%
16%
81%
13%
CD150
B
HAEC
HUVEC
***
*
CD150⁺ LSK cells (x10³)
16
12
8
4
0
1
3
5
7
Days of regeneration
C
GFP⁺
BM cells
GFP⁺
HAEC
HUVEC
Sort LSK cells
Transplant
D
HAEC LSK
HUVEC LSK
% Donor in Peripheral Blood
100
10
1
0.1
4
8
12
20
Weeks post Tx
E
4 weeks
20 weeks
% Donor-derived myeloid cells
100
10
1
0.1
HAEC
HUVEC
HAEC
HUVEC

Figure 4-2: HUVEC fail to regenerate long-term functional HSC. (A) Frequency of SLAM markers CD150 and CD48 on LSK cells co-cultured with HAEC or HUVEC for 7 days. Whereas CD48 was expressed on virtually all LSK cells in both conditions, HAEC co-cultured LSK cells expressed higher levels of CD150. (B) Kinetics of $CD150^{+}$LSK cell regeneration by HAEC and HUVEC indicating that HUVEC fail to support HSC expansion in co-culture. (C) Transplantation setup to compare long-term hematopoietic function of LSK cells regenerated by HAEC versus HUVEC. (D) Peripheral blood production by transplanted GFP^{+}LSK cells in n=7 recipients/group throughout the first 20 weeks of engraftment. HUVEC fail to support long-term functional HSC regeneration. (E) HUVEC co-cultured HSPC lose multi-lineage hematopoietic potential following long-term engraftment. Diamonds represent individual mice. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 for HAEC vs HUVEC groups.

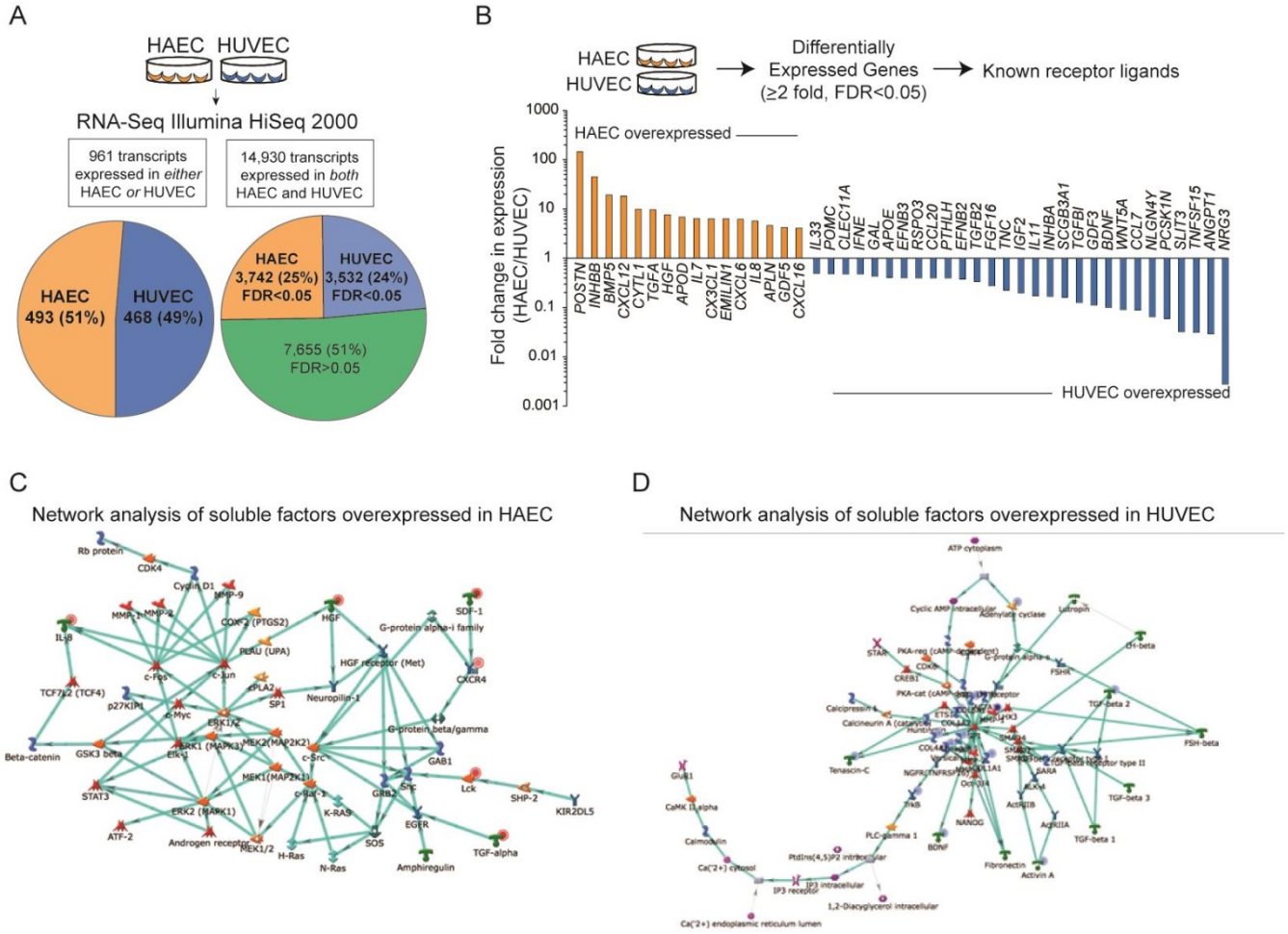

A
HAEC HUVEC
RNA-Seq Illumina HiSeq 2000
961 transcripts expressed in either HAEC or HUVEC
14,930 transcripts expressed in both HAEC and HUVEC
HAEC 493 (51%)
HUVEC 468 (49%)
HAEC 3,742 (25%) FDR<0.05
HUVEC 3,532 (24%) FDR<0.05
7,655 (51%) FDR>0.05
B
HAEC
HUVEC
Differentially Expressed Genes (≥2 fold, FDR<0.05)
Known receptor ligands
HAEC overexpressed
HUVEC overexpressed
Fold change in expression (HAEC/HUVEC)
1000
100
10
1
0.1
0.01
0.001
POSTN INHBB BMP5 CXCL12 CYTL1 TGFA HGF APOD IL7 CX3CL1 EMILIN1 CXCL6 IL8 APLN GDF5 CXCL16
IL33 POMC CLEC11A IFNE GAL APOE EFNB3 RSPO3 CCL20 PTHLH EFNB2 TGFB2 FGF16 TNC IGF2 IL11 INHBA SCGB3A1 TGFBI GDF3 BDNF WNT5A CCL7 NLGN4Y PCSK1N SLIT3 TNFSF15 ANGPT1 NRG3
C
Network analysis of soluble factors overexpressed in HAEC
Rb protein
CDK4
Cyclin D1
MMP-9
MMP-1
MMP-2
COX-2 (PTGS2)
HGF
SDF-1
G-protein alpha-i family
IL8
PLAU (UPA)
HGF receptor (Met)
CXCR4
c-Fos
c-Jun
cPLA2
SP1
Neuropilin-1
TCF7L2 (TCF4)
p27KIP1
c-Myc
ERK1/2
G-protein beta/gamma
Beta-catenin
GSK3 beta
ERK1 (MAPK3)
Elk-1
MEK2(MAP2K2)
c-Src
GAB1
MEK1(MAP2K1)
Src
Lck
SHP-2
KIR2DL5
STAT3
c-Raf-1
GRB2
ERK2 (MAPK1)
K-RAS
ATF-2
Androgen receptor
MEK1/2
H-Ras
SOS
EGFR
TGF-alpha
N-Ras
Amphiregulin
D
Network analysis of soluble factors overexpressed in HUVEC
ATP cytoplasm
Cyclic AMP intracellular
Adenylate cyclase
Leptropin
STAR
PKA-reg (cAMP-dependent)
G-protein alpha-s
CH-beta
FSHR
CREB1
PKA-cat (cAMP-dependent)
Calcipressin-1
Calcineurin A (catalytic)
TGF-beta 2
FSH-beta
Tenascin-C
Oct3/4
TGF-beta 3
GluR1
CaMK II alpha
FKB
ActRIIB
PLC-gamma 1
NANOG
ActRIIA
TGF-beta 1
Calmodulin
BDNF
Fibronectin
Activin A
Ca(2+) cytosol
Ptdlns(4,5)P2 intracellular
IP3 intracellular
IP3 receptor
1,2-Diacylglycerol intracellular
Ca(2+) endoplasmic reticulum lumen

Figure 4-3: Dynamic transcriptional changes occur in both HAEC and HUVEC during hematopoietic regeneration. (A) Overview of experiment to determine the endothelial cell transcriptome that mediates LT-HSC regeneration and investigate changes induced by injured BM cells in EC. (B) Box plots of all transcripts in HAEC and HUVEC in the absence or presence of BM cells. (C) GSEA was used to gene sets that were altered in HAEC and HUVEC in response to injured BM cells. Chemokine and G-protein coupled receptor related gene sets were highly induced in both HAEC and HUVEC by injured BM cells. (D) Representative leading edge chemokines induced in EC. (E) Soluble factors from HAEC and HUVEC conditioned media (CM) provide a survival benefit relative to cytokines alone, but are not sufficient for full hematopoietic regeneration. (G) Hematopoietic regeneration by HAEC depends on proximity between EC and IR-BM. *$p<0.05$ for comparisons indicated.

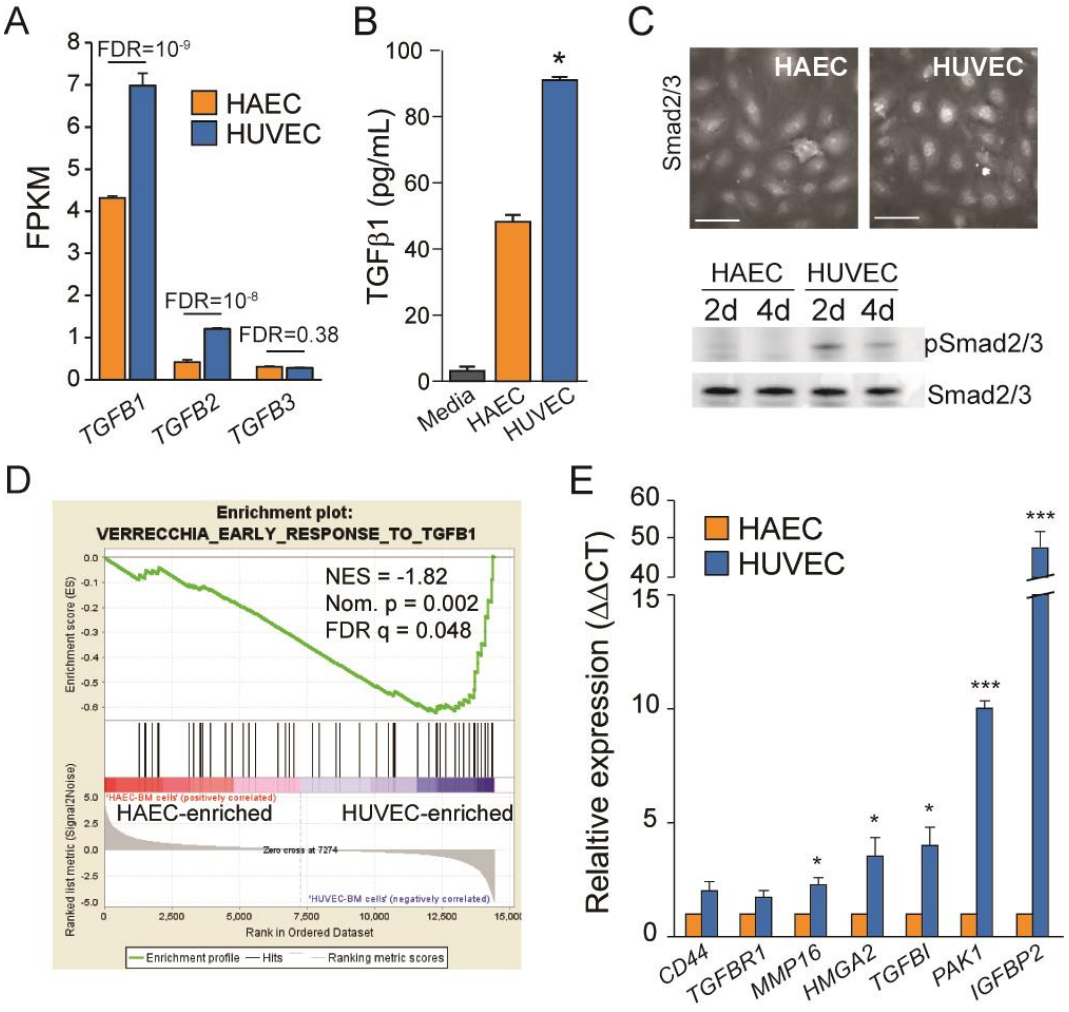

A
FPKM
FDR=10-9
FDR=10-8
FDR=0.38
HAEC
HUVEC
TGFB1
TGFB2
TGFB3
B
TGFβ1 (pg/mL)
*
Media
HAEC
HUVEC
C
Smad2/3
HAEC
HUVEC
HAEC HUVEC
2d 4d 2d 4d
pSmad2/3
Smad2/3
D
Enrichment plot:
VERRECCHIA_EARLY_RESPONSE_TO_TGFB1
NES = -1.82
Nom. p = 0.002
FDR q = 0.048
Enrichment score (ES)
HAEC-enriched
HUVEC-enriched
Zero cross at 7274
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
E
Relative expression (ΔΔCT)
HAEC
HUVEC
***
***
*
*
*
CD44
TGFBR1
MMP16
HMGA2
TGFBI
PAK1
IGFBP2

Figure 4-4: A TGF-β1 driven transcriptional program is hyperactive in HUVEC. (A) *TGFB1* and *TGFB2* transcripts are significantly overexpressed in HUVEC relative to HAEC. (n=4 replicates/group). (B) HUVEC secreted nearly 2-fold more active TGF-β1 into conditioned media after 4 days of culture. (C) HUVEC display greater nuclear Smad2/3 localization and a greater proportion of Smad2/3 is phosphorylated at Ser 423/5 relative to HAEC. (D) GSEA identification of TGF-β1 responsive gene programs highly enriched in HUVEC. (E) HUVEC overexpress a panel of TGF-β1 responsive genes by RNA-Seq and validated with qRT-PCR (not shown). *p<0.05, ***p<0.001 for comparison indicated.

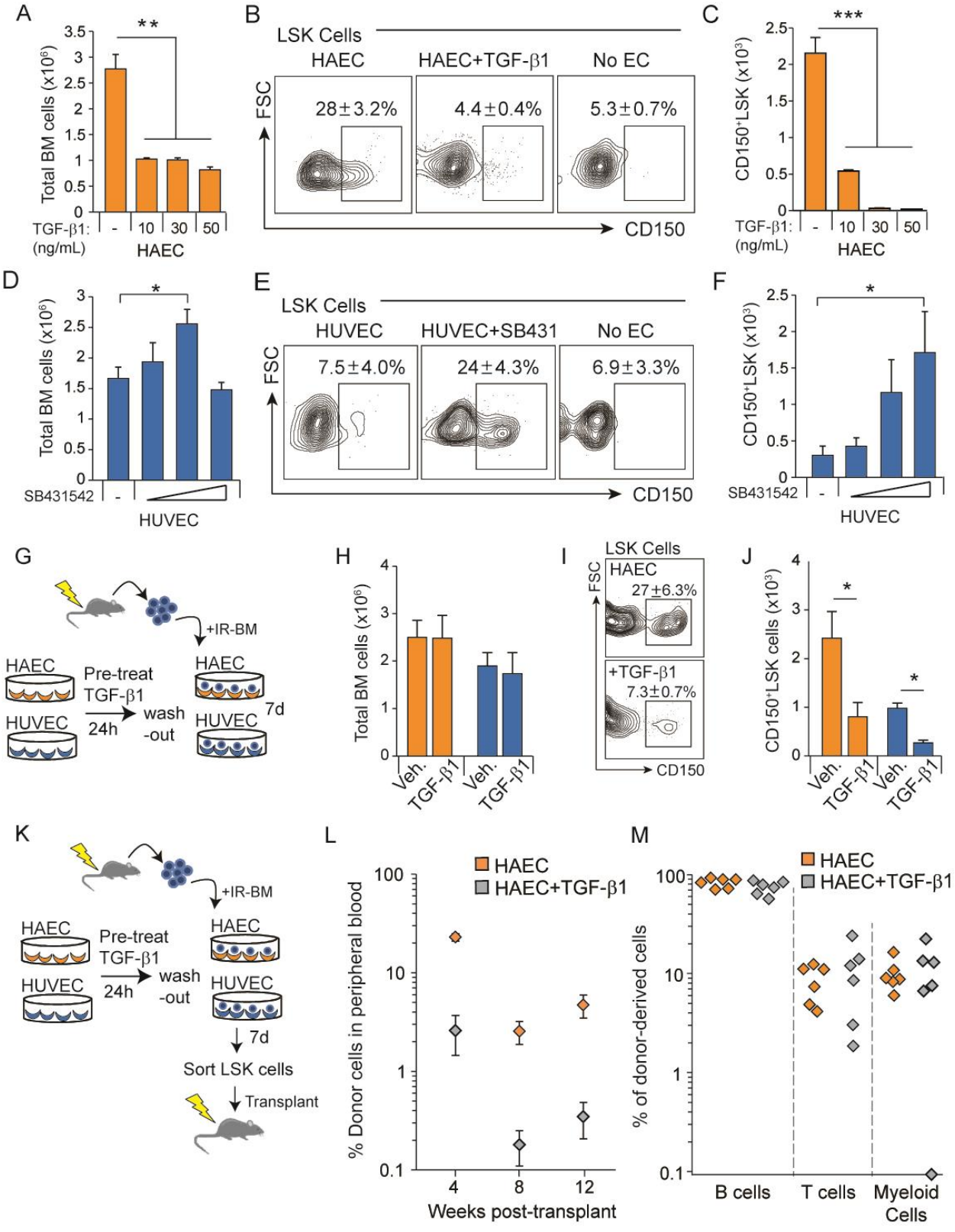

A
Total BM cells (x10^6)
TGF-β1: (ng/mL)
HAEC
B
LSK Cells
HAEC
HAEC+TGF-β1
No EC
28±3.2%
4.4±0.4%
5.3±0.7%
FSC
CD150
C
CD150+LSK (x10^3)
D
SB431542
HUVEC
E
HUVEC
HUVEC+SB431
7.5±4.0%
24±4.3%
6.9±3.3%
F
G
+IR-BM
Pre-treat TGF-β1
24h
wash -out
7d
H
Veh.
TGF-β1
I
27±6.3%
+TGF-β1
7.3±0.7%
J
K
Sort LSK cells
Transplant
L
% Donor cells in peripheral blood
Weeks post-transplant
M
% of donor-derived cells
B cells
T cells
Myeloid Cells

Figure 4-5: TGF-β1 suppresses endothelial-dependent HSC regeneration. Increasing doses of TGF-β1 in HAEC co-cultures resulted in (A) significantly reduced hematopoietic cell regeneration, (B) a significant reduction in the number of LSK cells expressing CD150, and (C) completely abolished phenotypic HSC regeneration. (D-F) Opposite effects were observed using the ALK5 inhibitor SB431542. TGF-β receptor signaling inhibition (D) increased total hematopoietic regeneration, (E) strongly enhanced the proportion of regeneration LSK cells that express CD150, and (F) increased HSC regeneration in a dose-dependent manner. (G) Schematic depicting pre-treatment model for testing EC-autonomous effects of TGF-β1. Pre-treating EC with TGF-β1 did not change total hematopoietic cell regeneration (H) but resulted in a significant decrease in $CD150^{+}$LSK cell regeneration (I) through reduced CD150 expression on LSK cells (J). This led to a significant decline in engraftment and repopulation potential of LSK cells sorted from HAEC regeneration co-cultures (K-L) without affecting multi-lineage hematopoietic potential (M) for up to 12 weeks following transplantation (n=6-7 mice/group, diamonds are individual mice). Results are mean ± SEM from 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ for comparison indicated.

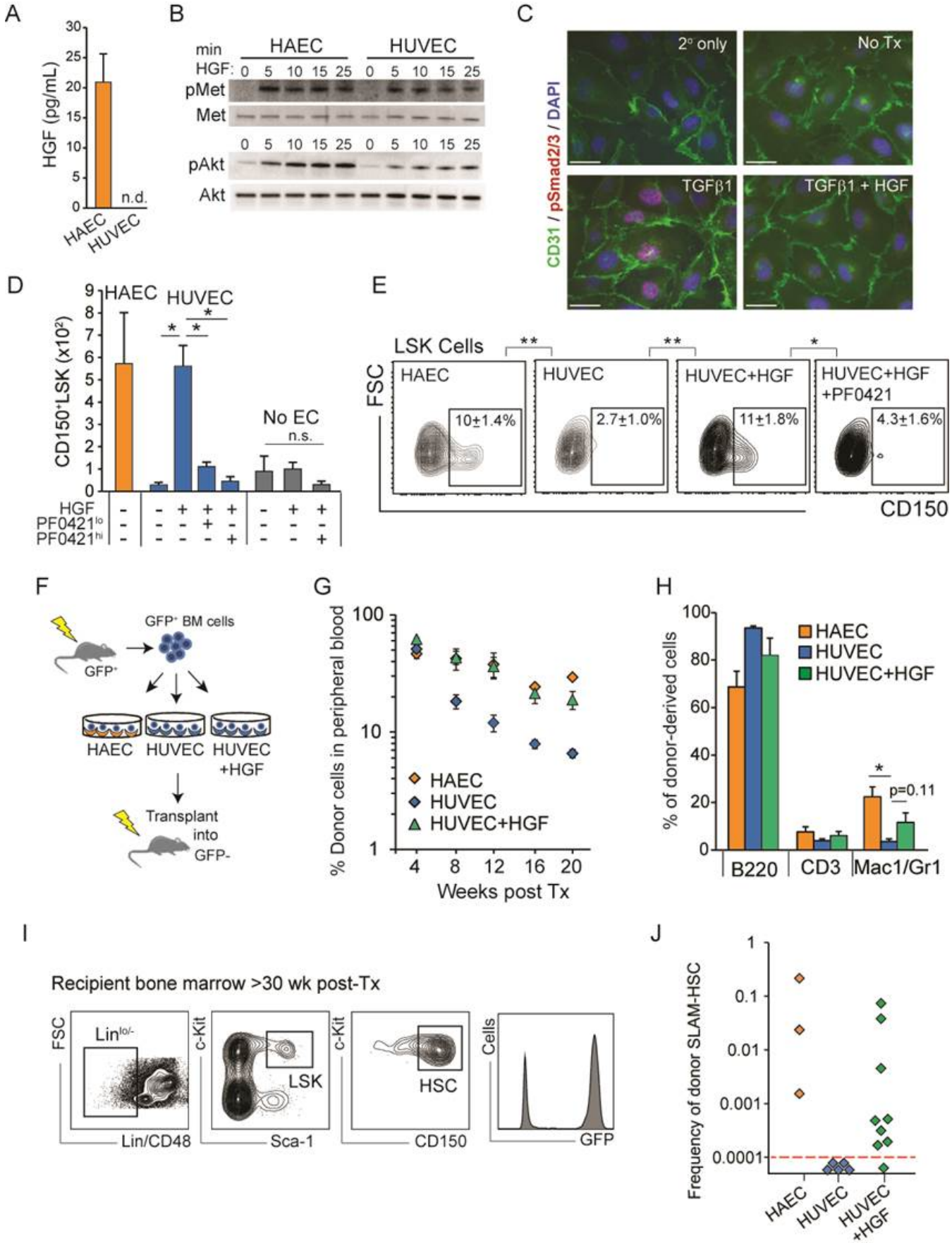

A
HGF (pg/mL)
n.d.
HAEC
HUVEC
B
min
HAEC
HUVEC
HGF: 0 5 10 15 25 0 5 10 15 25
pMet
Met
pAkt
Akt
C
2° only
No Tx
TGFβ1
TGFβ1 + HGF
CD31 / pSmad2/3 / DAPI
D
CD150+LSK (x10²)
HAEC
HUVEC
No EC
n.s.
HGF
PF0421lo
PF0421hi
E
LSK Cells
FSC
HAEC
HUVEC
HUVEC+HGF
HUVEC+HGF +PF0421
10±1.4%
2.7±1.0%
11±1.8%
4.3±1.6%
CD150
F
GFP+ BM cells
GFP+
HAEC
HUVEC
HUVEC +HGF
Transplant into GFP-
G
% Donor cells in peripheral blood
Weeks post Tx
HAEC
HUVEC
HUVEC+HGF
H
% of donor-derived cells
HAEC
HUVEC
HUVEC+HGF
p=0.11
B220
CD3
Mac1/Gr1
I
Recipient bone marrow >30 wk post-Tx
FSC
Lin lo/-
Lin/CD48
c-Kit
LSK
Sca-1
HSC
CD150
Cells
GFP
J
Frequency of donor SLAM-HSC
HAEC
HUVEC
HUVEC +HGF

Figure 4-6: Hepatocyte growth factor enhances endothelial-dependent LT-HSC regeneration. (A) HGF protein and transcript (not shown) are overexpressed in HAEC compared to HUVEC. (B) HGF rapidly activates c-Met and Akt HAEC and HUVEC. (C) HGF partially inhibits TGF-β1 induced Smad2/3 phosphorylation in HUVEC. (D) Treatment of HUVEC regeneration co-cutlures with HGF enhanced HSC regenreation in a c-Met dependent manner. Notably HGF did not alter HSC regeneration in the absence of EC. (E) HGF enhances hematopoietic regeneration by increasing CD150 expression on LSK cells (n=3 independent experiments). F-J: HGF enhances engraftment and long-term tri-lineage hematopoieiss by HUVEC-regenerated HSPC. (F) Transplantation schema to determine long-term in vivo function of HSC regenerated by HAEC, HUVEC, or HUVEC+HGF. Treatment with HGF during regeneration enhanced hematopoiesis (G) and restored multi-lineage hematopoietic potential (H) of HUVEC-regenerated HSPC. (I) Long-term HSC bone marrow engraftment was determined by flow cytometry for donor-derived CD150+CD48-LSK cells >30 wk after transplant. (I) Frequency of donor-derived HSC engrafted in recipient bone marrow after different regenerative conditions. Data are mean ± SEM. *$p<0.05$ for comparison indicated.

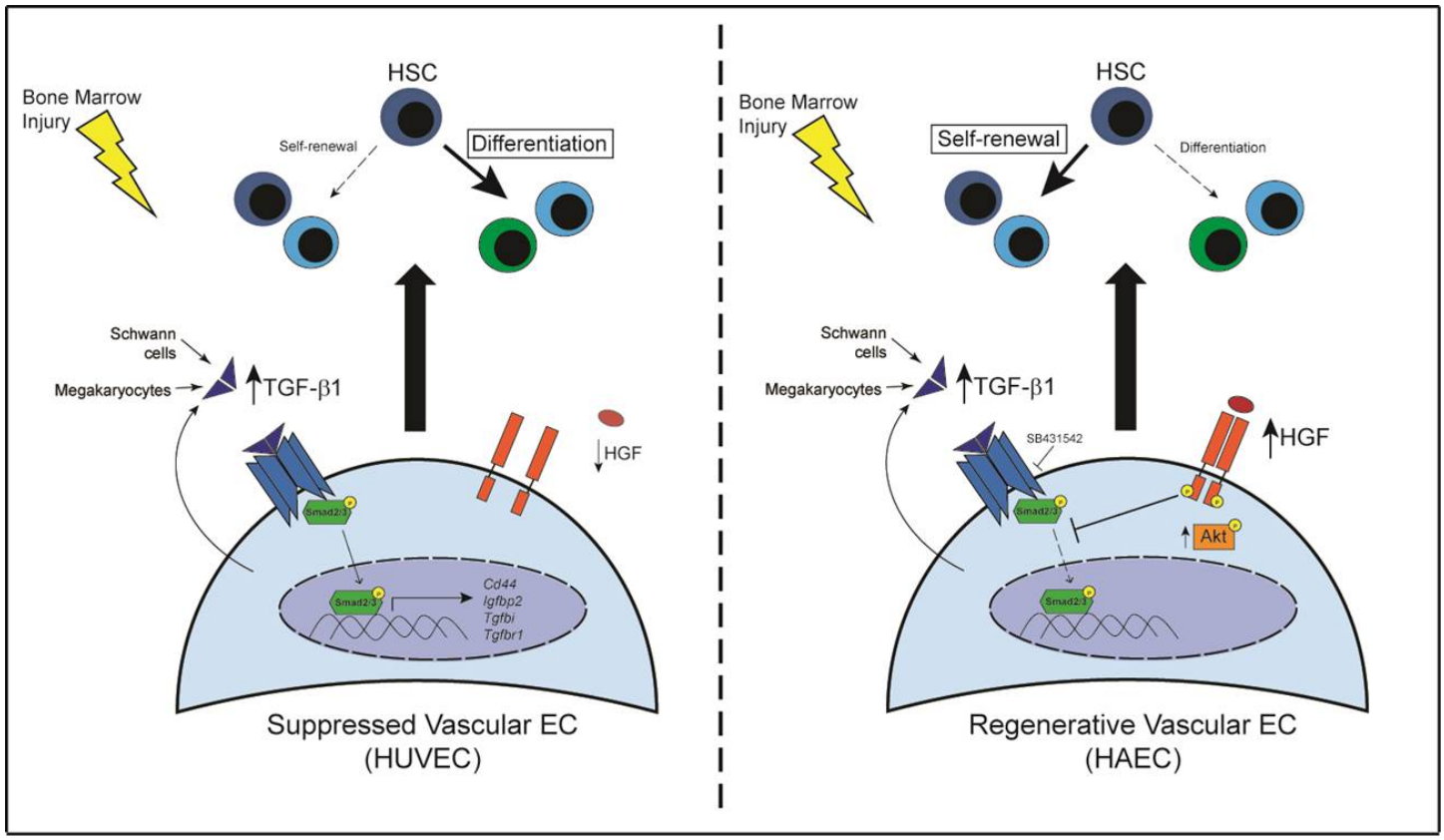

Bone Marrow Injury
HSC
Self-renewal
Differentiation
Schwann cells
Megakaryocytes
TGF-β1
HGF
Smad2/3
Cd44
Igfbp2
Tgfbi
Tgfbr1
Suppressed Vascular EC
(HUVEC)
Bone Marrow Injury
HSC
Self-renewal
Differentiation
Schwann cells
Megakaryocytes
TGF-β1
SB431542
HGF
Akt
Smad2/3
Regenerative Vascular EC
(HAEC)

Figure 4-7: Proposed model for the effects of TGF-β1 and HGF on HSC regeneration by vascular EC. Diagrams depicting the effects of TGF-β1 and HGF on EC-dependent regeneration as supported by these studies. *Left panel showing a suppressed vascular EC niche*: After bone marrow injury, TGF-β1 levels are elevated with several potential cellular sources including EC, megakaryocytes, and non-myelinating Schwann cells. TGF-β1 activates Smad2/3 phosphorylation and TGF-β1 responsive gene expression in EC. TGF-β1 signaling in EC prevents their support of HSC self-renewal and instead promotes $CD150^{+}$LSK cell differentiation. *Right panel depicting a regenerative vascular EC niche*: Elevations of TGF-β1 and TGF-β1 signaling in EC are offset by the production or treatment with pro-regenerative growth factors such as HGF. HGF activates c-Met and AKT signaling while also preventing Smad2/3 activation and nuclear translocation by TGF-β1. These effects enhance $CD150^{+}$LSK cell self-renewal and regeneration by a regenerative vascular EC niche.

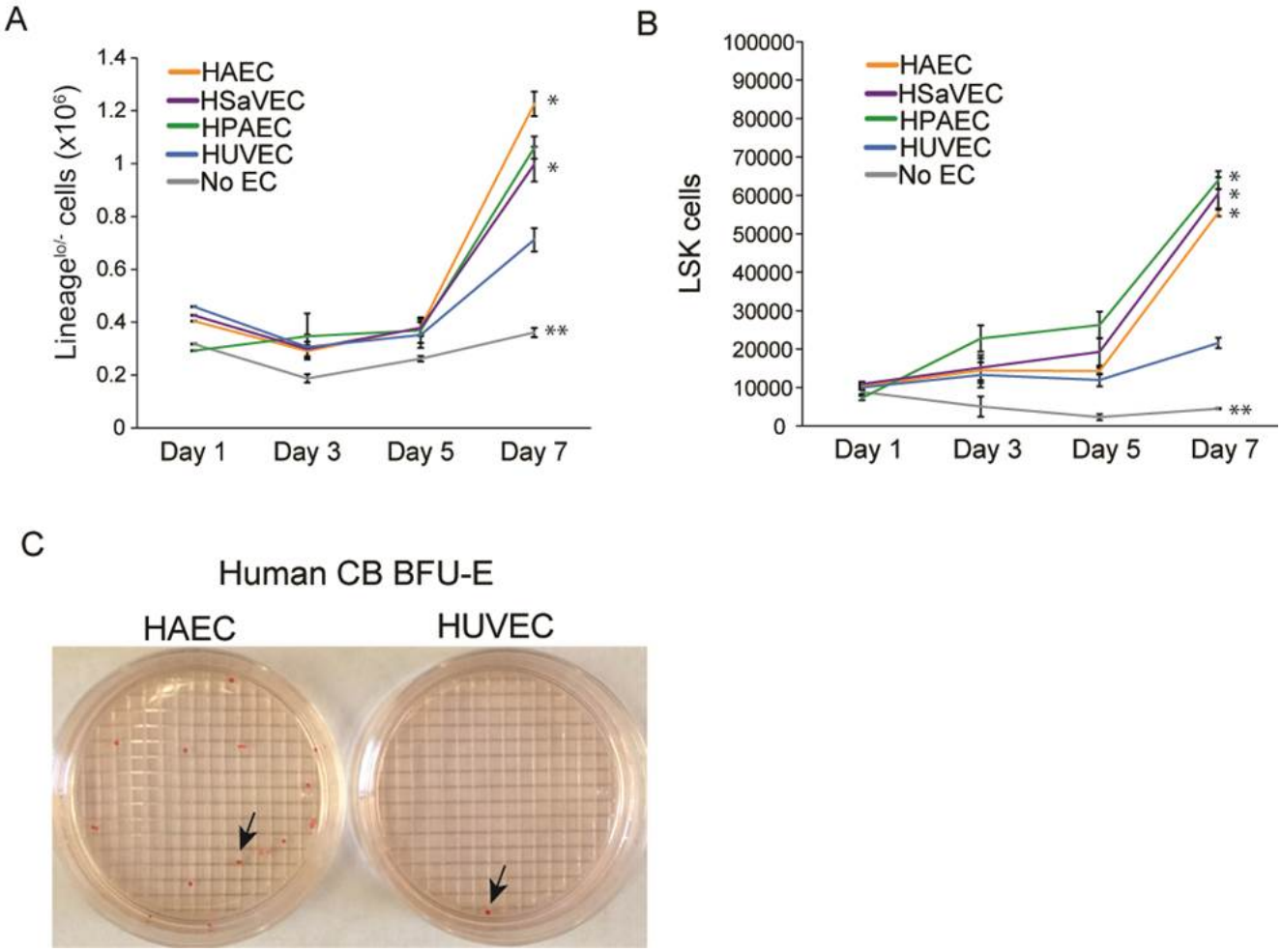
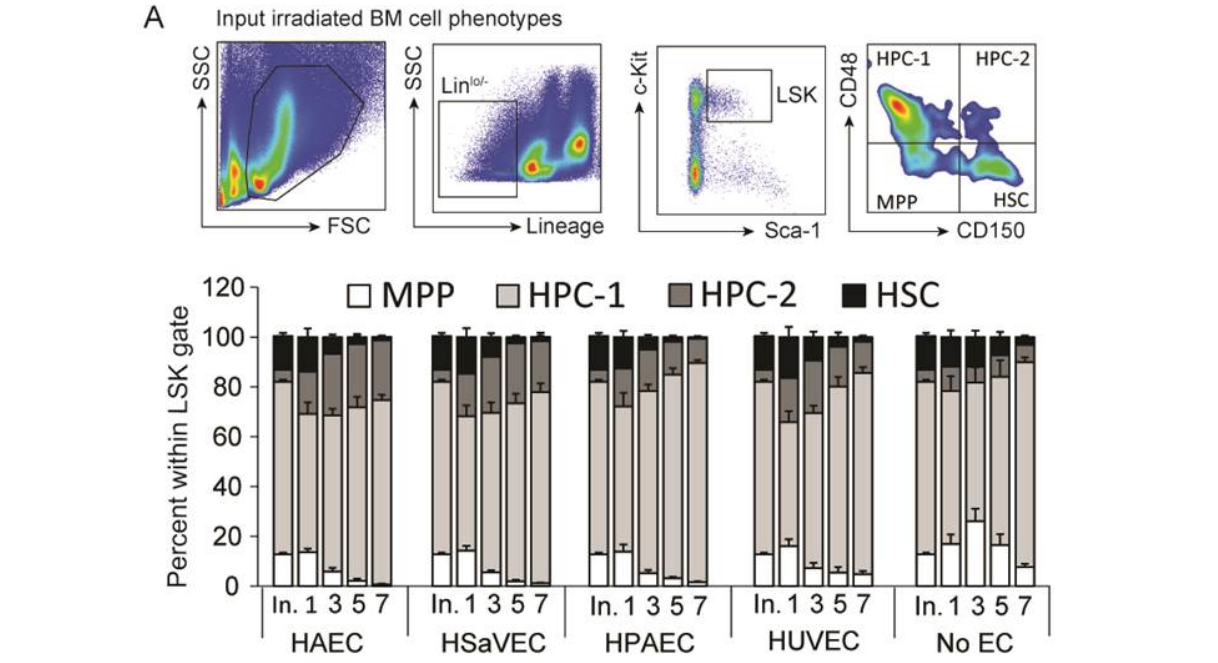

A
Lineage lo/- cells (x10^6)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HAEC
HSaVEC
HPAEC
HUVEC
No EC
*
*
**
Day 1
Day 3
Day 5
Day 7
B
LSK cells
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
HAEC
HSaVEC
HPAEC
HUVEC
No EC
*
*
*
**
Day 1
Day 3
Day 5
Day 7
C
Human CB BFU-E
HAEC
HUVEC

Figure 4-8, related to Figure 4-1. (A) Kinetics of Lineage$^{lo/-}$ hematopoietic cell regeneration with EC from aorta (HAEC), saphenous vein (HSaVEC), pulmonary artery (HPAEC), umbilical vein (HUVEC), or in the absence of EC (No EC). (B) Kinetics of LSK cell regeneration under same conditions as (A). Data are mean ± SEM from 4 independent experiments. (C) Representative results from colony formation assays with human cord blood (CB) progenitor cells showing increased erythroid blast forming units (BFU-E, arrows) after HAEC regeneration.


A
Input irradiated BM cell phenotypes
SSC
FSC
SSC
Lin^lo/-
Lineage
c-Kit
LSK
Sca-1
CD48
HPC-1
HPC-2
MPP
HSC
CD150
MPP
HPC-1
HPC-2
HSC
Percent within LSK gate
120
100
80
60
40
20
0
In. 1 3 5 7
HAEC
HSaVEC
HPAEC
HUVEC
No EC

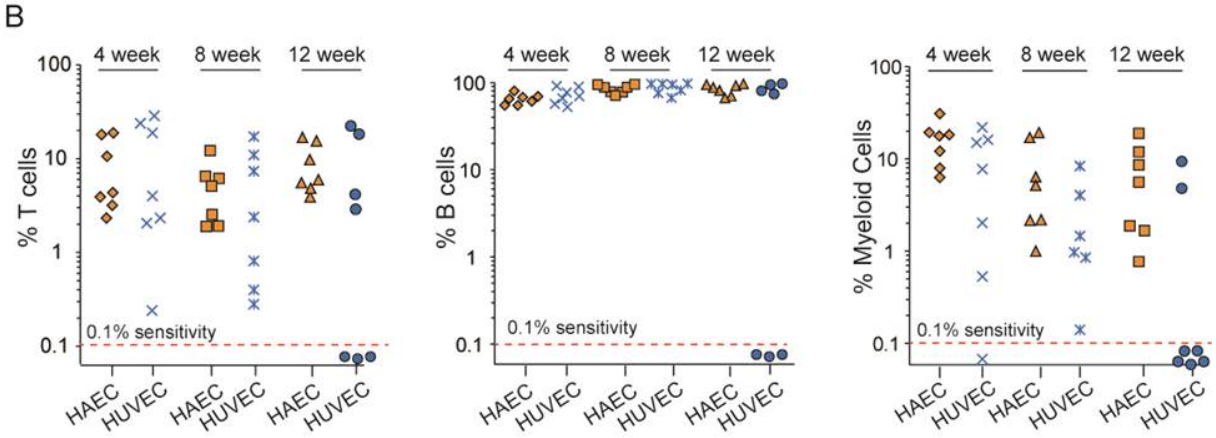

B
4 week
8 week
12 week
% T cells
% B cells
% Myeloid Cells
100
10
1
0.1
0.1% sensitivity
HAEC
HUVEC

Figure 4-9; related to Figure 4-2. (A) Frequencies of MPP, HPC-1, HPC-2, and SLAM-HSC at the onset of co-culture (Input, In.) and throughout regeneration (days 1,3,5, and 7). Results show mean ± SEM from 4 independent experiments. (B) Peripheral blood multi-lineage hematopoietic repopulation by HAEC or HUVEC–regenerated LSK cells. Data for individual recipient mice are shown (n=7/group). HUVEC-regenerated LSK cells were more variable in T cell and myeloid cell production. By 12 weeks of engraftment, 3/7 mice transplanted with HUVEC LSK cells had undetectable donor derived peripheral blood leukocytes, and an additional 2/7 mice lost myeloid cell repopulating capacity by week 12.

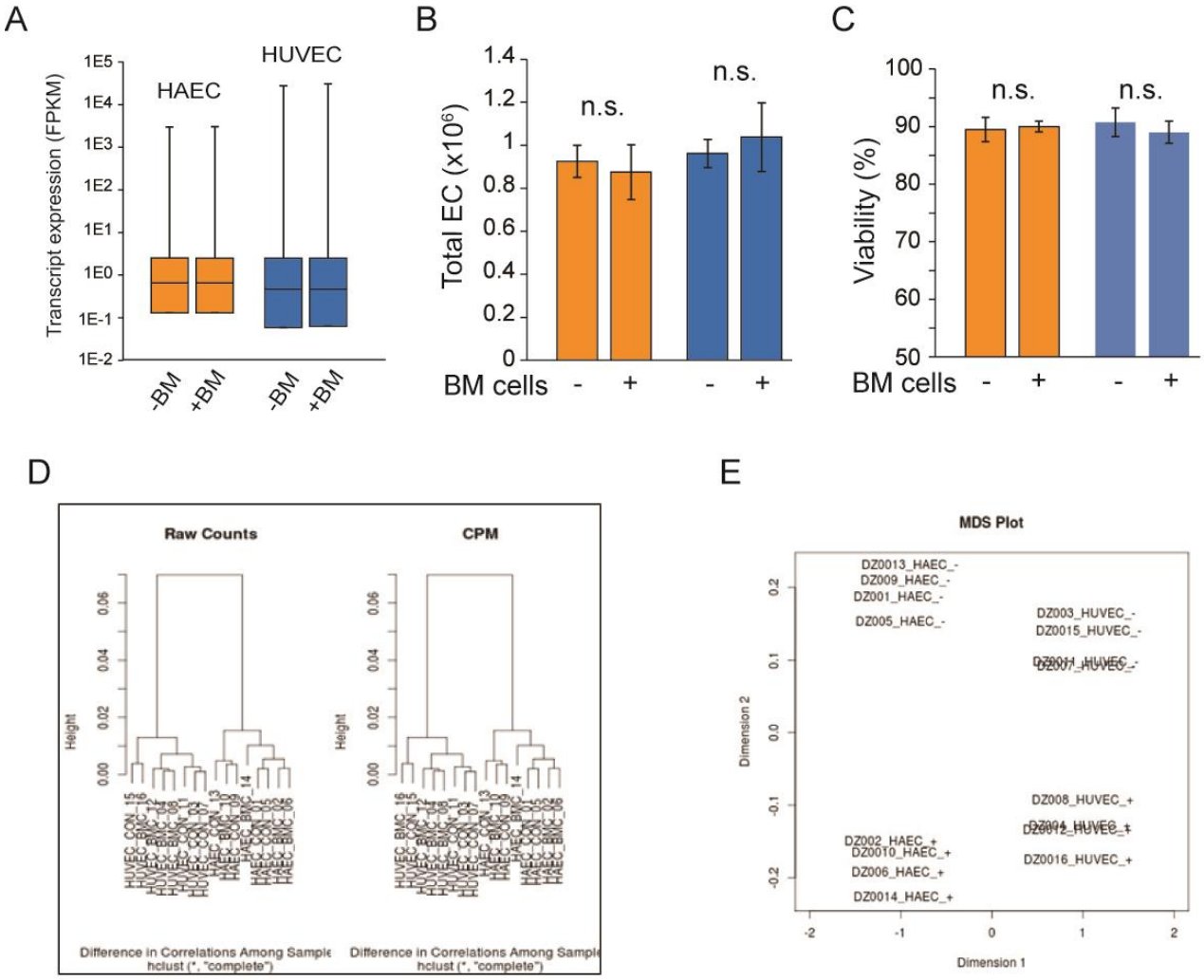

A
HAEC
HUVEC
Transcript expression (FPKM)
1E5
1E4
1E3
1E2
1E1
1E0
1E-1
1E-2
-BM
+BM
-BM
+BM
B
Total EC (x10^6)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n.s.
n.s.
BM cells - + - +
C
Viability (%)
100
90
80
70
60
50
n.s.
n.s.
BM cells - + - +
D
Raw Counts
CPM
Height
0.06
0.04
0.02
0.00
Difference in Correlations Among Sample
hclust (*, "complete")
Difference in Correlations Among Sample
hclust (*, "complete")
E
MDS Plot
DZ0013_HAEC_-
DZ009_HAEC_-
DZ001_HAEC_-
DZ005_HAEC_-
DZ003_HUVEC_-
DZ0015_HUVEC_-
DZ008_HUVEC_+
DZ002_HAEC_+
DZ0010_HAEC_+
DZ006_HAEC_+
DZ0014_HAEC_+
DZ0016_HUVEC_+
Dimension 2
0.2
0.1
0.0
-0.1
-0.2
-2
-1
0
1
2
Dimension 1

Figure 4-10; related to Figure 4-3. (A) Box and whisker plot of gene expresion in HAEC and HUVEC in the absence or presence of irradiated BM cells. There was no significant change in median gene expresion between conditions. There was no difference in (B) total EC numbers or (C) EC viability between the 4 different groups submitted for RNA Sequencing. (D) Hierarchical clustering and (E) multidimensional scaling (MDS) plot showing tight clustering between RNA Sequencing biological replicates.

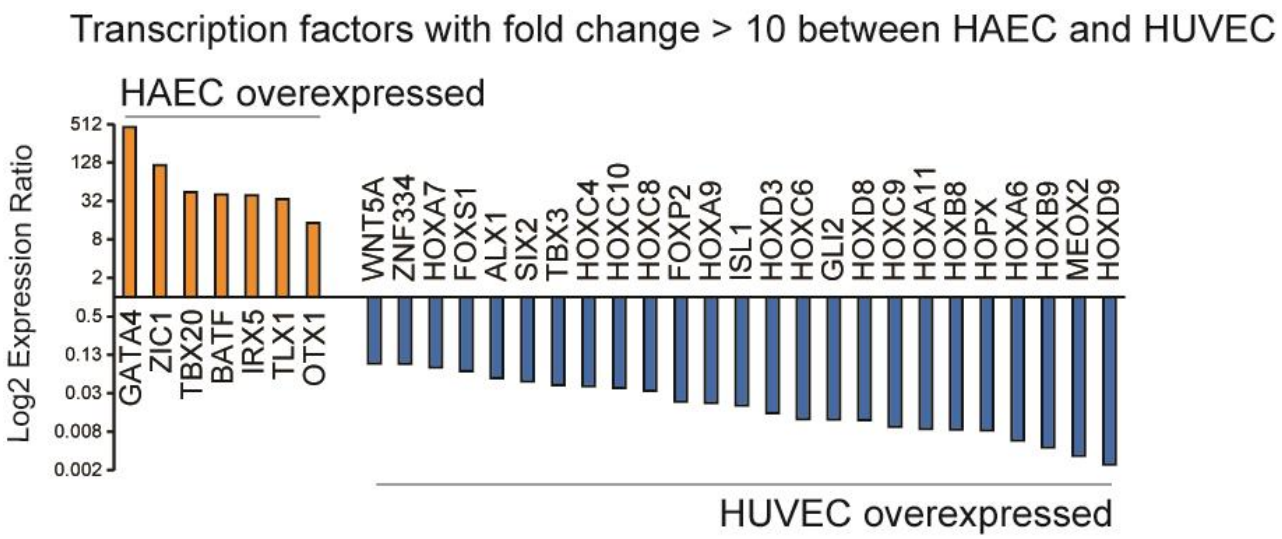

Transcription factors with fold change > 10 between HAEC and HUVEC
HAEC overexpressed
Log2 Expression Ratio
512
128
32
8
2
0.5
0.13
0.03
0.008
0.002
GATA4
ZIC1
TBX20
BATF
IRX5
TLX1
OTX1
WNT5A
ZNF334
HOXA7
FOXS1
ALX1
SIX2
TBX3
HOXC4
HOXC10
HOXC8
FOXP2
HOXA9
ISL1
HOXD3
HOXC6
GLI2
HOXD8
HOXC9
HOXA11
HOXB8
HOPX
HOXA6
HOXB9
MEOX2
HOXD9
HUVEC overexpressed

Figure 4-11; related to Figure 4-4. (A) Transcription factor profiling in HAEC and HUVEC showing the most highly differentially expressed transcription factors at a fold change >10. HUVEC heavily enriched for homeobox transcription factors expression.

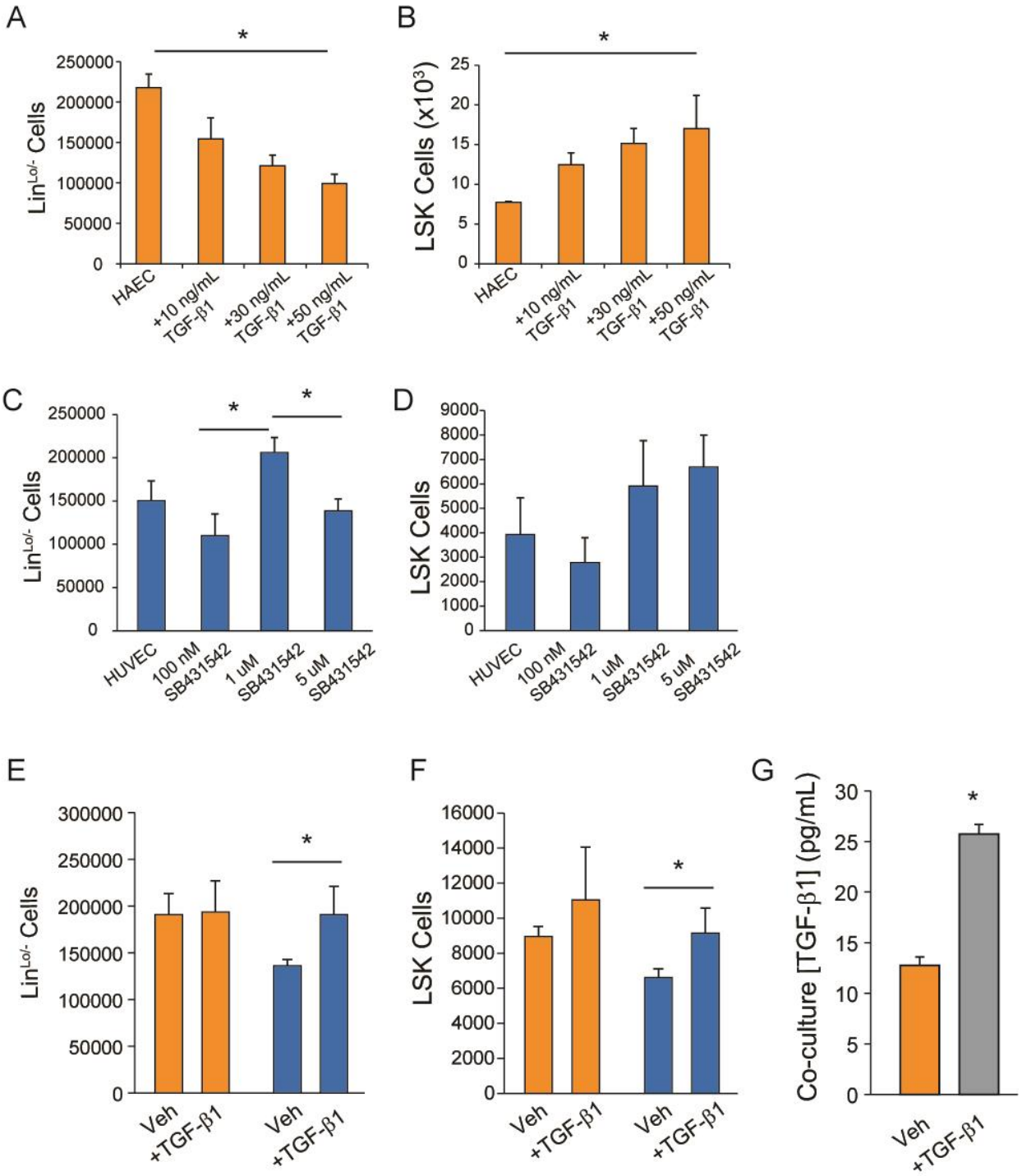

A
B
C
D
E
F
G
Lin^Lo/- Cells
LSK Cells (x10^3)
LSK Cells
Co-culture [TGF-β1] (pg/mL)
HAEC
+10 ng/mL TGF-β1
+30 ng/mL TGF-β1
+50 ng/mL TGF-β1
HUVEC
100 nM SB431542
1 uM SB431542
5 uM SB431542
Veh
+TGF-β1
*

Figure 4-12; related to Figure 4-5. (A) TGF-β1 decreased Lineagelo/- hematopoietic cell regeneration in HAEC co-cultures; however led to an increase of LSK cells (B) due to enrichment in c-Kit expression (data not shown). (C) Inhibition of TGFβ receptor signaling with SB431542 had dose-dependent effects on $Lin^{lo/-}$ cell regeneration and (D) LSK cell regeneration, potentially due to the dual effects of SB431542 on ALK1 and ALK5 signaling in EC. (E-F) $Lin^{lo/-}$ cell (E) and LSK cell (F) regeneration by HAEC and HUVEC in the EC TGF-β1 pre-treatment assay. TGF-β1 pretreatment led to small but significant increases in $Lin^{lo/-}$ regeneration and LSK cell regeneration by HUVEC. (G) Pre-treatment of HAEC with TGF-β1 led to significantly increased production of TGF-β1 in co-culture at day 5.

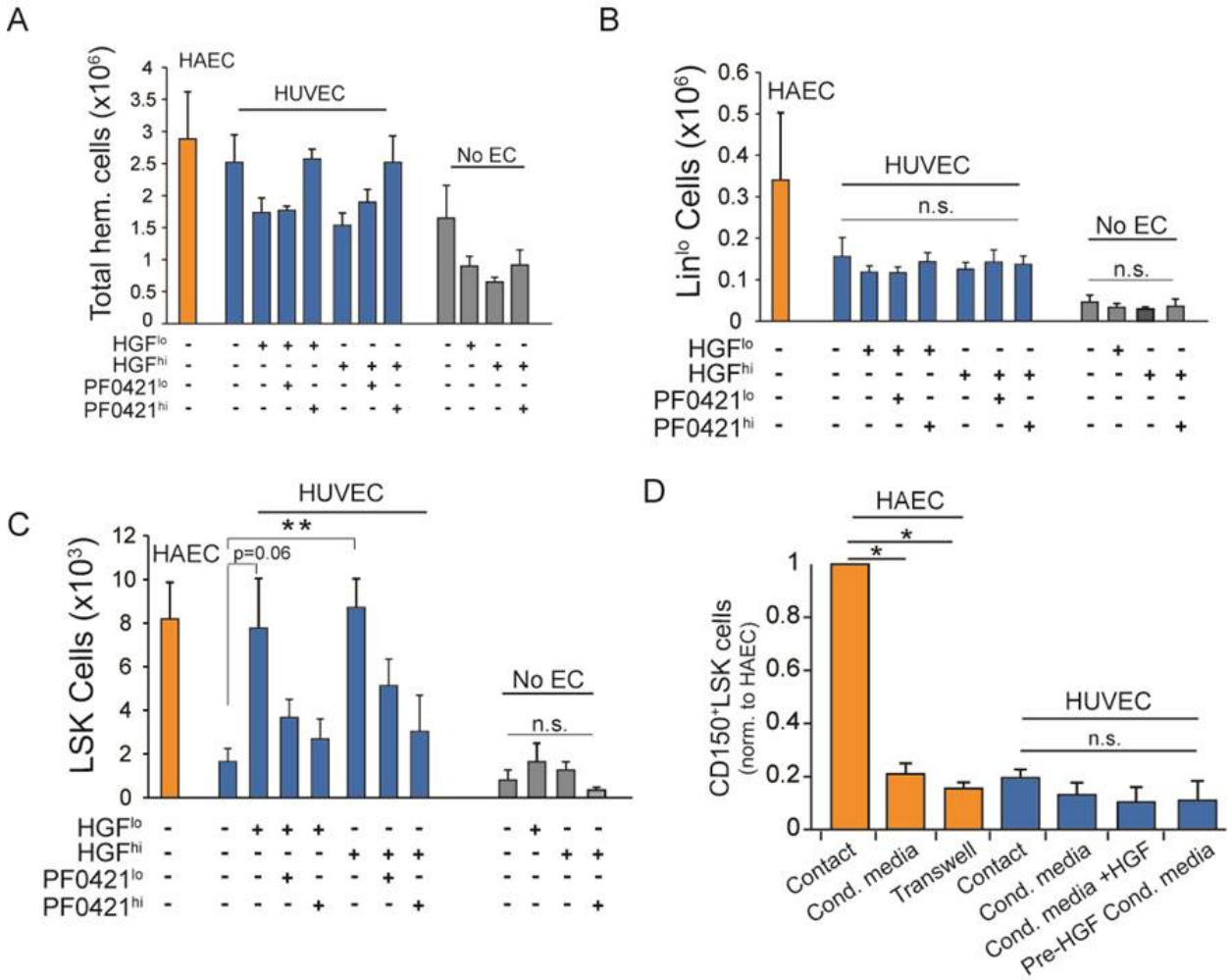

A
Total hem. cells (x10^6)
HAEC
HUVEC
No EC
HGF^lo
HGF^hi
PF0421^lo
PF0421^hi
B
Lin^lo Cells (x10^6)
n.s.
C
LSK Cells (x10^3)
**
p=0.06
D
CD150+LSK cells
(norm. to HAEC)
Contact
Cond. media
Transwell
Cond. media +HGF
Pre-HGF Cond. media

Figure 4-13, related to Figure 4-6. HGF had selective effects on stimulating HSPC regeneration. (A) total hematopoietic cells were largely unaffected and there was even a trend for decreasing total cellularity with the addition of HGF. (B) $Lin^{lo/-}$ cell regeneration was unaffected. (C) HGF treatment enhanced LSK cell regeneration by HUVEC. (D) Conditioned media studies indicate that HGF does not work in combination with soluble factors from HUVEC to enhance HSC regeneration. Data are mean ± SEM. * $p<0.05$ for comparison indicated.

Table 4-1: Gene sets overexpressed in HUVEC-BM versus HAEC-BM at FDRq < 0.05 by Gene Set Enrichment Analysis

NAME	SIZE	NES	FDR q-val
REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	53	-2.1314228	0.001021328
REACTOME_NCAM1_INTERACTIONS	21	-2.0809815	0.001550639
GU_PDEF_TARGETS_UP	65	-2.0672884	0.002072126
REACTOME_NCAM_SIGNALING_FOR_NEURITE_OUT_GROWTH	45	-1.9635826	0.01270328
HOELZEL_NF1_TARGETS_DN	74	-1.946526	0.015338915
GAUSSMANN_MLL_AF4_FUSION_TARGETS_B_UP	16	-1.9457092	0.012782428
SMID_BREAST_CANCER_RELAPSE_IN_BONE_UP	59	-1.9414734	0.011249037
MIKKELSEN_NPC_HCP_WITH_H3K27ME3	133	-1.9382583	0.010484016
PID_SYNDECAN_1_PATHWAY	36	-1.8982482	0.02357771
QI_HYPOXIA_TARGETS_OF_HIF1A_AND_FOXA2	34	-1.8897412	0.024429498
WIEDERSCHAIN_TARGETS_OF_BMI1_AND_PCGF2	52	-1.8830847	0.02521844
REACTOME_COLLAGEN_FORMATION	39	-1.8805418	0.02379958
NABA_COLLAGENS	26	-1.8782058	0.022842307
VERRECCHIA_RESPONSE_TO_TGFB1_C1	18	-1.8493396	0.03575853
YAMASHITA_METHYLATED_IN_PROSTATE_CANCER	33	-1.8432238	0.036887154
VERRECCHIA_EARLY_RESPONSE_TO_TGFB1	54	-1.8247418	0.04769902

Table 4-2: Gene sets enriched in HAEC+BM relative to HAEC-BM at FDR q < 0.05 by Gene Set Enrichment Analysis

NAME	SIZE	NES	FDR q-val
MIKKELSEN_MEF_HCP_WITH_H3K27ME3	203	-2.8949816	0
MIKKELSEN_MEF_ICP_WITH_H3K27ME3	69	-2.4742587	0.002530386
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	98	-2.3801057	0.003366857
MIKKELSEN_MEF_LCP_WITH_H3K27ME3	27	-2.3375478	0.003860466
MIKKELSEN_MEF_HCP_WITH_H3_UNMETHYLATED	98	-2.2821784	0.005417433
SENGUPTA_NASOPHARYNGEAL_CARCINOMA_DN	194	-2.2516778	0.006396599
REACTOME_GPCR_LIGAND_BINDING	145	-2.2433114	0.006023202
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	56	-2.2424703	0.005270301
KONG_E2F3_TARGETS	87	-2.2319574	0.005376341
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	93	-2.2038245	0.006435629
KEGG_RETINOL_METABOLISM	23	-2.1905475	0.006568219
FARMER_BREAST_CANCER_CLUSTER_2	30	-2.1739535	0.006516369
RICKMAN_HEAD_AND_NECK_CANCER_E	33	-2.1289093	0.009598078
NABA_SECRETED_FACTORS	150	-2.1023202	0.011710416
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	21	-2.0931966	0.011832712
KANG_DOXORUBICIN_RESISTANCE_UP	52	-2.0758982	0.01272622
REACTOME_POTASSIUM_CHANNELS	43	-2.0650175	0.013452877
REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE	33	-2.061627	0.013141053
MOLENAAR_TARGETS_OF_CCND1_AND_CDK4_DN	52	-2.019649	0.018121643
MIKKELSEN_ES_LCP_WITH_H3K4ME3	77	-1.9703134	0.027226772
RICKMAN_HEAD_AND_NECK_CANCER_C	48	-1.9263425	0.037672568

Table 4-3: Gene sets enriched in HUVEC+BM relative to HUVEC-BM at FDR q < 0.05 by Gene Set Enrichment Analysis

NAME	SIZE	NES	FDR q-val
MIKKELSEN_MEF_HCP_WITH_H3K27ME3	203	-2.7955582	0
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	21	-2.7709382	0
REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	98	-2.7131855	0
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	56	-2.6414888	8.03E-05
MIKKELSEN_MEF_ICP_WITH_H3K27ME3	69	-2.5214672	2.56E-04
REACTOME_GPCR_LIGAND_BINDING	145	-2.318188	0.003035582
MIKKELSEN_MEF_HCP_WITH_H3_UNMETHYLATED	98	-2.2930346	0.003742717
KONG_E2F3_TARGETS	87	-2.281643	0.003631766
NABA_SECRETED_FACTORS	150	-2.1864781	0.007901215
MIKKELSEN_MEF_LCP_WITH_H3K27ME3	27	-2.185623	0.007175563
KEGG_RETINOL_METABOLISM	23	-2.169977	0.007501005
WILENSKY_RESPONSE_TO_DARAPLADIB	16	-2.1608763	0.007562114
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	93	-2.1172256	0.01118026
MIKKELSEN_IPS_ICP_WITH_H3K27ME3	18	-2.1074882	0.011698635
GREENBAUM_E2A_TARGETS_UP	32	-2.0603082	0.016493987
MOLENAAR_TARGETS_OF_CCND1_AND_CDK4_DN	52	-2.0582554	0.015719363
MAHADEVAN_RESPONSE_TO_MP470_UP	19	-2.0409892	0.017389325
MIKKELSEN_ES_LCP_WITH_H3K4ME3	77	-2.029837	0.018351018
FARMER_BREAST_CANCER_CLUSTER_2	30	-2.0260918	0.018037423
SENGUPTA_NASOPHARYNGEAL_CARCINOMA_DN	194	-2.0170414	0.018656535
KANG_DOXORUBICIN_RESISTANCE_UP	52	-1.9747597	0.025487553
REACTOME_DEPOSITION_OF_NEW_CENPA_CONTAINING_NUCLEOSOMES_AT_THE_CENTROMERE	33	-1.9531001	0.029292729
NIKOLSKY_BREAST_CANCER_19Q13.1_AMPLICON	19	-1.9296727	0.03418717
RICKMAN_HEAD_AND_NECK_CANCER_E	33	-1.9267896	0.033476662
REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	82	-1.9198242	0.0339852

Chapter 5: "New Insights"
Discussion and Conclusions

Part 1: Introduction

Part 2: A platform to study endothelial-dependent hematopoietic regeneration
- Aortic endothelial cells regenerate and repair HSC in co-culture
- The clinical utility of EC-derived factors for HSC regeneration

Part 3: Vascular endothelial cell heterogeneity takes center stage
- HUVEC have inferior HSC regeneration activity

Part 4: TGF-β1: A negative regulator of endothelial-dependent HSC regeneration
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- Sources and signaling of TGF-β1 in the bone marrow microenvironment
- EC-autonomous effects of TGF-β1

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- Hepatocyte Growth Factor

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- HAEC and HUVEC as surrogates for bone marrow EC
  - *- Arterial versus venous vascular EC*
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- A paradigm shift is needed in designing therapies for hematopoietic injury
- A precautionary note on using EC-targeted therapy in hematologic malignancies

Part 1: Introduction

The goal of this work has been to better understand the mechanisms by which ECs promote HSC regeneration and to identify specific factors responsible for regulating this process. HSC rely heavily on vascular EC for homing, quiescence, self-renewal, and to promote hematopoietic regeneration after injury (Ding et al., 2012; Hooper et al., 2009; Sipkins et al., 2005). Importantly, vascular injury is also occurs in the settings of chemotherapy or exposure to ionizing radiation, which limits the regeneration of hematopoiesis (Hooper et al., 2009). It is predicted then, that regulators of the niche after will ultimately influence HSC regeneration and long-term function. The research presented here leveraged molecular and functional heterogeneity in vascular EC to identify factors that regulate endothelial-dependent hematopoietic regeneration. My studies have uncovered a novel role of TGF-β1 in suppressing HSC regeneration by blocking the function of vascular EC, and have identified several candidate EC-derived factors (including HGF) that may be used to enhance endothelial-dependent HSC regeneration. In this chapter, I discuss the implications of these findings with respect to hematopoietic regeneration in vivo. Specifically, I will cover the clinical utility of vascular EC-derived factors for hematopoietic regeneration, the cellular sources and effects of bone marrow TGF-β1 and HGF in vivo, and how this research informs the approach to therapy in hematopoietic regeneration and hematologic malignancies.

Part 2: A platform to study endothelial-dependent HSC regeneration

To better understand the mechanisms regulating hematopoietic regeneration, my initial studies were aimed at establishing a system that allowed precise investigation into the interactions between HSC and EC. To this end, a co-culture system was established and validated in vivo for functional HSC regeneration by vascular EC. The establishment of this assay was the focus of Chapter 3 of this dissertation and has been the basis for identifying and functionally testing EC derived factors that influence HSC regeneration.

Aortic endothelial cells regenerate and repair HSC in co-culture

Using the co-culture system we were able to establish several important findings. The regeneration of HSC in direct contact co-culture with HAEC resulted in a >20-fold expansion of HSC that were able provide long-term multi-lineage hematopoietic reconstitution in irradiated recipients. HAEC-regenerated HSC were self-renewing as evidenced by their serial repopulating capacity. Furthermore, these studies established for the first time that co-culture with vascular EC leads to a rapid resolution of DNA damage markers (γH2AX foci) and an overall reduction in DNA damage (Comet assay) in HSPC co-cultured with HAEC in comparison with hematopoietic cytokines only. To the best of my knowledge, this was the first data to show that the DNA damage response by HSC can regulated in a non-cell autonomous manner. Dormant HSC are known to accumulate DNA damage that is repaired on entry into the cell cycle (Beerman et al., 2014); however, using Ki67 and Hoechst33342 staining I have also shown that the proportion of HSPC in S/G_2/M phases of the cell cycle does not significantly increase in EC co-cultures relative to cytokines alone until 72 hours of regeneration, which is longer than these cells took to resolve DNA damage. Therefore, these data suggest that EC may stimulate DNA damage repair that is independent of the cell cycle status of HSC. Further

studies are needed to determine the type of DNA damage response injured HSCs utilize in the presence of EC. The relative utilization of NHEJ or HDR by regenerating HSC could be determined using HSC isolated from Ku70/80 or BRCA knockout mice, respectively. Furthermore, the potential link between HSC DNA damage repair to cell cycle status could be more precisely understood with tightly controlled temporal studies in co-culture.

The clinical utility of EC-derived factors for hematopoietic regeneration

As a part of these initial studies, the clinical utility of EC as tools for hematopoietic regeneration was also investigated. Remarkably, we found that there is at least a 48 hour window of opportunity following irradiation during which starting co-culture with EC can regenerate functional repopulating HSC. In addition to the potential role of EC or EC-derived factors in preventing cytopenias associated with cancer therapies, this finding strongly endorses their use as treatments for unintended ionizing radiation exposure, when it may take hours or days for an irradiated patient to receive care. I also directly compared the efficacy of HAEC-derived support to that of G-CSF on HSC regeneration. As discussed in Chapter 1, G-CSF is routinely used to prevent neutropenia in myelosuppressed patients. However, my studies showed that G-CSF treatment does not improve the regeneration of HSC directly, as treatment of regenerating BM cultures with G-CSF significantly depleted $CD150^{+}LSK$ cells relative to EC co-culture. These data suggest that optimal HSC recovery during *in vivo* treatment with G-CSF is dependent on support of HSC self-renewal by the niche. These findings are of particular importance, given the destructive effects of cytotoxic therapies on the bone marrow vasculature (Brenet et al., 2013; Hooper et al., 2009) and the microenvironment as a whole (Cao et al., 2011). Indeed, it was recently published that treatment with G-CSF in the context of ionizing radiation injury can exacerbate long-term HSC dysfunction (Li et al., 2015). Together with the

data from Chapter 3, these findings highlight the importance of improving current therapies for hematopoietic injury by restoring the function of the injured microenvironment and niches for HSC.

Part 3: Vascular endothelial cell heterogeneity takes center stage

The studies described above emphasize the essential role of vascular EC in HSC regeneration, and support the notion that identifying factors to regenerate HSC is a clinically important goal. With this in mind, my next set of studies was focused the identification of factors that regulate endothelial-dependent HSC regeneration. A difficulty in identifying these factors successfully is that vascular EC are functionally and molecularly heterogeneous. Whereas they were once thought to act only as passive conduits for nutrient delivery, vascular EC are now known to perform essential stem cell niche roles in tissues throughout the body (reviewed in (Rafii et al., 2016)]. Their regulation of a diverse set of processes across multiple organs requires that EC be functionally and molecularly dynamic cells (Aird, 2012). Gene expression profiling studies have shown that there is substantial transcriptional heterogeneity in EC isolated from different vascular beds throughout the body (Chi et al., 2003). Moreover, recent studies are beginning to uncover several types of vascular EC in the bone marrow, which are differentially regulated during hematopoietic regeneration and have distinct effects on HSC function (Itkin et al., 2016; Kusumbe et al., 2014). We hypothesized that a better understanding of this EC heterogeneity could lead to the identification of secretory products that regulate EC-dependent HSC regeneration.

<u>HUVEC have inferior HSC regeneration activity</u>

To identify the contribution of EC heterogeneity to HSC regeneration following radiation injury, I screened vascular EC derived from several sources using the co-culture assay. Whereas the majority of EC tested strongly promote HSC regeneration in co-culture, HUVEC had greatly reduced capacity to support hematopoietic regeneration. HUVEC were markedly deficient in regenerating phenotypic HSC ($CD150^{+}$LSK cells) relative to highly regenerative HAEC. These findings were supported functionally in transplantation studies, that showed HUVEC-regenerated HSC are largely unable to provide long-term hematopoiesis after

transplantation, with a specific deficit in myelopoiesis. To better understand some of the cellular mechanisms responsible, I performed conditioned media and transwell studies. The results indicated that although soluble factors from HAEC and HUVEC can provide HSPC with a similar survival advantage in co-culture, the full regenerative and self-renewal potential of HSC required direct cell contact with HAEC. The HSC-EC cell contact dependence is in agreement with previous findings (Kobayashi et al., 2010). In other studies however, soluble factors from brain-derived EC have been shown to promote HSC regeneration as efficiently as contact with EC (Himburg et al., 2010). These disparate findings are likely due to differences in EC secretomes based on their function within a specific tissue of origin.

Part 4: TGF-β1: A negative regulator of endothelial-dependent HSC regeneration

I hypothesized that there were two possible mechanisms to explain to why HUVEC were poor supporters of HSC regeneration relative to HAEC. Either HUVEC lacked the expression of factors present in HAEC and other EC types that support HSC regeneration and/ or HUVEC expressed repressors of HSC regeneration. To distinguish between these possibilities and to identify functionally important mediators of HSC regeneration, the transcriptomes of HAEC and HUVEC were evaluated. Our analysis of differential gene expression identified several members of the transforming growth factor (TGF)-β superfamily of receptor ligands that were differentially expressed. Specifically, the canonical TGF-β ligands *Tgfb1* and *Tgfb2* were overexpressed at low FDR in HUVEC relative to HAEC.

TGF-β family members have many roles in tissue homeostasis during development and disease [reviewed in (Massague, 2012; Massague and Xi, 2012)]. In the hematopoietic system, TGF-β ligands play important roles as ventralizing factors during mesodermal specification to hematopoietic precursors (Pardanaud and Dieterlen-Lievre, 1999), as well as in directly maintaining HSC quiescence in the adult [reviewed by (Blank and Karlsson, 2015)]. HSC express type I and type II TGF-β receptors and are directly responsive to TGF-β ligands (Blank and Karlsson, 2015). Interestingly, the TGF-β1 and β2 isoforms have differential effects on hematopoiesis, with TGF-β1 displaying 100 fold higher potency in the suppression of bone marrow progenitor cell activity (Ohta et al., 1987). TGF-β1 is necessary and sufficient to directly inhibit HSC cycling *in vitro* (Batard et al., 2000; Sitnicka et al., 1996; Soma et al., 1996). This inhibition is known to occur through several mechanisms including the induction of cell cycle inhibitor gene expression, downregulation of cytokine receptor expression, and inhibition of lipid raft clustering, which is important for cytokine receptor signal transduction (Cheng et al., 2001) (Cheng Shen 2001; Dubois Rescetti 1994; Scandura Boccuni 2004; Yamazaki Iwama 2009).

Although these effects are well documented in vitro, the specific effects of TGF-β1 on HSC *in vivo* have been difficult to pinpoint. In addition to lethality of knockout models, the complexity and redundancy of TGF-β1 signaling through TGFβ receptors by both Smads and non-Smad signaling pathways (Mu et al., 2012) has made genetic manipulation to study its precise *in vivo* function challenging (Larsson et al., 2003).

TGF-β1 inhibits HSC regeneration by vascular EC

To determine how TGF-β1 influences EC-dependent HSC regeneration, I investigated the effect of activating or inhibiting TGF-β signaling in our established co-culture system. Addition of TGF-β1 was sufficient to inhibit total hematopoietic cell regeneration and the regeneration of phenotypic HSC in bone marrow cells isolated from whole body irradiated mice and co-cultured with HAEC. Furthermore, TGF-β1 markedly reduced the proportion of HSPCs expressing the LT-HSC SLAM family marker, CD150, and enriched $Lin^{lo/-}$ cells that expressed high surface levels of c-Kit. These findings suggest that TGF−β1 induces the differentiation of CD150+ HSCs, yet simultaneously creates a bottleneck in the progenitor compartment that prevents downstream hematopoietic cell expansion. In HUVEC co-cultures, inhibition of TGFBR1 signaling with SB431542 increased total hematopoietic cell and HSC regeneration, with enrichment in HSPC expressing CD150. Overall, these studies show that TGF-β1 is a master regulator of endothelial-dependent HSC regeneration.

The effects of TGF-β1 on EC-dependent HSC regeneration had not been previously studied; however, the above results were supported by recent findings of other groups. Zhang et al., who showed that treatment of irradiated bone marrow mononuclear cells with SB431542 improved viability and increased CFU-GM activity and engraftment 8 weeks post-transplant (Zhang, Wang 2013). Blockade of TGF−β signaling has also been studied in the context of hematopoietic regeneration after chemotherapy. Brenet et al. found that neutralization of TGF-

β1 with a blocking antibody (1D11) sustains hematopoietic regeneration after 5-fluorouracil treatment by preventing $p57^{Kip2}$-induced HSC reentry into a quiescent state (Brenet, Scandura 2013). Recently a study by the D'Andrea lab demonstrated that both Smad3 knockdown and 1D11 treatment rescue HSPC from mitomycin-C induced apoptosis in mouse and human models of Fanconi Anemia (Zhang, Kozono 2016). Interestingly, inhibiting TGF-β in Fanconi cells resulted in a higher utilization of homologous recombination in comparison to NHEJ for DNA crosslink repair. Together, these studies support my findings that TGF-β1 is a dominant regulator of hematopoietic regeneration. Furthermore, they highlight that suppressing TGF-β1 production by EC and other cellular sources can maximize hematopoietic regeneration.

Sources of TGF−β in the bone marrow microenvironment

Several different hematopoietic and non-hematopoietic cells express TGF−β isoforms (Kunisaki et al., 2013; Kusumbe et al., 2014; Nolan et al., 2013; Zhao et al., 2014). Megakaryocytes are the major source of TGF−β1 and at steady state contribute to a quiescent niche for HSC (Zhao et al., 2014). Notably, levels of TGF−β1 are known to increase in the bone marrow after cytotoxic therapy (Brenet et al., 2013). All three canonical TGF−β ligands are expressed by bone marrow EC and may contribute to elevated TGF−β levels after injury. Specifically, Nolan et al. showed that *Tgfb2* expression is elevated in bone marrow microvascular EC 10 and 28 days following ionizing radiation exposure (Nolan et al., 2013). Furthermore, recent work from Ralf Adams' group has described a new type of sinusoidal EC, type H, which is responsible for repopulating the bone marrow sinusoidal network after injury and express high levels of *Tgfb1* and *Tgfb3* (Kusumbe et al., 2014). Together, these results show that vascular EC are a potential source of elevated TGF−β1 after bone marrow injury.

Another potential mechanism by which TGF−β1 may be elevated in the bone marrow after injury is through increased activation of its latent form. TGF−β1 is normally secreted into

the extracellular matrix in a non-covalently bound complex with its pro-domain and a latency associated peptide (Shi et al., 2011). Here, it is sequestered by ligand traps until active TGF−β1 is dissociated by proteolytic cleavage, reactive oxygen species, or integrin adhesion within the ECM [reviewed by (Horiguchi et al., 2012)]. The bone marrow microenvironment undergoes extensive remodeling for weeks after injury (Cao et al., 2011), which may contribute to excess release of TGF−β1 from the ECM and prolong its effects. A key cellular source of TGF−β1 activation has recently been identified as the non-myelinating Schwann cell (Yamazaki et al., 2009). Interestingly, these Schwann cells ensheath tyrosine hydroxylase expressing adrenergic neurons, which innervate the arteriolar vascular niche for HSC. It is conceivable that in the injury setting, excessive TGF−β1 release by matrix remodeling and non-myelinating Schwann cell activity may prevent dormant HSC from re-entering the cell cycle and repopulating the hematopoietic compartment.

EC-autonomous effects of TGF−β1

A major finding from my studies is that TGF−β has direct effects on EC that inhibit their ability to support HSC regeneration. We initially identified TGF−β1 as a candidate suppressor based on its overexpression HUVEC. The simplest interpretation of our findings was that EC-derived TGF-β1directly suppressed HSC; however, several other pieces of data indicated that TGF−β1 actually participates in an autocrine EC signaling loop. First, analysis of HAEC and HUVEC transcriptomes reveals that they express the expected components for differential TGF−β signaling. Whereas ALK5 and Smad3 are overexpressed by HUVEC, ALK1 and Smad1 are overexpressed by HAEC, suggesting that both HAEC and HUVEC are transcriptionally poised to signal through different arms of the TGF-β signaling pathway. Furthermore, both gene

set enrichment analysis and immunofluorescence studies indicated that TGF−β signaling is also hyperactive in HUVEC relative to HAEC.

The activation of TGF−β signaling and transcriptional programs in HUVEC led to the hypothesis that an EC-intrinsic effect of TGF−β might participate in suppressing HSC regeneration. To test this, I pre-treated HAEC with TGF−β1 the day prior to the addition of irradiated bone marrow cells only, and did not expose BM cells to exogenous TGF-β1. Interestingly, EC pre-treatment with TGF−β1 resulted in a 60% reduction in HSC regeneration by HAEC. This reduction in HSC content was attributable to a loss of CD150 expression in LSK cells, and was functionally significant. Specifically, LSK cells isolated from co-cultures established after HAEC pretreatment with TGF−β1 had 10-fold lower engraftment activity in vivo than LSK cells isolated from co-cultures established with untreated HAEC.

Presently, very little is known about the effects TGF−β may have on the regenerating hematopoietic microenvironment. My findings indicate that after injury, elevated TGF−β levels suppress the ability of bone marrow vascular EC to regenerate HSC. Our data suggest that blocking TGF−β signaling in EC should be considered as an important target for enhancing the regeneration and repair of HSC in vivo. TGFβ is essential factor for maintaining HSC quiescence and self-renewal potential under steady state as well as reestablishing HSC quiescence after injury, Thus, the timing of treatments targeting TGF−β is of notable concern. Clearly more work is needed to determine the related effects of TGF−β on vascular function, ECM remodeling, and reestablishment of hematopoietic homeostasis.

Part 5: Identification and functional significance of positive regulators of endothelial-dependent HSC regeneration

Hepatocyte Growth Factor

Transcriptome analysis of HUVEC and HAEC was also utilized to identify candidate factors that enhance HSC regeneration by HUVEC. Using this approach, hepatocyte growth factor (HGF) was determined to enhance EC-dependent HSC regeneration and long-term multi-lineage repopulating potential. In the absence of EC, HGF did not improve HSC regeneration. Furthermore, conditioned media experiments indicate that HGF in combination with soluble factors from EC were not sufficient to promote maximum HSC regeneration. Therefore, HGF may be enhancing stem cell regeneration by targeting EC. In support of this, HGF activated its receptor in HAEC and HUVEC. Moreover, HGF treatment resulted in rapid activation of Akt in ECs, which has been shown to be sufficient to drive HSC regeneration by EC in vitro and in vivo (Kobayashi et al., 2010). Together, these results suggest that HGF is also an autocrine mediator of endothelial-dependent HSC regeneration.

Hepatocyte growth factor (HGF) is a potent regenerative factor for many tissues, in part owing to its ability to stimulate motility, growth, and survival of regenerating progenitor cells at the site of injury (reviewed by Trusolino, 2010). To do this, HGF binds to and induces dimerization of its cognate receptor, c-Met, which has no other known ligands but interacts with many co-receptors including integrins, plexins, and the hyaluronan receptor, CD44. Through HGF binding and these associations, a wide variety of important downstream mediators including Erk, Jnk, PI3K/Akt, NF-κB, and STAT signaling pathways can be activated. In human hematopoietic cells, HGF increases the colony forming potential of total bone marrow mononuclear cells, but not $CD34^+$ cells, suggesting that it acts on more committed progenitors. In the same studies, c-Met was detected by flow cytometry on the surface of a subset of unfractionated bone marrow but was undetectable on $CD34^+$ cells (Takai, 1997). In agreement

with this, I found no effect of HGF on phenotypic HSC regeneration in the absence of EC. However, there was a small but significant increase in the percentage of LSK cells surviving in HGF treated no EC cultures, consistent with the notion that HGF has effects on the progenitor compartment.

There is compelling evidence to suggest that HGF can promote regeneration of the hematopoietic microenvironment. Levels of HGF protein are 6-fold higher in the bone marrow of radio-protected *Tie2-cre; Bax*$^{-/-}$*Bak*$^{fl/-}$ mice following exposure to ionizing radiation (Doan, 2013), and HGF protects vascular EC from ionizing radiation-induced apoptosis in culture (Hu, 2009). Interestingly, treatment with HGF and VEGF activates chemokine and G-protein coupled receptor gene expression in HUVEC (Gerritsen, 2003), which is in surprisingly close agreement with the effects of adding irradiated bone marrow cells to both HAEC and HUVEC in my experiments. In other studies, forced expression of HGF in smooth muscle cells increased VEGFR2 and CD31 expression on bone marrow derived endothelial progenitor cells in co-culture, and enhances their proliferation and migratory capacity in vitro (Zhu, 2010). As discussed above, ionizing radiation and chemotherapy cause the regression of bone marrow sinusoids, the regeneration from which has been shown to depend on VEGFR2 and Tie2 (angiopoietin receptor) reactivation, respectively (Hooper, 2009; Kopp, 2005). Taken in context, these data suggest that HGF may play a role in stimulating angiogenesis or neovascularization during bone marrow regeneration. I was not able to generate reproducible data indicating improved hematopoietic outcomes after HGF administration to irradiated mice (data not shown); however, I did not evaluate bone marrow angiogenesis in these experiments.

Another possible mechanism of action for HGF could be the activation of the vascular niche for HSC. In combination with VEGF (which is included in endothelial growth medium in my experiments), HGF treatment increases gene expression of SCF, IL-11, and intracellular adhesion molecule (ICAM)-1 in HUVEC (Gerritsen, 2003). These effects on EC could be

important for the engraftment and reconstitution of circulating HSC as they home from peripheral sites to the bone marrow niche.

Part 6: Heal the soil: Targeting the niche to enhance hematopoietic regeneration

HAEC and HUVEC as surrogates for bone marrow EC

To date, bone marrow-derived EC have been difficult to identify, isolate, and culture *ex vivo*. For this reason we used primary EC derived from other vascular sources to study hematopoietic regeneration. Ideally, this approach would result in the identification of factors that have independently been implicated in regulating hematopoietic regeneration *in vivo*. As discussed above, TGF-β1 and HGF have both been identified as factors that either regulate hematopoietic regeneration or bone marrow EC. This provides validity for the use of HAEC and HUVEC to study bone marrow HSC regeneration, and enhances the translational importance of these findings.

One question when considering an in vitro approach used to identify factors is: Do HAEC and HUVEC resemble specific EC subsets or recapitulate bone marrow EC during different stages of hematopoietic regeneration? As described in Chapter 2, the diversity of vascular EC in bone marrow has only been partially uncovered within the last few years (Itkin et al., 2016; Kusumbe et al., 2014). The heterogeneity between different bone marrow EC subsets has not yet been fully determined at the transcriptome level; therefore a direct comparison with HAEC and HUVEC is not yet possible. However, there are several characteristics of HAEC and HUVEC that resemble bone marrow EC and may help to explain their differential effects on hematopoietic regeneration.

Arterial versus Venous Vascular Endothelial Cells

HAEC and HUVEC are derived from very distinct vascular sources. Whereas HAEC are conditioned by a high shear, adult vessel; HUVEC derive from an embryonic source with lower perfusion pressures. Gene expression studies have shown that EC derived from several arterial vessel sources tend to cluster distinctly from venous vessels (Chi et al., 2003); therefore, it is

possible that HAEC more closely recapitulate bone marrow arteriolar EC. In support of this, HAEC adhere more firmly to plastic and have higher baseline levels of endothelial nitric oxidie synthase (eNOS) activation (data not shown). These are consistent with increased pericyte coverage, cell-cell adhesion, and shear stress in arteriolar bone marrow EC relative to more distal sinusoids (Itkin et al., 2016). However, studies of arteriolar EC have indicated that they provide a niche for quiescent HSC (Kunisaki et al., 2013), whereas my studies show that HAEC induce proliferation of HSC in co-culture. Notably, HAEC induce proliferation of both irradiated and non-irradiated HSC, indicating it is a fundamental property of the HSC-EC crosstalk and not an effect of radiation.

Bone Marrow Type H Endothelial Cells

Although the full molecular landscape of the bone marrow vasculature has not been elucidated, TGF-β isoforms are known to be differentially expressed between EC subsets. Specifically, *Tgfb1* and *Tgfb3* are overexpressed by $CD31^{hi}$, $Endomucin^{hi}$ "type-H" endothelial cells, which localize to the marginal region of the bone marrow transition zone, where arteriolar vessels transition to looping capillary beds (Kusumbe et al., 2014). During hematopoietic regeneration, type H vessels have been shown to proliferate, and ultimately regenerate the more distal, type L capillaries, which subsequently downregulate TGF-β ligand expression (Kusumbe et al., 2014). Interestingly, HUVEC have both higher levels of TGF-β isoform expression and expand more rapidly than HAEC in growth culture. Therefore, HUVEC may be more representative of type H EC when compared with HAEC. With respect to effects hematopoietic regeneration, the predominance of TGF-β producing, type H vessels following radiation injury would be predicted to suppress regeneration while the bone marrow microvasculature is repopulated. Interestingly, a similar biphasic effect of organ regeneration has been described in mouse liver (Ding et al., 2010), where hepatocyte proliferation precedes

vascular EC proliferation. Although a biphasic effect of hemato-endothelial regeneration has not been studied in bone marrow, HSC are known to leave the bone marrow following radiation injury, and the hematopoietic nadir 1-2 weeks after insult overlaps with the timing of bone marrow sinusoidal repopulation by type H EC (Kusumbe et al., 2014). Therefore, a TGF-β driven switch could be responsible for regenerating the hematopoietic microenvironment prior to the regeneration of hematopoiesis.

Acute and Long-term Vascular Injury

In addition to recapitulating characteristics of different bone marrow EC subsets, my data also suggest that HAEC and HUVEC may resemble the vascular EC profile during acute and chronic bone marrow injury. The molecular response of bone marrow EC to injury from ionizing radiation exposure has recently been described at different times of recovery (Himburg et al., 2016; Nolan et al., 2013). Endothelial cell *Hgf* expression increases 2-3 fold between 6 and 24 hr following exposure to 5 Gy irradiation, and this increase in expression correlates with a maximum of HGF protein content in bone marrow of nearly 3 ng/mL (Himburg et al., 2016). Taken together with previously published data that HGF is elevated in radioprotected bone marrow, these findings indicate that HGF is likely to be an important factor driving the early vascular recovery from radiation injury *in vivo*. These results indicate that HAEC may more closely resemble the vascular response to acute bone marrow injury, and suggest that the soluble factors produced by HAEC may stimulate early vascular regeneration.

Conversely, longer-term studies on the vascular response to bone marrow irradiation have shown that *Tgfb2* expression is elevated in bone marrow EC at 10 and 28 days following a 7.5 Gy dose of irradiation (Nolan et al., 2013), raising the possibility that HUVEC recapitulate the bone marrow vasculature after long-term or chronic injury. In support of this, TGF-β isoforms are known to mediate long-term vascular dysfunction and organ fibrosis through endothelial-to-

mesenchymal transition (Ranchoux et al., 2015; Xu et al., 2015). Therefore, it is possible that HUVEC recapitulate a chronically injured bone marrow vasculature that lacks the ability to fully regenerate the hematopoietic system.

A paradigm shift is needed in designing therapies for hematopoietic injury

There is a growing body of evidence that bone marrow vascular EC are essential regulators of stem cell maintenance, regeneration, and aging. This work and that of others shows that vascular EC are potent mediators of HSC regeneration *in vivo*, and can even promote HSC DNA damage repair *in vitro* (Chute et al., 2004; Li et al., 2010; Zachman et al., 2013). However, current therapies for hematopoietic regeneration target hematopoietic stem and progenitor cells directly, rather than their niche. I see two major problems with this approach: First, current therapies (e.g. G-CSF) will stimulate the regeneration of hematopoiesis and the proliferation of HSC within a dysfunctional microenvironment. My early work comparing EC and G-CSF mediated HSC regeneration, in combination with the more recent TGF–β1 findings, suggest that this will cause HSC attrition. As mentioned above a recent report has shown that G-CSF treatment after irradiation can exacerbate long-term HSC injury (Li et al., 2015), although the mechanisms were not investigated. Secondly, targeting only hematopoietic cells assumes that the niche does not sustain injury from cytotoxic exposures. We now know that this is incorrect, and that injury to the niche does impact HSC regeneration (Cao et al., 2011; Hooper et al., 2009; Scandura et al., 2004). Therefore, an overarching goal for the development of novel therapies for hematopoietic regeneration should be to stimulate vascular recovery in the bone marrow.

A precautionary note on using EC-targeted therapies in hematologic malignancies

Targeting the regeneration or survival of bone marrow vascular EC may not always be beneficial. Although the field is fairly new, more work is being published on the putative ability of

vascular EC to support leukemia drug resistance, which may contribute to disease relapse by promoting quiescence of leukemia-initiating cells (Bosse et al., 2016; Cogle et al., 2014). Our data show that EC can reduce DNA damage in LSK cells after radiation injury. Therefore, in the context of both hematologic malignancies and solid tumors, much more investigation is needed to determine the potential risks and benefits of treating hematopoietic suppression in the setting of anti-tumor therapy.

References from the Literature

Acar, M., Kocherlakota, K.S., Murphy, M.M., Peyer, J.G., Oguro, H., Inra, C.N., Jaiyeola, C., Zhao, Z., Luby-Phelps, K., and Morrison, S.J. (2015). Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature *526*, 126-130.

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature *439*, 599-603.

Aird, W.C. (2012). Endothelial cell heterogeneity. Cold Spring Harb Perspect Med *2*, a006429.

Alvarez-Errico, D., Vento-Tormo, R., Sieweke, M., and Ballestar, E. (2015). Epigenetic control of myeloid cell differentiation, identity and function. Nat Rev Immunol *15*, 7-17.

Anderson, L.A., Pfeiffer, R.M., Landgren, O., Gadalla, S., Berndt, S.I., and Engels, E.A. (2009). Risks of myeloid malignancies in patients with autoimmune conditions. Br J Cancer *100*, 822-828.

Aoki, M., Morishita, R., Taniyama, Y., Kida, I., Moriguchi, A., Matsumoto, K., Nakamura, T., Kaneda, Y., Higaki, J., and Ogihara, T. (2000). Angiogenesis induced by hepatocyte growth factor in non-infarcted myocardium and infarcted myocardium: up-regulation of essential transcription factor for angiogenesis, ets. Gene Ther *7*, 417-427.

Azzam, E.I., Jay-Gerin, J.P., and Pain, D. (2012). Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett *327*, 48-60.

Bai, J., Guo, X.G., and Bai, X.P. (2012). Epidermal growth factor receptor-related DNA repair and radiation-resistance regulatory mechanisms: a mini-review. Asian Pac J Cancer Prev *13*, 4879-4881.

Baldridge, M.T., King, K.Y., Boles, N.C., Weksberg, D.C., and Goodell, M.A. (2010). Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature *465*, 793-797.

Batard, P., Monier, M.N., Fortunel, N., Ducos, K., Sansilvestri-Morel, P., Phan, T., Hatzfeld, A., and Hatzfeld, J.A. (2000). TGF-(beta)1 maintains hematopoietic immaturity by a reversible negative control of cell cycle and induces CD34 antigen up-modulation. J Cell Sci *113 (Pt 3)*, 383-390.

Becker, A.J., McCulloch, E., and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature *197*, 452-454.

Beerman, I., Seita, J., Inlay, M.A., Weissman, I.L., and Rossi, D.J. (2014). Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. Cell Stem Cell *15*, 37-50.

Bendall, L.J., and Bradstock, K.F. (2014). G-CSF: From granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine Growth Factor Rev *25*, 355-367.

Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y., and Traver, D. (2010). Haematopoietic stem cells derive directly from aortic endothelium during development. Nature *464*, 108-111.

Bhatia, S. (2013). Therapy-related myelodysplasia and acute myeloid leukemia. Semin Oncol *40*, 666-675.

Blank, U., and Karlsson, S. (2015). TGF-beta signaling in the control of hematopoietic stem cells. Blood *125*, 3542-3550.

Boisset, J.C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature *464*, 116-120.

Bosse, R.C., Wasserstrom, B., Meacham, A., Wise, E., Drusbosky, L., Walter, G.A., Chaplin, D.J., Siemann, D.W., Purich, D.L., and Cogle, C.R. (2016). Chemosensitizing AML cells by targeting bone marrow endothelial cells. Exp Hematol *44*, 363-377 e365.

Boulais, P.E., and Frenette, P.S. (2015). Making sense of hematopoietic stem cell niches. Blood *125*, 2621-2629.

Bradley, T.R., and Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci *44*, 287-299.

Brenet, F., Kermani, P., Spektor, R., Rafii, S., and Scandura, J.M. (2013). TGFbeta restores hematopoietic homeostasis after myelosuppressive chemotherapy. J Exp Med *210*, 623-639.

Bronchud, M.H., Scarffe, J.H., Thatcher, N., Crowther, D., Souza, L.M., Alton, N.K., Testa, N.G., and Dexter, T.M. (1987). Phase I/II study of recombinant human granulocyte colony-stimulating factor in patients receiving intensive chemotherapy for small cell lung cancer. Br J Cancer *56*, 809-813.

Butler, J.M., Nolan, D.J., Vertes, E.L., Varnum-Finney, B., Kobayashi, H., Hooper, A.T., Seandel, M., Shido, K., White, I.A., Kobayashi, M.*, et al.* (2010). Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell *6*, 251-264.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R.*, et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature *425*, 841-846.

Cao, X., Wu, X., Frassica, D., Yu, B., Pang, L., Xian, L., Wan, M., Lei, W., Armour, M., Tryggestad, E.*, et al.* (2011). Irradiation induces bone injury by damaging bone marrow microenvironment for stem cells. Proc Natl Acad Sci U S A *108*, 1609-1614.

Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature *473*, 298-307.

Ceccaldi, R., Rondinelli, B., and D'Andrea, A.D. (2016). Repair Pathway Choices and Consequences at the Double-Strand Break. Trends Cell Biol *26*, 52-64.

Chabanon, A., Desterke, C., Rodenburger, E., Clay, D., Guerton, B., Boutin, L., Bennaceur-Griscelli, A., Pierre-Louis, O., Uzan, G., Abecassis, L.*, et al.* (2008). A cross-talk between stromal cell-derived factor-1 and transforming growth factor-beta controls the quiescence/cycling switch of CD34(+) progenitors through FoxO3 and mammalian target of rapamycin. Stem Cells *26*, 3150-3161.

Challen, G.A., Boles, N.C., Chambers, S.M., and Goodell, M.A. (2010). Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell Stem Cell *6*, 265-278.

Chan, C.K., Chen, C.C., Luppen, C.A., Kim, J.B., DeBoer, A.T., Wei, K., Helms, J.A., Kuo, C.J., Kraft, D.L., and Weissman, I.L. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. Nature *457*, 490-494.

Chao, N.J. (2007). Accidental or intentional exposure to ionizing radiation: biodosimetry and treatment options. Exp Hematol *35*, 24-27.

Chen, J., Ellison, F.M., Keyvanfar, K., Omokaro, S.O., Desierto, M.J., Eckhaus, M.A., and Young, N.S. (2008). Enrichment of hematopoietic stem cells with SLAM and LSK markers for the detection of hematopoietic stem cell function in normal and Trp53 null mice. Exp Hematol *36*, 1236-1243.

Chen, M.J., Yokomizo, T., Zeigler, B.M., Dzierzak, E., and Speck, N.A. (2009). Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature *457*, 887-891.

Chen, S., Lewallen, M., and Xie, T. (2013). Adhesion in the stem cell niche: biological roles and regulation. Development *140*, 255-265.

Cheng, T., Shen, H., Rodrigues, N., Stier, S., and Scadden, D.T. (2001). Transforming growth factor beta 1 mediates cell-cycle arrest of primitive hematopoietic cells independent of p21(Cip1/Waf1) or p27(Kip1). Blood *98*, 3643-3649.

Cheshier, S.H., Prohaska, S.S., and Weissman, I.L. (2007). The effect of bleeding on hematopoietic stem cell cycling and self-renewal. Stem Cells Dev *16*, 707-717.

Chi, J.T., Chang, H.Y., Haraldsen, G., Jahnsen, F.L., Troyanskaya, O.G., Chang, D.S., Wang, Z., Rockson, S.G., van de Rijn, M., Botstein, D*., et al.* (2003). Endothelial cell diversity revealed by global expression profiling. Proc Natl Acad Sci U S A *100*, 10623-10628.

Chitteti, B.R., Cheng, Y.H., Poteat, B., Rodriguez-Rodriguez, S., Goebel, W.S., Carlesso, N., Kacena, M.A., and Srour, E.F. (2010). Impact of interactions of cellular components of the bone marrow microenvironment on hematopoietic stem and progenitor cell function. Blood *115*, 3239-3248.

Chua, H.L., Plett, P.A., Sampson, C.H., Joshi, M., Tabbey, R., Katz, B.P., MacVittie, T.J., and Orschell, C.M. (2012). Long-term hematopoietic stem cell damage in a murine model of the hematopoietic syndrome of the acute radiation syndrome. Health Phys *103*, 356-366.

Chute, J.P., Fung, J., Muramoto, G., and Erwin, R. (2004). Ex vivo culture rescues hematopoietic stem cells with long-term repopulating capacity following harvest from lethally irradiated mice. Exp Hematol *32*, 308-317.

Chute, J.P., Muramoto, G.G., Fung, J., and Oxford, C. (2005). Soluble factors elaborated by human brain endothelial cells induce the concomitant expansion of purified human BM CD34+CD38- cells and SCID-repopulating cells. Blood *105*, 576-583.

Chute, J.P., Muramoto, G.G., Salter, A.B., Meadows, S.K., Rickman, D.W., Chen, B., Himburg, H.A., and Chao, N.J. (2007). Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. Blood *109*, 2365-2372.

Cogle, C.R., Goldman, D.C., Madlambayan, G.J., Leon, R.P., Al Masri, A., Clark, H.A., Asbaghi, S.A., Tyner, J.W., Dunlap, J., Fan, G.*, et al.* (2014). Functional integration of acute myeloid leukemia into the vascular niche. Leukemia *28*, 1978-1987.

Dainiak, N. (2010). Rationale and recommendations for treatment of radiation injury with cytokines. Health Phys *98*, 838-842.

Ding, B.S., Nolan, D.J., Butler, J.M., James, D., Babazadeh, A.O., Rosenwaks, Z., Mittal, V., Kobayashi, H., Shido, K., Lyden, D.*, et al.* (2010). Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. Nature *468*, 310-315.

Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature *495*, 231-235.

Ding, L., Saunders, T.L., Enikolopov, G., and Morrison, S.J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. Nature *481*, 457-462.

Doan, P.L., Himburg, H.A., Helms, K., Russell, J.L., Fixsen, E., Quarmyne, M., Harris, J.R., Deoliviera, D., Sullivan, J.M., Chao, N.J.*, et al.* (2013a). Epidermal growth factor regulates hematopoietic regeneration after radiation injury. Nat Med.

Doan, P.L., Himburg, H.A., Helms, K., Russell, J.L., Fixsen, E., Quarmyne, M., Harris, J.R., Deoliviera, D., Sullivan, J.M., Chao, N.J.*, et al.* (2013b). Epidermal growth factor regulates hematopoietic regeneration after radiation injury. Nat Med *19*, 295-304.

Doan, P.L., Russell, J.L., Himburg, H.A., Helms, K., Harris, J.R., Lucas, J., Holshausen, K.C., Meadows, S.K., Daher, P., Jeffords, L.B.*, et al.* (2013c). Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. Stem Cells *31*, 327-337.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell Stem Cell *10*, 120-136.

Drouet, M., and Herodin, F. (2010). Radiation victim management and the haematologist in the future: time to revisit therapeutic guidelines? Int J Radiat Biol *86*, 636-648.

Dzierzak, E., and Speck, N.A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol *9*, 129-136.

Eaves, C.J. (2015). Hematopoietic stem cells: concepts, definitions, and the new reality. Blood *125*, 2605-2613.

Ellis, S.L., Grassinger, J., Jones, A., Borg, J., Camenisch, T., Haylock, D., Bertoncello, I., and Nilsson, S.K. (2011). The relationship between bone, hemopoietic stem cells, and vasculature. Blood *118*, 1516-1524.

Fliedner, T.M., Graessle, D., Paulsen, C., and Reimers, K. (2002). Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. Cancer Biother Radiopharm *17*, 405-426.

Fukada, M., Fujikawa, A., Chow, J.P., Ikematsu, S., Sakuma, S., and Noda, M. (2006). Protein tyrosine phosphatase receptor type Z is inactivated by ligand-induced oligomerization. FEBS Lett *580*, 4051-4056.

Gilman, A., and Philips, F.S. (1946). The biological actions and therapeutic applications of the B-chloroethyl amines and sulfides. Science *103*, 409-415.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med *183*, 1797-1806.

Goodman, L.S., Wintrobe, M.M., and et al. (1946). Nitrogen mustard therapy; use of methyl-bis (beta-chloroethyl) amine hydrochloride and tris (beta-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. J Am Med Assoc *132*, 126-132.

Goumans, M.J., Lebrin, F., and Valdimarsdottir, G. (2003). Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. Trends Cardiovasc Med *13*, 301-307.

Greenbaum, A., Hsu, Y.M., Day, R.B., Schuettpelz, L.G., Christopher, M.J., Borgerding, J.N., Nagasawa, T., and Link, D.C. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature *495*, 227-230.

Guezguez, B., Campbell, C.J., Boyd, A.L., Karanu, F., Casado, F.L., Di Cresce, C., Collins, T.J., Shapovalova, Z., Xenocostas, A., and Bhatia, M. (2013). Regional localization within the bone marrow influences the functional capacity of human HSCs. Cell Stem Cell *13*, 175-189.

Harfouche, G., Vaigot, P., Rachidi, W., Rigaud, O., Moratille, S., Marie, M., Lemaitre, G., Fortunel, N.O., and Martin, M.T. (2010). Fibroblast growth factor type 2 signaling is critical for DNA repair in human keratinocyte stem cells. Stem Cells *28*, 1639-1648.

Hasselbalch, H.C. (2012). Perspectives on chronic inflammation in essential thrombocythemia, polycythemia vera, and myelofibrosis: is chronic inflammation a trigger and driver of clonal evolution and development of accelerated atherosclerosis and second cancer? Blood *119*, 3219-3225.

Herodin, F., and Drouet, M. (2005). Cytokine-based treatment of accidentally irradiated victims and new approaches. Exp Hematol *33*, 1071-1080.

Himburg, H.A., Harris, J.R., Ito, T., Daher, P., Russell, J.L., Quarmyne, M., Doan, P.L., Helms, K., Nakamura, M., Fixsen, E.*, et al.* (2012). Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche. Cell Rep *2*, 964-975.

Himburg, H.A., Muramoto, G.G., Daher, P., Meadows, S.K., Russell, J.L., Doan, P., Chi, J.T., Salter, A.B., Lento, W.E., Reya, T.*, et al.* (2010). Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat Med *16*, 475-482.

Himburg, H.A., Sasine, J., Yan, X., Kan, J., Dressman, H., and Chute, J.P. (2016). A Molecular Profile of the Endothelial Cell Response to Ionizing Radiation. Radiat Res *186*, 141-152.

Himburg, H.A., Yan, X., Doan, P.L., Quarmyne, M., Micewicz, E., McBride, W., Chao, N.J., Slamon, D.J., and Chute, J.P. (2014). Pleiotrophin mediates hematopoietic regeneration via activation of RAS. J Clin Invest *124*, 4753-4758.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K.*, et al.* (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell Stem Cell *4*, 263-274.

Horiguchi, M., Ota, M., and Rifkin, D.B. (2012). Matrix control of transforming growth factor-beta function. J Biochem *152*, 321-329.

Hu, S.Y., Duan, H.F., Li, Q.F., Yang, Y.F., Chen, J.L., Wang, L.S., and Wang, H. (2009). Hepatocyte growth factor protects endothelial cells against gamma ray irradiation-induced damage. Acta Pharmacol Sin *30*, 1415-1420.

Itkin, T., Gur-Cohen, S., Spencer, J.A., Schajnovitz, A., Ramasamy, S.K., Kusumbe, A.P., Ledergor, G., Jung, Y., Milo, I., Poulos, M.G.*, et al.* (2016). Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N.*, et al.* (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997-1002.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y.*, et al.* (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med *12*, 446-451.

Ivanov, V.K., Tsyb, A.F., Khait, S.E., Kashcheev, V.V., Chekin, S.Y., Maksioutov, M.A., and Tumanov, K.A. (2012). Leukemia incidence in the Russian cohort of Chernobyl emergency workers. Radiat Environ Biophys *51*, 143-149.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071-1078.

Jaffredo, T., Gautier, R., Eichmann, A., and Dieterlen-Lievre, F. (1998). Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development *125*, 4575-4583.

Jasin, M., and Rothstein, R. (2013). Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol *5*, a012740.

Keller, J.R., McNiece, I.K., Sill, K.T., Ellingsworth, L.R., Quesenberry, P.J., Sing, G.K., and Ruscetti, F.W. (1990). Transforming growth factor beta directly regulates primitive murine hematopoietic cell proliferation. Blood *75*, 596-602.

Kesminiene, A., Evrard, A.S., Ivanov, V.K., Malakhova, I.V., Kurtinaitis, J., Stengrevics, A., Tekkel, M., Anspaugh, L.R., Bouville, A., Chekin, S.*, et al.* (2008). Risk of hematological malignancies among Chernobyl liquidators. Radiat Res *170*, 721-735.

Khan, J.A., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y., Arnal-Estape, A., Pinho, S., Ciero, P., Nakahara, F., Ma'ayan, A., *et al.* (2016). Fetal liver hematopoietic stem cell niches associate with portal vessels. Science *351*, 176-180.

Kiel, M.J., Acar, M., Radice, G.L., and Morrison, S.J. (2009). Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance. Cell Stem Cell *4*, 170-179.

Kiel, M.J., and Morrison, S.J. (2008). Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol *8*, 290-301.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. Cell Stem Cell *1*, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005a). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005b). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kissa, K., and Herbomel, P. (2010). Blood stem cells emerge from aortic endothelium by a novel type of cell transition. Nature *464*, 112-115.

Kobayashi, H., Butler, J.M., O'Donnell, R., Kobayashi, M., Ding, B.S., Bonner, B., Chiu, V.K., Nolan, D.J., Shido, K., Benjamin, L., *et al.* (2010). Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. Nat Cell Biol *12*, 1046-1056.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., and Rafii, S. (2005a). The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) *20*, 349-356.

Kopp, H.G., Avecilla, S.T., Hooper, A.T., Shmelkov, S.V., Ramos, C.A., Zhang, F., and Rafii, S. (2005b). Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. Blood *106*, 505-513.

Krenning, G., Zeisberg, E.M., and Kalluri, R. (2010). The origin of fibroblasts and mechanism of cardiac fibrosis. J Cell Physiol *225*, 631-637.

Kristinsson, S.Y., Bjorkholm, M., Hultcrantz, M., Derolf, A.R., Landgren, O., and Goldin, L.R. (2011). Chronic immune stimulation might act as a trigger for the development of acute myeloid leukemia or myelodysplastic syndromes. J Clin Oncol *29*, 2897-2903.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., *et al.* (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. Nature *502*, 637-643.

Kusumbe, A.P., Ramasamy, S.K., and Adams, R.H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature *507*, 323-328.

Kusumbe, A.P., Ramasamy, S.K., Itkin, T., Mae, M.A., Langen, U.H., Betsholtz, C., Lapidot, T., and Adams, R.H. (2016). Age-dependent modulation of vascular niches for haematopoietic stem cells. Nature.

Larsson, J., Blank, U., Helgadottir, H., Bjornsson, J.M., Ehinger, M., Goumans, M.J., Fan, X., Leveen, P., and Karlsson, S. (2003). TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood *102*, 3129-3135.

Li, B., Bailey, A.S., Jiang, S., Liu, B., Goldman, D.C., and Fleming, W.H. (2010). Endothelial cells mediate the regeneration of hematopoietic stem cells. Stem Cell Res *4*, 17-24.
Li, C., Lu, L., Zhang, J., Huang, S., Xing, Y., Zhao, M., Zhou, D., Li, D., and Meng, A. (2015). Granulocyte colony-stimulating factor exacerbates hematopoietic stem cell injury after irradiation. Cell Biosci *5*, 65.

Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., and Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood *98*, 3241-3248.

Lieber, M.R. (2010). The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem *79*, 181-211.

Lindahl, T., and Barnes, D.E. (2000). Repair of endogenous DNA damage. Cold Spring Harb Symp Quant Biol *65*, 127-133.

Lo Celso, C., Wu, J.W., and Lin, C.P. (2009). In vivo imaging of hematopoietic stem cells and their microenvironment. J Biophotonics *2*, 619-631.

Lord, B.I., and Hendry, J.H. (1972). The distribution of haemopoietic colony-forming units in the mouse femur, and its modification by x rays. Br J Radiol *45*, 110-115.

Lord, B.I., Testa, N.G., and Hendry, J.H. (1975). The relative spatial distributions of CFUs and CFUc in the normal mouse femur. Blood *46*, 65-72.

MacVittie, T.J., Farese, A.M., and Jackson, W., 3rd (2005). Defining the full therapeutic potential of recombinant growth factors in the post radiation-accident environment: the effect of supportive care plus administration of G-CSF. Health Phys *89*, 546-555.

Mah, L.J., El-Osta, A., and Karagiannis, T.C. (2010). gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia *24*, 679-686.

Maier, C.S., Chavez, J., Wang, J., and Wu, J. (2010). Protein adducts of aldehydic lipid peroxidation products identification and characterization of protein adducts using an aldehyde/keto-reactive probe in combination with mass spectrometry. Methods Enzymol *473*, 305-330.

Marenzana, M., and Arnett, T.R. (2013). The Key Role of the Blood Supply to Bone. Bone Res *1*, 203-215.

Martins, A., Han, J., and Kim, S.O. (2010). The multifaceted effects of granulocyte colony-stimulating factor in immunomodulation and potential roles in intestinal immune homeostasis. IUBMB Life *62*, 611-617.

Massague, J. (2012). TGF-beta signaling in development and disease. FEBS Lett *586*, 1833.

Massague, J., and Xi, Q. (2012). TGF-beta control of stem cell differentiation genes. FEBS Lett *586*, 1953-1958.

Mauch, P., Constine, L., Greenberger, J., Knospe, W., Sullivan, J., Liesveld, J.L., and Deeg, H.J. (1995). Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. Int J Radiat Oncol Biol Phys *31*, 1319-1339.

McAllister, K.A., Grogg, K.M., Johnson, D.W., Gallione, C.J., Baldwin, M.A., Jackson, C.E., Helmbold, E.A., Markel, D.S., McKinnon, W.C., Murrell, J., *et al.* (1994). Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet *8*, 345-351.

McCulloch, E.A., and Till, J.E. (1960). The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res *13*, 115-125.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature *466*, 829-834.

Meng, A., Wang, Y., Brown, S.A., Van Zant, G., and Zhou, D. (2003a). Ionizing radiation and busulfan inhibit murine bone marrow cell hematopoietic function via apoptosis-dependent and -independent mechanisms. Exp Hematol *31*, 1348-1356.

Meng, A., Wang, Y., Van Zant, G., and Zhou, D. (2003b). Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. Cancer Res *63*, 5414-5419.

Mercier, F.E., and Scadden, D.T. (2015). Not All Created Equal: Lineage Hard-Wiring in the Production of Blood. Cell *163*, 1568-1570.

Metcalf, D. (2008). Hematopoietic cytokines. Blood *111*, 485-491.

Metcalf, D., and Nicola, N.A. (1983). Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. J Cell Physiol *116*, 198-206.

Miyake, T., Kung, C.K., and Goldwasser, E. (1977). Purification of human erythropoietin. J Biol Chem *252*, 5558-5564.

Mohrin, M., Bourke, E., Alexander, D., Warr, M.R., Barry-Holson, K., Le Beau, M.M., Morrison, C.G., and Passegue, E. (2010). Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell *7*, 174-185.

Moldovan, G.L., and D'Andrea, A.D. (2009). How the fanconi anemia pathway guards the genome. Annu Rev Genet *43*, 223-249.

Montfort, M.J., Olivares, C.R., Mulcahy, J.M., and Fleming, W.H. (2002). Adult blood vessels restore host hematopoiesis following lethal irradiation. Exp Hematol *30*, 950-956.

Morgan, W.F., and Sowa, M.B. (2007). Non-targeted bystander effects induced by ionizing radiation. Mutat Res *616*, 159-164.

Morrison, S.J., and Kimble, J. (2006). Asymmetric and symmetric stem-cell divisions in development and cancer. Nature *441*, 1068-1074.

Morrison, S.J., and Scadden, D.T. (2014). The bone marrow niche for haematopoietic stem cells. Nature *505*, 327-334.

Morton, L.M., Dores, G.M., Tucker, M.A., Kim, C.J., Onel, K., Gilbert, E.S., Fraumeni, J.F., Jr., and Curtis, R.E. (2013). Evolving risk of therapy-related acute myeloid leukemia following cancer chemotherapy among adults in the United States, 1975-2008. Blood *121*, 2996-3004.

Mu, Y., Gudey, S.K., and Landstrom, M. (2012). Non-Smad signaling pathways. Cell Tissue Res *347*, 11-20.

Muramoto, G.G., Chen, B., Cui, X., Chao, N.J., and Chute, J.P. (2006). Vascular endothelial cells produce soluble factors that mediate the recovery of human hematopoietic stem cells after radiation injury. Biol Blood Marrow Transplant *12*, 530-540.

Nakamura-Ishizu, A., and Suda, T. (2013). Hematopoietic stem cell niche: an interplay among a repertoire of multiple functional niches. Biochim Biophys Acta *1830*, 2404-2409.
Neptune, E.R., Frischmeyer, P.A., Arking, D.E., Myers, L., Bunton, T.E., Gayraud, B., Ramirez, F., Sakai, L.Y., and Dietz, H.C. (2003). Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet *33*, 407-411.

Nolan, D.J., Ginsberg, M., Israely, E., Palikuqi, B., Poulos, M.G., James, D., Ding, B.S., Schachterle, W., Liu, Y., Rosenwaks, Z.*, et al.* (2013). Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Dev Cell *26*, 204-219.

Nombela-Arrieta, C., Pivarnik, G., Winkel, B., Canty, K.J., Harley, B., Mahoney, J.E., Park, S.Y., Lu, J., Protopopov, A., and Silberstein, L.E. (2013). Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nat Cell Biol *15*, 533-543.

Notta, F., Zandi, S., Takayama, N., Dobson, S., Gan, O.I., Wilson, G., Kaufmann, K.B., McLeod, J., Laurenti, E., Dunant, C.F.*, et al.* (2016). Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science *351*, aab2116.

Ogo, T., Chowdhury, H.M., Yang, J., Long, L., Li, X., Torres Cleuren, Y.N., Morrell, N.W., Schermuly, R.T., Trembath, R.C., and Nasim, M.T. (2013). Inhibition of overactive transforming growth factor-beta signaling by prostacyclin analogs in pulmonary arterial hypertension. Am J Respir Cell Mol Biol *48*, 733-741.

Oguro, H., Ding, L., and Morrison, S.J. (2013). SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell *13*, 102-116.

Ohta, M., Greenberger, J.S., Anklesaria, P., Bassols, A., and Massague, J. (1987). Two forms of transforming growth factor-beta distinguished by multipotential haematopoietic progenitor cells. Nature *329*, 539-541.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044-3050.

Olive, P.L., and Banath, J.P. (2006). The comet assay: a method to measure DNA damage in individual cells. Nat Protoc *1*, 23-29.

Ono, N., Ono, W., Mizoguchi, T., Nagasawa, T., Frenette, P.S., and Kronenberg, H.M. (2014). Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. Dev Cell *29*, 330-339.

Orkin, S.H. (2003). Priming the hematopoietic pump. Immunity *19*, 633-634.

Orkin, S.H., and Zon, L.I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Papayannopoulou, T., and Scadden, D.T. (2008). Stem-cell ecology and stem cells in motion. Blood *111*, 3923-3930.

Pardanaud, L., and Dieterlen-Lievre, F. (1999). Manipulation of the angiopoietic/hemangiopoietic commitment in the avian embryo. Development *126*, 617-627.

Paul, F., Arkin, Y., Giladi, A., Jaitin, D.A., Kenigsberg, E., Keren-Shaul, H., Winter, D., Lara-Astiaso, D., Gury, M., Weiner, A.*, et al.* (2015). Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. Cell *163*, 1663-1677.

Perie, L., Duffy, K.R., Kok, L., de Boer, R.J., and Schumacher, T.N. (2015). The Branching Point in Erythro-Myeloid Differentiation. Cell *163*, 1655-1662.

Plett, P.A., Sampson, C.H., Chua, H.L., Joshi, M., Booth, C., Gough, A., Johnson, C.S., Katz, B.P., Farese, A.M., Parker, J.*, et al.* (2012). Establishing a murine model of the hematopoietic syndrome of the acute radiation syndrome. Health Phys *103*, 343-355.

Plzak, L.F., Fried, W., Jacobson, L.O., and Bethard, W.F. (1955). Demonstration of stimulation of erythropoiesis by plasma from anemic rats using Fe59. J Lab Clin Med *46*, 671-678.

Poulos, M.G., Guo, P., Kofler, N.M., Pinho, S., Gutkin, M.C., Tikhonova, A., Aifantis, I., Frenette, P.S., Kitajewski, J., Rafii, S.*, et al.* (2013). Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. Cell Rep *4*, 1022-1034.

Rafii, S., Butler, J.M., and Ding, B.S. (2016). Angiocrine functions of organ-specific endothelial cells. Nature *529*, 316-325.

Ranchoux, B., Antigny, F., Rucker-Martin, C., Hautefort, A., Pechoux, C., Bogaard, H.J., Dorfmuller, P., Remy, S., Lecerf, F., Plante, S*., et al.* (2015). Endothelial-to-mesenchymal transition in pulmonary hypertension. Circulation *131*, 1006-1018.

Richman, C.M., Weiner, R.S., and Yankee, R.A. (1976). Increase in circulating stem cells following chemotherapy in man. Blood *47*, 1031-1039.

Rossi, D.J., Seita, J., Czechowicz, A., Bhattacharya, D., Bryder, D., and Weissman, I.L. (2007). Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. Cell Cycle *6*, 2371-2376.

Sabin, F.R. (2002). Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. 1917. J Hematother Stem Cell Res *11*, 5-7.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M*., et al.* (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell *131*, 324-336.

Salter, A.B., Meadows, S.K., Muramoto, G.G., Himburg, H., Doan, P., Daher, P., Russell, L., Chen, B., Chao, N.J., and Chute, J.P. (2009). Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. Blood *113*, 2104-2107.

Sattler, M., Winkler, T., Verma, S., Byrne, C.H., Shrikhande, G., Salgia, R., and Griffin, J.D. (1999). Hematopoietic growth factors signal through the formation of reactive oxygen species. Blood *93*, 2928-2935.

Scandura, J.M., Boccuni, P., Massague, J., and Nimer, S.D. (2004). Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. Proc Natl Acad Sci U S A *101*, 15231-15236.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

Schultze, J.L., and Beyer, M. (2016). Myelopoiesis Reloaded: Single-Cell Transcriptomics Leads the Way. Immunity *44*, 18-20.

Shao, L., Luo, Y., and Zhou, D. (2014). Hematopoietic stem cell injury induced by ionizing radiation. Antioxid Redox Signal *20*, 1447-1462.

Shen, H., Yu, H., Liang, P.H., Cheng, H., XuFeng, R., Yuan, Y., Zhang, P., Smith, C.A., and Cheng, T. (2012). An acute negative bystander effect of gamma-irradiated recipients on transplanted hematopoietic stem cells. Blood *119*, 3629-3637.

Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T.A. (2011). Latent TGF-beta structure and activation. Nature *474*, 343-349.

Siena, S., Bregni, M., Brando, B., Ravagnani, F., Bonadonna, G., and Gianni, A.M. (1989). Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated

patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor. Blood *74*, 1905-1914.

Siminovitch, L., McCulloch, E.A., and Till, J.E. (1963). The Distribution of Colony-Forming Cells among Spleen Colonies. J Cell Physiol *62*, 327-336.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Cote, D., Means, T.K., Luster, A.D., Scadden, D.T., and Lin, C.P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature *435*, 969-973.

Sitnicka, E., Ruscetti, F.W., Priestley, G.V., Wolf, N.S., and Bartelmez, S.H. (1996). Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. Blood *88*, 82-88.

Sivaraj, K.K., and Adams, R.H. (2016). Blood vessel formation and function in bone. Development *143*, 2706-2715.

Soma, T., Yu, J.M., and Dunbar, C.E. (1996). Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of transforming growth factor-beta but not macrophage inflammatory protein-1 alpha activities. Blood *87*, 4561-4567.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity *25*, 977-988.

Taniyama, Y., Morishita, R., Hiraoka, K., Aoki, M., Nakagami, H., Yamasaki, K., Matsumoto, K., Nakamura, T., Kaneda, Y., and Ogihara, T. (2001). Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat diabetic hind limb ischemia model: molecular mechanisms of delayed angiogenesis in diabetes. Circulation *104*, 2344-2350.

Till, J.E., and McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res *14*, 213-222.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C.*, et al.* (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Tsushima, H., Iwanaga, M., and Miyazaki, Y. (2012). Late effect of atomic bomb radiation on myeloid disorders: leukemia and myelodysplastic syndromes. Int J Hematol *95*, 232-238.
Urao, N., and Ushio-Fukai, M. (2013). Redox regulation of stem/progenitor cells and bone marrow niche. Free Radic Biol Med *54*, 26-39.

van Galen, P., Kreso, A., Wienholds, E., Laurenti, E., Eppert, K., Lechman, E.R., Mbong, N., Hermans, K., Dobson, S., April, C.*, et al.* (2014). Reduced lymphoid lineage priming promotes human hematopoietic stem cell expansion. Cell Stem Cell *14*, 94-106.

van Os, R.P., Dethmers-Ausema, B., and de Haan, G. (2008). In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods Mol Biol *430*, 143-157.

Visnjic, D., Kalajzic, Z., Rowe, D.W., Katavic, V., Lorenzo, J., and Aguila, H.L. (2004). Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood *103*, 3258-3264.

Wang, J., Sun, Q., Morita, Y., Jiang, H., Gross, A., Lechel, A., Hildner, K., Guachalla, L.M., Gompf, A., Hartmann, D.*, et al.* (2012). A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. Cell *148*, 1001-1014.

Wang, L., Benedito, R., Bixel, M.G., Zeuschner, D., Stehling, M., Savendahl, L., Haigh, J.J., Snippert, H., Clevers, H., Breier, G.*, et al.* (2013). Identification of a clonally expanding haematopoietic compartment in bone marrow. EMBO J *32*, 219-230.

Wang, L.D., and Wagers, A.J. (2011). Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol *12*, 643-655.

Wang, Y., Liu, L., Pazhanisamy, S.K., Li, H., Meng, A., and Zhou, D. (2010). Total body irradiation causes residual bone marrow injury by induction of persistent oxidative stress in murine hematopoietic stem cells. Free Radic Biol Med *48*, 348-356.

Wang, Y., Schulte, B.A., LaRue, A.C., Ogawa, M., and Zhou, D. (2006). Total body irradiation selectively induces murine hematopoietic stem cell senescence. Blood *107*, 358-366.
Waselenko, J.K., MacVittie, T.J., Blakely, W.F., Pesik, N., Wiley, A.L., Dickerson, W.E., Tsu, H., Confer, D.L., Coleman, C.N., Seed, T.*, et al.* (2004). Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. Ann Intern Med *140*, 1037-1051.

Watson, G.E., Lorimore, S.A., Macdonald, D.A., and Wright, E.G. (2000). Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionizing radiation. Cancer Res *60*, 5608-5611.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Wu, A.M., Till, J.E., Siminovitch, L., and McCulloch, E.A. (1967). A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J Cell Physiol *69*, 177-184.

Xu, X., Friehs, I., Zhong Hu, T., Melnychenko, I., Tampe, B., Alnour, F., Iascone, M., Kalluri, R., Zeisberg, M., Del Nido, P.J.*, et al.* (2015). Endocardial fibroelastosis is caused by aberrant endothelial to mesenchymal transition. Circ Res *116*, 857-866.

Yahata, T., Takanashi, T., Muguruma, Y., Ibrahim, A.A., Matsuzawa, H., Uno, T., Sheng, Y., Onizuka, M., Ito, M., Kato, S.*, et al.* (2011). Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood *118*, 2941-2950.

Yamazaki, S., Iwama, A., Takayanagi, S., Eto, K., Ema, H., and Nakauchi, H. (2009). TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. Blood *113*, 1250-1256.

Ye, M., Iwasaki, H., Laiosa, C.V., Stadtfeld, M., Xie, H., Heck, S., Clausen, B., Akashi, K., and Graf, T. (2003). Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. Immunity *19*, 689-699.

Zachman, D.K., Leon, R.P., Das, P., Goldman, D.C., Hamlin, K.L., Guha, C., and Fleming, W.H. (2013). Endothelial cells mitigate DNA damage and promote the regeneration of hematopoietic stem cells after radiation injury. Stem Cell Res *11*, 1013-1021.

Zeisberg, E.M., Potenta, S.E., Sugimoto, H., Zeisberg, M., and Kalluri, R. (2008). Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. J Am Soc Nephrol *19*, 2282-2287.

Zeisberg, E.M., Tarnavski, O., Zeisberg, M., Dorfman, A.L., McMullen, J.R., Gustafsson, E., Chandraker, A., Yuan, X., Pu, W.T., Roberts, A.B.*, et al.* (2007). Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med *13*, 952-961.

Zhang, H., Kozono, D.E., O'Connor, K.W., Vidal-Cardenas, S., Rousseau, A., Hamilton, A., Moreau, L., Gaudiano, E.F., Greenberger, J., Bagby, G.*, et al.* (2016). TGF-beta Inhibition Rescues Hematopoietic Stem Cell Defects and Bone Marrow Failure in Fanconi Anemia. Cell Stem Cell.

Zhang, H., Wang, Y.A., Meng, A., Yan, H., Wang, X., Niu, J., Li, J., and Wang, H. (2013a). Inhibiting TGFbeta1 has a protective effect on mouse bone marrow suppression following ionizing radiation exposure in vitro. J Radiat Res *54*, 630-636.

Zhang, H., Zhai, Z., Wang, Y., Zhang, J., Wu, H., Wang, Y., Li, C., Li, D., Lu, L., Wang, X.*, et al.* (2013b). Resveratrol ameliorates ionizing irradiation-induced long-term hematopoietic stem cell injury in mice. Free Radic Biol Med *54*, 40-50.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhao, J.L., and Baltimore, D. (2015). Regulation of stress-induced hematopoiesis. Curr Opin Hematol *22*, 286-292.

Zhao, M., Perry, J.M., Marshall, H., Venkatraman, A., Qian, P., He, X.C., Ahamed, J., and Li, L. (2014). Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med *20*, 1321-1326.

Zhou, B.O., Yue, R., Murphy, M.M., Peyer, J.G., and Morrison, S.J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell Stem Cell *15*, 154-168.

Zon, L.I. (2008). Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. Nature *453*, 306-313.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C.*, et al.* (2008). Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell *3*, 625-636.

Curriculum Vitae

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Education

2009 – Present	Oregon Health & Science University Medical Scientist Training Program Current: 8th year student (3rd year Medical)
2003 – 2009	University of Colorado, Boulder Department of Integrative Physiology Degree: Concurrent BA/MS

Dissertation

Dissertation Title:	Regulation of Endothelial Cell-Dependent Hematopoietic Stem Cell Regeneration
Committee Members:	William H. Fleming MD/PhD (Mentor), Melissa Wong PhD (Chair), Markus Grompe MD, Philip Streeter PhD, Jeffrey Tyner PhD, George Giraud MD/PhD
Department:	Cell Developmental & Cancer Biology Program in Molecular and Cellular Biosciences Oregon Health & Science University
Date of defense:	May 17, 2016

Awards, Fellowships, and Grants

2003	Milton McPike Achievement Award
2003	James D. Farley Scholarship
2005-2007	Undergraduate Research Opportunities Program Research Award, CU Boulder (HHMI funded)
2007	*Summa cum laude* undergraduate honors, University of Colorado, Boulder CO *Research Project: 'Role of calcium-independent phospholipase A_2 in cardiolipin remodeling in the failing rat heart'*
2011-2013	Ruth L. Kirschstein T32 Training Grant in Molecular Hematology (T32HL007781)
2013	Tartar Trust Fellowship, Oregon Health & Science University
2013	Radiologic Society of North America Medical Student Award (RMS1436)
2014-present	Ruth L. Kirschstein F30 National Research Service Award (F30HL123205)

Publications and Presentaitons

Peer-reviewed Journal Articles

1. **Zachman, D.K**.*, Das, P.*, Leon, R.P.*, Goldman, D.C., Hamlin, K.L., Guha, C., Fleming W.H. Endothelial cells mitigate DNA damage and promote the regeneration of hematopoietic stem cells after radiation injury. *Stem Cell Research*. 2013. 11(3): 1013-21. PMCID: PMC4066846. *Equal contribution.
2. Rees, M.L., Gioscia-Ryan, R.A., McCune, S.A., Browder, J.C., **Zachman, D.K.**, Chicco, A.J., Johnson, C.A., Murphy, R.C., Moore R.L., Sparagna, G.C. The AIN-76A diet accelerates the development of heart failure in SHHF rats: a cautionary note on its use in cardiac studies. *J Anim Physiol Anim Nutr*. 2014. 98(1): 56-64. PMID: 23298172.
3. **Zachman, D.K.**, Chicco, A.J., McCune, S.A., Murphy, R.C., Moore, R.L., Sparagna, G.C Role of calcium-independent phospholipase A2 in cardiolipin remodeling in the spontaneously hypertensive heart failure rat heart. *J Lipid Res*. 2010. 51(3): 525-34. PMCID: PMC2817582.
4. Edwards, A.G., Rees, M.L., Gioscia, R.A., **Zachman, D.K**., Lynch, J.M., Browder, J.C., Chicco, A.J. Moore, R.L. PKC-permitted elevation of sarcolemmal KATP concentration may explain female-specific resistance to myocardial infarction. *J Physiol*. 2009. 587(Pt 23): 5723-37. PMCID: PMC2805381.
5. Tsao, F.H., Shanmuganayagam, D., **Zachman, D.K.**, Khosravi, M., Folts, J.D., Meyer, K.C. A continuous fluorescence assay for the determination of calcium-dependent secretory phospholipase A2 activity in serum. *Clin Chim Acta.* 2007. 379(1-2): 119-26. PMID: 17292873.

Abstracts and Presentations

1. **Zachman, D.K.,** Goldman, D.C., Hamlin, K.L., Guha, C., Fleming W.H. Role of hepatocyte growth factor in endothelial-dependent hematopoietic stem cell regeneration. *Blood*. 2014. 124(21): 4369. Abstract.
2. **Zachman DK**, Leon RP, Das P, Goldman DC, Hamlin KL, Guha C, Fleming WH: Endothelial cells mitigate DNA damage and restore function in irradiated hematopoietic stem cells. 55th Annual Meeting of the American Society of Radiation Oncology (ASTRO), Atlanta. Oral Presentation.

Teaching and Leadership Experience

2007	Graduate Teaching Assistant, University of Colorado Boulder, Boulder CO
2008-2009	Professional Research Assistant, University of Colorado Boulder, Boulder CO
2014	Research Mentor, Continuing Umbrella of Research Education (CURE) Intern Program, Oregon Health & Science University, Portland OR
2008-Present	Camp Counselor, Camp Wapiyapi (for children with cancer and their siblings)

Research Experience and Interest

Mechanisms of KATP-dependent cardioprotection from ischemia/reperfusion injury

One way an ischemic myocardium resists injury is by activating ATP-sensitive potassium channels (K_{ATP}), which hyperpolarize the sarcolemmal membrane and limit action potential duration. These channels are in part responsible for Delayed Preconditioning, a

cardioprotective phenomenon that can be induced acutely by a variety of stimuli but sustained only by exercise training or sex-specific levels of 17-β-estradiol. As a part of Russell Moore's group at the University of Colorado, I helped to uncover the role of Protein Kinase C activation in K_{ATP} trafficking from endosomes to the sarcolemmal membrane during ischemia/reperfusion injury using Langendorff perfused rat hearts. This work identified a putative drug target for enhancing KATP function and enhancing myocardial protection from ischemia/reperfusion injury.

Role of cardiac cardiolipin metabolism in the development of heart failure

My major research focus in Russell Moore's lab at the University of Colorado was the role of the mitochondrial membrane phospholipid, cardiolipin, in cardiac ATP production and heart failure. Cardiolipins are unique diphospholipids that are important for the proper functioning of energy transducing membranes. In healthy mammals, over 90% of cardiolipins have all 4 acyl-moieties occupied with the w-6 fatty acid, linoleic acid (18:2). This species of cardiolipin, $(18:2)_4$CL, which is believed to provide an optimal physiochemical environment for the function of electron transport proteins within the inner mitochondrial membrane, is lost in both infantile (Barth Syndrome) and adult heart failure. Because of this, we were interested in the biochemical pathways responsible for the production and degradation of $(18:2)_4$CL. Given my background in studying secretory phospholipase A_2, I sought to determine the role of mitochondrial phospholipases in cardiolipin metabolism. I found that 18:2 incorporation into $(18:2)_4$CL was mediated in part by calcium-independent phospholipase A_2 g (iPLA_2g), and that overall 18:2 incorporation into cardiolipin was attenuated in cardiac myocytes isolated from a rat model of heart failure, relative to non-failing hearts. In an effort to drive 18:2 into cardiolipin and ultimately improve cardiac energetics, we studied the effects of dietary 18:2 supplementation on the progression of heart failure in this rat model and showed that 18:2 supplementation delayed heart failure development but only in the absence of a western-type background diet.

Mechanisms of endothelial-dependent hematopoietic stem cell regeneration

Hematopoietic stem cells (HSC) are exquisitely sensitive to injury by genotoxic agents such as ionizing radiation or chemotherapy. HSC injury suppresses hematopoiesis and can require transplantation, for which allogenic matching is often difficult or limited by the availability of donor HSC. The purpose of my PhD research is to identify treatments that promote HSC regeneration from injury and therefore reduce the need for transplantation. To do this, I am studying the role of the bone marrow microenvironment in supporting HSC. Endothelial cells are essential components of the microenvironment and provide survival signals for injured HSC; however, the specific identities of these signals are largely unknown. To identify endothelial factors that promote HSC regeneration, I first helped develop an *in vitro* system to study endothelial-dependent HSC regeneration. Using this model, we have identified several candidate factors that may regulate HSC survival, repair, and self-renewal. Recently, I have shown that Transforming Growth Factor (TGF)-β1 is a potent suppressor of endothelial-dependent HSC regeneration and Hepatocyte Growth Factor enhances the potential of endothelial cells to regenerate HSC after radiation injury. Furthermore, these factors modulate the function HSC *in vivo* following regeneration and transplantation. In the future I am interested in pursuing the consequences of aging on endothelial-dependent stem cell dysfunction, and whether preventive measures such as diet and exercise are able to improve long-term stem cell function and longevity by improving vascular health.