Activation of Developmental Signaling Pathways in Hematopoietic Stem Cell Regeneration

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

The homeostatic hematopoietic stem cell compartment is comprised of quiescent long term self renewing stem cells and cycling short term stem cells with finite renewal potential. To study the molecular mechanisms governing self renewal of hematopoietic cells we must force the quiescent population to proliferate. One approach to accomplish this goal is to damage the hematopoietic compartment with ionizing radiation or cytotoxic chemotherapy. Such injuries ablate mature blood cells and drive the primitive stem cells into cycle. We have elected to use a simple model of hematopoietic damage and regeneration to study the molecular mechanisms controlling self renewal and regeneration in hematopoietic stem cells. At the beginning of this project it was unclear whether developmental signaling pathways which homeostatically control self renewal are utilized during injury repair. In particular, there is very little understanding of the signals required for regeneration after radiation damage. We hypothesized extracellular signal transduction pathways provided by the microenvironment are critical mediators of the stem cell repair process. To address these topics, we have chosen to pursue a candidate approach focusing on the Wnt and Notch developmental signaling pathways.

In order to examine the activation and requirement for each signaling cascade after radiation and chemotherapy damage we used a combination of loss of function and reporter mouse models. To this end, we have conducted the majority of experiments for the Wnt project in animals deficient in β–catenin, a key transcriptional component required in the pathway. Our investigations revealed the Wnt pathway is turned on
within regenerating stem cells and loss of β-catenin impairs regeneration of the stem cell compartment after both radiation and chemotherapy injury.

Using a Transgenic Notch Reporter mouse to investigate the role of Notch signaling following hematopoietic damage we determined the Notch pathway is also activated during regeneration. Furthermore, using a live imaging approach we discovered Notch activated cells change their fate choice during regeneration. To determine if Notch gain of function provides radio-protection we infected stem cells with an active form of Notch prior to radiation and then scored self renewal potential in vitro. This led us to the conclusion that Notch gain of function can provide a self renewal benefit to irradiated hematopoietic stem cells.

Cumulatively, we have investigated the roles of Wnt and Notch signaling in the process of hematopoietic regeneration. Our results show these pathways are activated by stem cells as a consequence of damage. Functional analysis reveals loss of Wnt impairs the ability of stem cells to proliferate following damage independent of the injury type. We have also determined activation of Notch signaling within stem cells changes their division pattern during regeneration. Our overall model of regeneration involves the coordinated action of both Wnt and Notch inputs provided by the stem cell micro-environment. Further studies into whether other developmental signals impact the regeneration process will provide additional insight into the overall process, and may allow for establishment of a therapeutic intervention to accelerate regeneration.
Dedication

I dedicate this work to my loving parents, Bill and Donna for their many years of sacrifice for my benefit and my brother Robert, for his constant good spirits. I would also like to thank all my friends both near and far, for their unwavering support and continued inspiration.
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1. Introduction

1.1 Overview

In contrast to the majority of somatic cells, stem cells possess the unique ability to develop into a diverse array of differentiated cell types while maintaining a reserve of primitive progenitors. Stem cells have been prospectively identified in a variety of distinct tissue types, including the intestines, skin, muscle and blood [1]. Functionally these cell types have been rigorously tested by a combination of lineage tracing experiments, DNA label retention or transplantation assays. Characterizations of stem cells from other organ systems are under investigation, particularly in the lung and trachea [2]. In the following sections each of these stem cell systems will be reviewed in order to establish some of the common properties known about adult stem cells including the following requirements: a physical niche in vivo, a quiescent population, and self renewal ability. Subsequently, I will provide a brief section focused on embryonic stem cells and iPS cells to highlight the potential of this evolving segment of stem cell biology.

1.2 Adult stem cells and iPS

Intestinal stem cells

Intestinal stem cells (ISCs) were originally postulated to be located at the +4 position of the crypt adjacent to Paneth cells based on label retention studies [3]. More recently however, this view has been expanded by the use of LGR5 transgenic reporter mice that identified crypt columnar cells located at the base of the crypt as ISCs [4].
LGR5 is a G-protein coupled receptor protein and target of Wnt signaling shown to be highly expressed in human colon cancers. As such LGR5 expression serves as a marker for Wnt activity. The LGR5 model of ISC was further supported by lineage tracing experiments that showed all 4 intestinal cell lineages (Paneth, enteroendocrine, goblet and enterocytes) were formed from cycling LGR5+ cells [4]. Clevers and colleagues then went on to develop an in vitro LGR5+ ISC culture system and demonstrated de novo formation of entire crypts-villus organoids from single LGR5+ cells without the need for stromal cell support [5].

Hair follicle stem cells

Another well studied epithelial stem cell is the hair follicle stem cell located in the bulge region adjacent to the hair shaft. Hair follicle stem cells are label retaining cells identified based on a doxycycline inducible histone-GFP expression in the bulge following a chase period [6]. Bulge stem cells are responsible for generating germ cells that migrate down the hair follicle into the dermal papilla during anagen phase of the hair cycle. Germ cells then give rise to transit amplifying cells which generate the hair follicle. After the hair is retracted in catagen phase the follicle rests for telogen phase while the dermal papilla relocates toward the bulge. At the beginning of the next anagen bulge stem cells proliferate to form more germ cells and the cycle repeats. Interestingly, the Wnt target LGR5 is also a marker of hair follicle stem cells, consistent with its association in intestinal stem cells [7]. More recently, LGR6 was identified as a marker
of bulge adjacent cells able to form the sebaceous gland, interfollicular epidermis and the hair follicle itself during embryonic development [8]. However, LGR6 cells lose this potential in adult animals suggesting a dynamic stem cell pool is involved in follicle generation.

*Satellite cells*

Stem cells in muscle, known as satellite cells, constitute nearly 30% of the muscle cell nuclei at birth but diminish to only 5% by adulthood [9]. Satellite cells are located on the surface of muscle myofibers under the basal lamina. While generally a quiescent stem cell population, satellite cells are induced into cycle by both injury and exercise. Proliferating satellite cells produce myogenic precursor cells which can divide before fusing with the existing myofiber. Self renewal in satellite cells was determined *in vitro*, by assessing myotube formation potential. Genetic analysis of this process revealed the transcription factor Pax 7 as a highly upregulated gene involved in the self renewal of muscle satellite cells in embryonic and early postnatal muscle [9]. Strikingly Pax7 deficient animals are unable to specify satellite cells or generate myoblasts. Using a Cre recombinase mediated genetic deletion of the Pax7 gene, it was determined Pax7 is only required for establishment of muscle satellite cells and not for homeostatic muscle function or regeneration after damage [10]. Satellite muscle precursor cells (SMPs) can be isolated by flow cytometry cell based on the surface marker phenotype CD45^-Sca-1^-
Mac-1^CXCR4^β1integrin^+ and are able to integrate into muscle fibers of damaged muscle [11, 12].

More recently, a non-satellite cell muscle progenitor was identified that does not express Pax7 and is required for postnatal muscle growth [13]. These cells termed Pax7-interstitial cells are unable to participate in myogenesis and accumulate at the expense of satellite cells. Interestingly, this population is not derived from a satellite cell lineage. Currently it is unclear how these two different stem cell pools cooperate in the transition from early postnatal muscle development to regulation of adult muscle proliferation.

*iPS cells*

Beyond simply investigating specific tissue resident stem cells, an emerging new field of induced pluripotent stem cells (iPS) through nuclear reprogramming with a cocktail of defined transcription factors (Oct4, KLF4, Sox2 and cMyc) has shown great potential as a therapeutic source of stem cells for treatment of disease or to repair damaged tissue [14]. These same factors also successfully reprogram human cells to the pluripotent state, conferring the embryonic stem cell like properties of continuous self renewal, Nanog expression and remodeled chromatin architecture [14, 15]. This concept shifts the paradigm of conventional stem cell biology and allows for the possibility to directionally differentiate iPS cells into specific lineages of interest.

Since iPS cells were developed a tremendous amount of information about their generation and properties have been learned, and several groups have begun to address
specific diseases with the use of iPS cells. Using a murine model of sickle cell anemia, Jaenisch and colleagues were able to generate and transplant hematopoietic progenitor cells with a corrected β-globin gene to restore normal erythroid function in recipients [16]. To extend this concept Daley and colleagues generated patient-specific iPS cells from individuals with a variety of genetic disorders including muscular dystrophy, Gaucher disease, Huntington’s disease and a variety of other diseases [17]. This will permit investigation into clinical therapies to treat these genetic disorders as well as permit drug screening and autologous stem cell transplants without immune rejection.

One major complication with the iPS system is the potential teratoma formation from iPS cells failing to properly differentiate. To address this issue Yamanaka has tested a variety of iPS cell lines to determine how to distinguish a clone with safe transplantation potential from a clone with teratoma forming properties [18]. Neurospheres derived from various iPS lines were transplanted into NOD/SCID animals to evaluate teratoma formation. The ‘safe’ iPS lines do not form teratomas and are able to generate all 3 neural lineages when introduced into a spinal cord injury mouse model. However, ‘unsafe’ iPS cells form teratomas and do not contribute to neural repair in transplantation, conversely they caused locomotor loss and neural dysfunction. Collectively, these data establish iPS as a new branch of stem cell biology and suggest diseases and injuries could someday be repaired by differentiation of iPS into a specific lineage for transplantation.
1.3 Hematopoietic stem cells and self renewal

Since the discovery of the hematopoietic stem cell by Till and McCulloch in the 1960’s blood forming stem cells have been at the forefront of stem cell biology [19] [20]. Their contribution, the definitive proof of clonality within spleen colony forming units, sparked a massive scientific effort to understand the fundamentals of stem cell function and properties. Nearly fifty years since this discovery the field of hematopoietic stem cell biology flourishes. There are currently several major objectives being pursued by hematopoietic stem cell labs. In broad terms, the goal of HSC research is to understand the cell and molecular biology blood cell differentiation and stem cell self renewal. To this end, intensive research is focused on ontogeny of the hematopoietic system, identifying genes required for self renewal and determining micro-environmental niche contributions that regulate HSC function. Furthermore, by elucidating the basic mechanisms of HSC self renewal we might also better understand how hematologic and other malignancies are propagated, whether it may be driven by a tissue resident stem cell with a mutation such as is found in chronic myelogenous leukemia, or by a progenitor which has reacquired self renewal capabilities or activated renewal signaling pathways such as in AML [21].

Ontogeny of hematopoiesis

Murine hematopoiesis is a complex process involving the yolk sac, aorta-gonad-mesonephros (AGM), fetal liver, bone marrow, spleen and thymus [22]. Hematopoiesis
is first initiated in the mouse yolk sac at E7.5dpc and placenta and AGM regions at E9dpc and E8.5dpc respectively [23]. By E10 the yolk sac, placenta and fetal liver are all colonized by myeloid-erythroid progenitors. Fetal hematopoiesis is mainly responsible for production of fetal erythrocytes throughout development. Progenitors migrate through the fetal blood vessels and colonize the fetal liver. The fetal liver serves as the major location for definitive hematopoietic progenitors by E12, and it retains the greatest number of repopulating stem cells until birth when the bone marrow becomes fully functional [24]. HSCs derived from the fetal liver are marked by a unique set of cell surface receptors which are distinct from those of adult HSCs. The main difference between fetal and adult HSC identification is fetal expression of the AA4.1 antigen and low level of Mac-1 expression (CD11b) [23]. HSCs residing in the fetal liver also express the conventional KLS phenotype and are CD150^+48^244^- [25]. HSCs from fetal liver are multipotent and can therefore reconstitute multilineage hematopoiesis when transplanted into lethally irradiated recipients[25].

The stem cell hierarchy

HSCs are responsible for the daily production of hundreds of millions of new blood cells to compensate for the destruction of old and dying cells. However, in murine bone marrow the long term HSC (LT-HSC) represents only 0.0013% of cells [26]. These long term HSCs are quiescent with less than 2% of cells in the S/G2M phase of the cell cycle based on BrdU incorporation. As HSCs differentiate they lose self renewal potential and
enter the cell cycle to become more proliferative multipotent progenitor cells (MPP). As MPPs progress through stages of increased differentiation they undergo corresponding increase in cycling frequency [26]. It is noteworthy that in this model of HSC to MPP transition the short term HSC (ST-HSC), which does retain limited self renewal potential is considered to be MPP1 stage of differentiation.

Commitment of the ST-HSC yields MMP2 cells which are committed out of the stem cell population. Proliferation of MMP pools ensures there are many progenitors available to sustain homeostatic hematopoiesis. Upon differentiation MPPs can make a fate choice to become either myeloid or lymphoid primed progenitors, the CMP or CLP respectively. Within the myeloid arm of the hematopoietic hierarchy the common myeloid progenitor (CMP) generates two more committed progenitors with increased lineage restriction [27]. The granulocyte-monocyte progenitor (GMP) produces neutrophils and macrophages which are the mainstay of the innate immune system and clear bacterial or viral infections in a nonspecific manner. The other population produced by the CMP is the megakaryocyte-erythroid progenitor (MEP). MEP cells are responsible for the production of platelets and red blood cells (RBCs). In an adult mouse there are approximately $1 \times 10^7$ RBCs per microliter of blood.

Key transcription factors involved in the specification of the myeloid lineage are PU.1, CEBPα, growth factor independent 1 (GFI-1) and interferon regulatory factor 8 (IRF8) [28]. Genes critical for specification of myeloid cells that function at the HSC
level include runt-related transcription factor 1 (Runx1 or AML1), and stem cell leukemia factor (SCL1) [29, 30].

CMP progenitors also generate lymphoid lineage committed cells for producing B-cells and T-cells of the adaptive immune system. The common lymphoid progenitor (CLP) is lymphoid restricted but still multipotent and can give rise to B-cells T-cells and natural killer cells [31]. Interestingly, lymphoid fate can be reversed by exogenous introduction of myeloid cell cytokine receptors IL-2 and GM-CSF in lymphoid restricted progenitors [32]. Furthermore, at the branch point between CMP and CLP, these cytokine receptors are turned off in cells destined for lymphoid lineage to prevent unauthorized lineage switching.

While the HSC-MPP-CMP/CLP linear progression makes logical sense, there are some convincing reports to challenge this model. In particular Adolfsson and colleagues have revised this model to suggest the MEP is directly derived from ST-HSCs [33]. This hypothesis also includes the concept of a lymphoid primed multipotent progenitor with the capacity to form both CLPs and GMPs. Resolution of the true hierarchy of progenitor development will lend great insight into the actual plasticity of stem and progenitor cells. It is also conceivable for each of these progenitors to be found in specific niches that influence their fate choices and proliferative potential. Currently however, there is limited evidence to support this hypothesis for normal hematopoiesis but this phenomenon may occur in the context of hematopoietic malignancy [34].
Hematopoietic stem cell niches

HSCs can differentiate into all of the blood forming and immune cell lineages while perpetuating a population of long-term stem cells. The preponderance of HSCs exist in a resting quiescent state, where they remain in G0 until they enter the cell cycle and choose either a fate of self-renewal or differentiation [26]. In adult animals this process occurs in the bone marrow of the long bones, where HSCs reside within a specialized niche microenvironment. Two competing yet simultaneously supported hypotheses exist on the location of the HSC niche. The first view suggests the bone lining spindle shaped N-Cadherin osteoblast constitutes the adult bone marrow niche [35]. Additional supporting evidence comes from analysis of mutant mouse models defective in osteoblast production such as the parathyroid hormone receptor null mouse [36]. Since the original findings implicating the endosteal osteoblast as the principal niche resident cell type, further examination has bolstered this hypothesis. Evidence supporting the endosteal niche came from studies using fetal liver hematopoietic stem cells deficient in the calcium sensing receptor required for homing to osteoblasts [37]. The fetal liver HSC population is normal however the animals die after birth due to bone marrow hypocellularity. Additional insight into the osteoblast niche came from Weissman and colleagues who prospectively identified the niche resident cell type by FACS sorting CD45⁻Tie2⁻αv⁺CD105⁺Thy1.1⁻ and transplanting these cells into the kidney capsule of adult mice to recreate bone marrow niches [38]. The niche progenitors generated ectopic bone and formed blood vessels which recruited hematopoietic stem cells to form a bone
marrow cavity. In contrast CD45\(^+\)Tie2\(^-\)α\(_v\)CD105\(^+\)Thy1.1\(^+\) progenitors were unable to form a bone marrow cavity in this model.

Niche osteoblasts function by providing factors to regulate HSC quiescence, retain stem cells in the niche, and enhance self renewal. Osteoblasts produce angiopoietin family ligands and thrombopoietin factors to maintain quiescence as well as SDF-1 (stromal derived factor 1 or CXCL12) to retain HSCs within the niche [39, 40]. In response to parathyroid hormone osteoblasts have been shown to up-regulate expression of Jagged ligands to enhance self renewal through mediating the Notch signaling pathway [36, 41]. Niche osteoblasts also help to prevent the excessive accumulation of HSCs by producing inhibitors of self renewal such as the adhesion molecule osteopontin (Opn). Mice deficient for Opn have an increased HSC compartment suggesting the expression of Opn negatively regulates HSC expansion [42].

The alternative hypothesis to the endosteal niche suggests the niche is comprised of sinusoidal endothelial cells. There is also a compelling argument to support this model of the HSC niche. One significant example suggesting osteoblasts are not required as the niche comes from developmental hematopoiesis. As described earlier, primitive hematopoiesis originates in yolk sack, AGM and placenta before seeding the fetal liver. Stem cells from the fetal liver then migrate to the bone marrow prior to birth. Clearly none of these organs contain osteoblast cells to form supportive microenvironments for HSC development, and because definitive HSCs reside in the fetal liver, osteoblasts are not a required constituent at this stage.
Recent additional insights into the vascular niche have strengthened the contribution of endothelial cells to niche biology. Studies to examine how endothelial cells contribute to hematopoietic regeneration after damage identified sinusoidal endothelial cells (SEC) as a critical cell type in bone marrow. While SECs are uniquely marked by the VEGFR3 receptor they are accompanied by a Sca-1^+ marrow arteriole population [43]. Conditional deletion of VEGFR2^+ which marks both SECs and arterioles blocks bone marrow regeneration after sub-lethal irradiation damage. Direct co-culture of bone marrow endothelial cells with LT-HSCs effectively expands stem cells in serum free \textit{in vitro} conditions [44]. This expansion effect was mediated by endothelial expression of Notch ligands as Notch1/Notch2 null HSCs did not expand in this context. \textit{In vivo}, Transgenic Notch Reporter (TNR) stem cells are found localized next to SECs within the bone marrow.

While both the endosteal and vascular niche concepts are still under development it is possible both niches exist \textit{in vivo}. In fact it has been postulated the osteoblast niche is the quiescent niche while the endothelial niche is the active cycling niche. However, there is currently no definitive data to show HSCs actually migrate between niches to enter or exit quiescence. While controversy remains as to what niche supports the HSC and when in its proliferation cycle it may be located at a particular location, it is documented that HSCs reside in a hypoxic environment as determined by perfusion of the Hoechst vital dye [45]. Whether the hypoxic microenvironment for LT-HSCs is to
prevent generation of reactive oxygen species, maintain quiescence or regulate metabolism is still unclear.

**Hematopoietic stem cells**

HSCs can be isolated in several ways, including separation based on expression of a combination of cell surface markers: c-kit receptor and Sca-1 antigen, low levels of Thy 1.1 and the absence of lineage markers for mature hematopoietic cell types (c-kit Thylow linlow/- Sca-1+ termed KTLS) [46, 47]. Cells identified as KTLS represent approximately 0.05% of total bone marrow cells but are functionally able to reconstitute the hematopoietic system of lethally irradiated mice. Residing within the KTLS population are both short term (c-kit+ Thy 1.1low linlow Sca-1+) HSCs which contribute to initial radioprotection via generation of myeloid and erythroid progenitors, and long term (c-kit+ Thy 1.1low lin Sca-1+) HSCs that provide sustained reconstitution and maintenance of hematopoiesis. In mouse strains lacking Thy 1.1, KLS CD34− cells (c-kit+ linlow/- Sca-1+ CD34−) can be used since they contain both short term and long term HSCs. HSC can also be purified based on functional properties such as Hoechst dye efflux by multi drug resistance transporters [48]. More recently, other methods have been developed to purify HSCs from the bone marrow and fetal liver, in particular the SLAM family markers CD150+, CD48−, CD244−. However these are not pan-stem cell markers and are therefore specific to hematopoietic stem cells [25].
The functional assessment of a true LT-HSC is relatively straightforward in vivo. Only a LT-HSC can provide multilineage reconstitution for greater than 16 weeks when transplanted into a lethally irradiated recipient. Short term HSCs and MPP cells can generate multilineage engraftment but fail to sustain consistent blood production indefinitely. To more rigorously test self renewal ability HSCs can be subjected to serial transplantation to determine the how many recipients can be reconstituted before the stem cell pool exhausts[49]. Using transplantation models a limiting dilution assay can be used to calculate the frequency of long term HSC in a given population by determining the engraftment potential of cells transplanted into lethally irradiated mice at various dilutions. Other in vivo assays to gauge self renewal are CFU-S$_8$ and CFU-S$_{12}$ to determine splenic colonies formed 8 or 12 days after transplantation. This is the assay originally utilized by Till and McCulloch to identify the clonality of spleen colony forming cells when they discovered the HSC [20].

**Self renewal**

Self renewal is the process by which a mother cell generates at least one undifferentiated daughter cell, in essence making a duplicate cell without losing self renewal potential or committing to a particular fate. The daughter cells produced in a self renewing division are also able to self renew, providing a mechanism for maintaining a stem cell pool. This unique property allows many tissues to be maintained for the lifetime of an organism. Elucidating the basic requirements for self renewal in HSCs will have
applications in hematopoiesis and cancer biology, and it may provide insight into age related tissue degeneration. For instance, HSCs lose lymphoid potential with age, and the molecular basis of this phenomenon is poorly understood. Recently, separate pools of lineage primed cells were identified within the LT-HSC population, and the expansion of myeloid primed HSCs may contribute to in vivo myeloid skew [50]. New evidence is also emerging to suggest there are also micro-environmental changes occurring within the niche and neutralization of insulin like growth factor 1 can restore youthful hematopoietic function in an aged animal [51].

The molecular mechanisms governing self renewal of HSCs in vivo are intensively investigated. Many specific genes have been identified using loss of function mouse models to assay HSC self renewal. There is a vast spectrum of genes involved in regulating HSC self renewal ranging from chromatin remodeling proteins like the Polycomb group gene Bmi-1, to the c-myb transcription factor [52, 53]. This suggests renewal is controlled at many independent points and is the product of a complex interplay. Microarray analysis of quiescent and cycling HSCs have revealed unique gene expression patterns for each phase of the stem cell, termed the quiescence signature, and the proliferation signature, respectively [54]. Using this approach the authors identified 5 phases of the HSC proliferation cycle following hematopoietic stress induced by 5-Fluorouracil (5-FU) treatment and 3-7 genes associated with each phase.

In addition to HSC intrinsic regulators of self renewal, extracellular signaling pathways also control stem cell fate. The developmental signaling pathways in particular
seem to have a powerful effect on the renewal process. All of the major developmental signaling cascades including BMP/TGFβ, Shh, Wnt and Notch have established roles in HSC renewal [41, 55-57]. Remarkably, these pathways mediate stem cell renewal in multiple stem cell populations independent of tissue type or embryonic germ layer, implying there is a fundamentally conserved requirement for activation of developmental pathways in orchestrating the renewal program. In the next sections an overview of the Wnt and Notch signaling pathways will be provided.

1.4 Signaling pathways and self renewal

The Wnt signaling pathway

As a potent cellular morphogen, Wnt ligands orchestrate tissue specific processes from proliferation and differentiation to cell migration throughout development. The wnt family was originally identified during a Drosophila melanogaster screen conducted by Nusslein-Volhard and Wieschaus to determine genes regulating larval segments and polarity. This screen determined wingless mutants have the appropriate number of segments but incorrect denticle bands (segment polarity mutant) [58]. Shortly thereafter, the mouse mammary tumor virus (MMTV) was determined to integrate into a locus termed Int-1, and cloning of this site led to the identification of Int-1 as a wingless homolog [59]. In cells lines int-1 transduction led to transformation of mammary epithelial cells confirming Int was a proto-oncogene [60].
Wnt acts by binding to the Frizzled receptors and LRP 5/6 co-receptor proteins on the cell surface [61]. In the absence of Wnt signal β-catenin is associated with a large multi-protein destruction complex which includes the scaffold proteins Axin and adenomatous polyposis coli (APC), the serine/threonine kinases glycogen synthase kinase-3β (GSK3-β) and casein kinase Iα (CKIα) [62]. This destruction complex enables GSK3-β and CKIα to phosphorylate β-catenin on a series of four serine and threonine residues, targeting it for ubiquitination by the E3 ubiquitin ligase β-TRCP and degradation by the proteosome [63]. When soluble Wnt ligands bind to the Frizzled-LRP receptors, the intercellular protein Dishevelled (Dvl) is phosphorylated and recruits Axin to the cell membrane, dissociating the β-catenin destruction complex [64]. Thus Axin, APC, and GSK3-β are unable to bind β-catenin and mediate its destruction, resulting in β-catenin stabilization and cytosolic accumulation. Stabilized β-catenin translocates into the nucleus where it binds to members of the TCF/Lef family of transcription factors and relieves transcriptional repression by the TCF/Lef binding protein Groucho, allowing for transcription of Wnt responsive target genes including c-myc and cyclin D1[65] [66].

Wnt function in hematopoiesis

Wnt signaling is a critical component intimately involved in maintaining stem cell self-renewal in multiple stem and progenitor cell populations including intestinal and hematopoietic stem cells [28]. In the hematopoietic system the Wnt pathway functions at
many levels to control lymphocyte proliferation, differentiation and apoptosis, particularly in T cells [67, 68]. Wnt signaling also has a role in myeloid cell function and loss of Wnt signaling impairs chronic myelogenous leukemia development [49].

Wnt/β-catenin signaling also has a well defined role in normal HSC self renewal and proliferation in vitro and in vivo. Retroviral over expression of β-catenin in murine KTLS cells causes significantly increased proliferation compared to vector transduced cells in vitro and provides 100% reconstitution in vivo [57]. Additionally, exogenous treatment of KTLS cells with purified Wnt3a ligand results in a six-fold increase in total cells derived from a single HSC. Conversely, this striking growth and self renewal effect is abrogated with inhibition of the pathway by forced Axin expression. Mouse models with constitutive activation of the Wnt pathway were developed to test the role of Wnt in the stem cell compartment in vivo. Using an Mx1 Cre driven β-catenin S33Y mutant (active) knock in mouse it was shown by 2 groups that continuous Wnt signaling impairs stem cell differentiation and leads to HSC exhaustion [69, 70]. Furthermore the stem cell dysfunction was associated with loss of quiescence and improper gene expression. Other studies of Wnt signaling have revealed a collaboration between Wnt signaling in stem cell proliferation and the Notch pathway in preventing differentiation during execution of a self renewing division [71].

Negative regulators of Wnt signaling are found in the bone marrow to inhibit pathway activation [72]. Bone marrow from SFRP -/- mice have dysregulated hemostasis and increased stem and progenitor cell compartments [72]. Interestingly, specific ligands
for the Wnt pathway perform non-redundant functions in hematopoiesis and HSCs derived from Wnt3a -/- fetal livers harbor an irreversible self renewal defect [73]. Direct ablation of β-catenin in HSCs results in normal homeostatic peripheral blood and bone marrow but impaired stem cell self renewal after transplantation [49].

In addition to the canonical Wnt pathway outlined above, some Wnt ligands can signal through β-catenin independent pathways including the Ca\(^{2+}\) release pathway, and the Drosophila planar cell polarity pathway [74]. In these non-canonical pathways, Wnt ligand binds to Frizzled receptors but GSK-3β is not inhibited to stabilize β-catenin. Instead the non-canonical Wnt pathways either trigger Ca\(^{2+}\) release to activate the CaMK pathway or activate RhoA and Rac to control the cytoskeleton, cell polarity and cell migration. The non-canonical Wnt5a is reported to induce self renewal of both ST and LT-HSCs by maintaining cells in G\(_0\) phase of the cell cycle [75].

Bone marrow microenvironment stromal cell types and stem/progenitor cells produce canonical and non-canonical Wnts [76]. The Wnt ligands produced by bone marrow stromal cells and mature hematopoietic cells include Wnt2b, Wnt5a and Wnt 10b [77]. Thus the outcome of stem cell division is the product of multiple inputs from canonical and non-canonical Wnt ligands and Wnt inhibitory factors. Collectively, Wnt signaling is a crucial pathway throughout development that functions in cell fate determination and cell proliferation. Furthermore the canonical Wnt/β-catenin pathway has a powerful influence in directing self renewal in HSCs and is available within the bone marrow. Specifically how Wnt signaling is involved in hematopoietic regeneration
after injury is unclear, but given the many functional roles of Wnt throughout hematopoiesis it is a possible candidate for contributing to the overall process or regeneration at the stem and progenitor cell levels.

*The Notch signaling pathway*

The Notch signaling cascade is an evolutionarily conserved pathway with diverse roles in cell fate specification, development and malignancy. The Notch pathway was originally identified in the early 1900’s in mutant Drosophila and was named Notch based on the aberrant wing phenotype of mutant flies. In mammals, there are four single-pass transmembrane cell surface Notch receptors which are activated by direct contact with integral membrane ligands of the Delta/Serrate/Lag2 family [78]. At the cell surface both receptors and ligands are subject to extracellular glycosylation by the glycosyltransferase fringe, and this modification inhibits Notch activation by Delta but not Serrate ligands [79]. This receptor-ligand interaction initiates a series of proteolytic cleavage events to produce an intracellular Notch fragment (ICN), which then translocates to the nucleus and associates with the transcriptional repressor CBF-1 (also known as CSL) [80]. When the ICN-CBF-1 complex associates with the transcriptional co-activator mastermind like 1 (MAML1), CBF-1 is converted into a transcriptional activator and drives expression of Notch target genes including Hes/Hey family transcription factors, c-myc and deltex1.
Notch signaling is regulated at several levels, beginning with processing of the Notch protein in the Golgi to form the active Notch heterodimer. Following receptor-ligand interaction, ligand endocytosis by the ligand presenting cell produces a conformational shift in the Notch receptor to allow access of two sequential proteases, TACE (TNF-α converting enzyme) and γ-secretase, which are required for liberation of ICN [81]. Intercellular regulation of Notch is mediated by the E3 ubiquitin ligase deltex and the proteins numb and numb-like. Numb has been shown to directly bind to the intercellular domain of the Notch receptor and recruit α-adaptin, a protein involved in receptor mediated endocytosis [82].

Functionally, activation of Notch signaling is involved in lateral inhibition and cell fate choice throughout development. In Drosophila sensory organ precursor cells that form sensory hairs, Numb asymmetric distribution regulates Notch activation and thus cell fate [83]. Loss of Numb in SOP completely alters the development of the sensory bristle and no neuron or sheath cell forms.

_Notch function in HSCs_

Notch signaling effects HSC self renewal and the specification of terminal cell lineages vertebrates. In zebrafish, the Notch-Runx pathway specifies adult HSCs and Notch mutants fail to establish adult stem cells [84]. Studies of Notch function using a Transgenic Notch Reporter (TNR) mouse to drive GFP expression in cells actively signaling through the Notch pathway, identified a reporter positive stem cell population.
in vivo [71]. Furthermore, reporter positive cells form significantly more CFU-S_{12} colonies in vivo and perform more self renewal divisions in vitro suggesting Notch active HSCs have enhanced self renewal compared to reporter negative HSCs [85]. How the specific expression levels of genes regulated by Notch are regulated during a self renewal fate or turned off during a stem cell commitment remain unknown. Interestingly, adult deletion of CSL does not impact stem cell function in transplantation suggesting the Notch signal is dispensable or effectively compensated for in vivo [86].

Clinically, activation of the Notch pathway in culture with a chimeric Delta-IgG fusion protein agonist has successfully been applied to cord blood units prior to transplantation. This culture approach can yield up to a 400-fold expansion of progenitors prior to transplantation and improved engraftment of CD34^{+} cells in both NOD/SCID xenografts and human patients [41].

**Notch in lineage differentiation**

Notch signaling in T-cell development is well documented, and Notch mutations are found in 50-70% of human T-ALLs [87]. Retroviral expression of Notch ICN induces CD4^{+}CD8^{+} T-ALL and suppresses B-cell development but does not impair myeloid production [88]. This data indicates Notch provides an instructive signal to produce immature T-cells at the expense of developing B-cells. In the thymus Notch regulates the transition from CD4^{-}CD8^{-} double negative (DN) stage 2 to DN3 [89]. Notch appears to also have a role in specifying megakaryocytes directly from HSCs in vitro when cultured
with OP-9 Delta like 1 stromal cells [90]. The development of CD41+ cells in these cultures could be inhibited with transduction of dnMAML or treatment with the γ-secretase inhibitor Compound E. Collectively, this data demonstrates the diverse roles of Notch signaling throughout the hematopoietic system, from stem cell self renewal to fate specification of terminally differentiated blood cells. Despite the existing data suggesting Notch is involved in many aspects of cell fate and self renewal, the role for Notch in hematopoietic regeneration is unknown. Thus we are interested in pursuing this pathway as a potential candidate for regulating regeneration.

1.5 Radiation and chemotherapy damage in hematopoiesis

Radiation

The hematopoietic system can be injured with toxic chemotherapeutic agents or ionizing radiation. Both chemical and radiation injury destroy proliferating progenitor cells and mature cycling cells of the hematopoietic system. Following 2-8 Gray (Gy) irradiation exposure, damage to the hematopoietic compartment can be expected. In the low dose ranges pancytopenia and anemia may occur and at higher dosages (4-8Gy) peripheral blood and HSC injury occur with significant pancytopenia, marrow aplasia and spontaneous bleeding [91]. Although little is understood about the molecular mechanisms regulating hematopoietic regeneration, the basis of long-term damage to the stem cell pool has been identified. Ionizing radiation specifically affects LT-HSC self-renewal
through persistent expression of the cell senescence markers p16\(^{\text{INK4A}}\), SA-\(\beta\)-gal, p21 and p19\(^{\text{Arf}}\) which collectively inhibit complete regeneration of the normal HSC pool [92].

Radiation damage to the stem cell compartment is reversible in some contexts. For example, \textit{ex-vivo} culture strategies have shown lethally irradiated bone marrow can be expanded in co-culture with endothelial cells to provide long-term hematopoietic reconstitution in a competitive transplant [93]. One \textit{in vivo} approach to protect HSC function after radiation was recently identified as a soluble factor produced by supportive endothelial cells. Administration of the recombinant protein pleiotrophin following TBI restores stem cell transplantation engraftment, suggesting self renewal potential remains in irradiated HSCs if up-regulation of senescence associated genes can be prevented [94].

Other approaches to accelerating regeneration following radiation exposure are limited in TBI settings. Agonists to the Toll-like receptor 5 such as peptides derived from bacterial flagellin can activate the nuclear factor-\(\kappa\)b signaling and prevent the damage of lethal radiation [95]. Other less mechanistically understood approaches have been identified to rescue lethally irradiated animals. For instance, administration of IL-12 prior to lethal radiation protects animals from death, but the IL-12 receptor is lacking on HSCs [96]. Genetic approaches such as over expression of the anti-apoptotic gene BCL-2, is sufficient to confer resistance to lethal radiation exposure by permitting survival of the stem cell pool [97].
Chemotherapy

An alternative approach to creating a hematopoietic damage and regeneration is to treat the bone marrow with systemic chemotherapy such as cyclophosphamide or 5-FU. Chemotherapeutic damage is sufficient to ablate the bone marrow and drive hematopoietic progenitors into cycle [54]. In contrast to radiation, chemical injury to the hematopoietic compartment can be regenerated in less than 2 weeks, and HSCs from chemical ablation are functional in transplantation. Thus we have two clinically utilized treatment options available to study hematopoietic regeneration. Using these complementary approaches will allow us to use the best experiment to accomplish our research goal of understanding the molecular signaling cascades activated during regeneration.
2. β-catenin is required for hematopoietic stem cell regeneration

2.1 Introduction

Hematopoietic stem cell (HSC) transplantation is a clinically utilized therapy for a variety of hematological malignancies and autoimmune disorders. Recipient preparation prior to transplantation requires delivery of a lethal radiation dose, which is administered as a series of sub-lethal fractionated doses totaling 1200-1350cGy. This conditioning approach ablates the immune system and provides bone marrow space for the donor graft to expand. Presently, there are few clinical options for enhancing bone marrow expansion after transplantation and current G-CSF cytokine therapy has only marginal success at improving engraftment [98-100]. Despite the frequent usage of radiation in clinical therapies, the kinetics of endogenous hematopoietic stem cell regeneration after radiation damage are poorly defined, and the overall molecular mechanisms of stem cell expansion following injury are unknown.

The current molecular understanding of radiation biology in HSCs is principally focused on identifying radiation protection mechanisms through inhibition of apoptosis and examining gene expression changes following radiation damage. Early studies demonstrated loss of the p53 tumor suppressor effectively inhibits radiation induced apoptosis [101]. Gain of function approaches using constitutive expression of the anti-apoptotic BCL2 proto-oncogene have shown this strategy also provides radiation
resistance in an HSC cell autonomous manner [97]. More recently, a role for the BH3-only protein Puma was identified as a key initiator of apoptosis in HSCs after total body irradiation [102]. It has also been reported that radiation increases expression of the senescence associated gene p16 specifically in the HSC compartment [92]. Currently however, micro-environmental contributions that affect HSC radiation resistance and subsequent regeneration within the stem cell niche are unknown. Therefore, identification of signaling pathways activated by the either the irradiated microenvironment or produced stem cell autonomously would provide therapeutic targets to exogenously accelerate the regeneration process.

Activation of the Wnt signaling pathway is one potential candidate for enhancing regeneration. The Wnt pathway has been identified as a key regulator of regeneration in multiple injury contexts and in diverse organ systems. Production of Wnt 7b ligands by tissue resident macrophages enhances epithelial regeneration in a kidney ischemia reperfusion model of damage [103]. In skin Wnt ligands are expressed during de novo hair follicle regeneration from epithelial cells in full thickness skin excisions [104]. Furthermore, β-catenin activity within the dermal papilla regulates the regeneration of the hair shaft and β-catenin deletion inhibits stem cell cycling in the niche [105]. In addition to the reports suggesting Wnt is involved in modulating mammalian regeneration, the Wnt pathway is involved in regeneration in other genetic organisms. In a zebrafish model of fin amputation Wnt8 increases proliferation of progenitor cells, and regeneration occurs faster in Axin1 heterozygous animals which have hyper-activated Wnt signaling.
β-catenin mediated Wnt signaling also regulates limb regeneration in adult axolotl and chick embryo forelimb [107]. While suggestive of a role for Wnt in general regeneration, there are currently no reports that provide direct genetic evidence of a requirement for β-catenin in mammalian hematopoietic regeneration.

To determine if consistent loss of the Wnt pathway has functional consequences in regeneration we examined a genetic β-catenin deletion mouse model. Irradiated HSCs isolated from β–catenin null animals have impaired proliferation and self renewal. Furthermore, fractionated radiation damage induces bone marrow hypocellularity and reduces the stem cell compartment in β-catenin null animals. Finally, we investigated whether Wnt signaling is broadly required in hematopoietic regeneration using a chemotherapeutic model of 5-Fluorouracil induced injury. Importantly, we find β-catenin is required for maintenance of the stem cell pool and for normal regeneration after 5-FU injury. Collectively, our data suggest the Wnt pathway may have a general role in mediating regeneration. Thus the Wnt pathway may serve as a potential candidate for enhancing or accelerating HSC regeneration in immune compromised individuals or bone marrow transplant recipients.

2.2 Materials and Methods

Mice

The loxP-β-catenin and Vav-cre transgenic mice used were in the C57Bl/6J background. All mice were bred and maintained on acidified water in the animal care
facility at Duke University Medical Center. 5-FU was injected inter-peritoneal at 120mg/kg in PBS. Radiation was delivered by a Shepherd Cs\(^{137}\)\(\gamma\)-iradiator at a dose rate of 6Gy/minute. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

**Isolation and FACS of HSCs**

HSCs were sorted and analyzed from mouse bone marrow based on surface marker expression of c-Kit and Sca-1, and low to negative expression of lineage markers (Lin) and CD34 using an antibody cocktail as previously described [49]. All antibodies were purchased from Pharmingen or eBioscience. Analysis and cell sorting were carried out on a FACS Vantage (Becton Dickinson) at the Duke Cancer Center FACS facility. For whole bone marrow analysis 1x 10\(^6\) cells were stained with KLS and CD150 and CD48 as described [108]. Annexin -V apoptosis assays were performed by staining cells with annexin-V and 7-AAD (BD, Biosciences).

**In vitro proliferation and methylcellulose assays**

For liquid culture freshly purified KLS34\(^-\) cells were plated in medium (XVivo15, BioWhittaker) supplemented with 50 \(\beta\)M 2-mercaptoethanol, 2\% FCS and SCF (50 ng/ml) and Flt-3 (30ng/ml). After culture cells were counted for total live cells by trypan blue exclusion. For methylcellulose assays, 500 KLS34\(^-\) cells were plated in complete methylcellulose medium (StemCell Technologies, catalog number M3434). Colony
numbers were initially counted 8-10 days after plating. Subsequently cells were harvested, counted and 5000 cells were replated into new 12-well plates.

*Immunofluorescence staining of cytospins and bone sections*

Freshly purified KLS34<sup>-</sup> cells were sorted by FACS and cytospins were fixed in 4% paraformaldehyde for 20 minutes. Slides were then washed in PBS +0.1% Tween, blocked with 20% normal goat serum and stained with mouse anti-active β-catenin (Upstate) or mouse IgG isotype control (BD Pharmingen). Slides were washed in PBS-T and stained with Donkey anti-mouse IgG-TRITC or IgG-Alexa Fluor 488 (Jackson Immunoresearch) (Invitrogen) and DAPI (Molecular Probes). Confocal Images were taken with a Zeiss 410 Axiovert Microscope.

For bone staining fresh bone specimens from control and irradiated mice were decalcified, infiltrated with sucrose, and embedded in OCT medium. Frozen sections were fixed in acetone, washed in PBS-T and blocked with 20% normal donkey serum (Jackson Immunoresearch). Sections were stained with Anti-Wnt10b (Santa Cruz) or the appropriate isotype control followed by Donkey anti-goat Alexa Fluor 594 (Invitrogen) The nuclear dye DAPI (Molecular Probes) was included in all stains.

*Statistical analysis*

Two-tailed student’s t-test and 1-way ANOVA were utilized to determine statistical significance. *P* values less than 0.05 were considered significant.
Cohorts of adult BA mice were given a single 4.5Gy radiation dose and bled from the sub-mandibular vein at the indicated time point. Control mice were mock irradiated before bleeding. One way ANOVA p<0.0001 for WBCs, lymphocytes and neutrophils, p=0.016 for platelets.
2.3 Results

Development of a radiation regeneration model of hematopoiesis

To better understand the regeneration kinetics following sub-lethal radiation damage we examined peripheral blood regeneration by automated complete blood cell count (CBC) after a single 4.5Gy γ-irradiation exposure. Our data indicate the 4.5Gy injury requires approximately 90 days to complete repopulation of total white blood cells (Figure 1).

We also analyzed whole bone marrow cell counts and determined restoration of whole bone marrow requires approximately 40 days (Figure 2). Finally we evaluated the regeneration of the stem cell compartment directly by analyzing lin− ckit+ hematopoietic progenitors and KLSCD34− (KLS34−) stem cells by flow cytometry (Figure 3). The stem and progenitor cell pools are significantly reduced compared to controls at day 7 post injury. A statistically significant expansion in progenitor cells occurs between day 7 and day 21. The KLS34− population regenerates differently than the progenitor lin− ckit+ population and after day 21 both the frequency and absolute number of KLS34− cells are reduced consistently until day 180.
Cohorts of adult BA mice were given a single 4.5Gy radiation dose and sacrificed at the indicated time point. Control mice were mock irradiated before sacrifice. Top panels: representative H&E staining of femur sections from mice after 4.5Gy. Bottom: Whole bone marrow cellularity from 4 long bones of the legs. n=3-9 mice per group. One way ANOVA p<0.0001.
Mice were irradiated with 4.5Gy and sacrificed at the indicated time points. FACS profile of whole bone marrow pregated on live lin' CD34' cells (A). Quantitation of lin' CD34' ckit+ hematopoietic progenitors in (B), and KLS stem cells panel (C). ANOVA p<0.001 for both KL and KLS.

Figure 3: KLS34- stem cell regeneration time course following 4.5Gy
While we now understand the kinetics of stem and progenitor cell regeneration in the 4.5Gy model the mechanism for this expansion is unknown. In order to address how KLS34⁻ stem cells respond to radiation we analyzed cell cycle status of regenerating HSCs. Irradiated KLS34⁻ cells are significantly reduced in the frequency of G0/G1 and significantly increased in actively cycling cells in both the S-phase and G2-M phases from days 7-30 post injury (Figure 4).

Collectively, we have examined how radiation damage and the subsequent hematopoietic regeneration affect bone marrow stem and progenitor cell content. Our data demonstrate there is only a partial regeneration of KLS34⁻ cells injured bone marrow and accordingly there is a loss in absolute numbers of KLS34⁻ stem cells as a result of sub-lethal radiation injury. However, in contrast to the stem cell compartment, lin⁻ckit⁺ progenitor cells regenerate to control levels. Our model also suggests that peripheral blood is the last component to complete regeneration. This regeneration model will allow further investigation of the signaling events activated after injury.

Irradiation injury activates Wnt signaling in hematopoietic stem cells

With the basic radiation injury model described above available for examining stem cell regeneration questions we next tested whether conventional self renewal mechanisms were involved in the repair process.
KLS34- stem cells were sorted from irradiated animals at the indicated time points. Cell cycle analysis was performed using propidium iodine staining for DNA content (A). Quantitation of the G₀ and S-G₂-M phases of the cell cycle at the indicated time points (B). One- way ANOVA, p< 0.001 for the G₀ and S-G₂-M.
Many previous studies have implicated the Wnt pathway in mediating homeostatic stem cell renewal [57, 69, 70, 109]. To test if canonical Wnt signaling is relevant to regeneration we examined β-catenin stabilization in purified KLS34− cells from irradiated bone marrow 7 days after a single 4.5Gy irradiation injury. Staining for non-phosphorylated nuclear β-catenin indicated irradiation induces an increase in the frequency of β-catenin positive HSCs (Figure 5). To determine whether irradiation affects Wnt signaling pathway components in the bone marrow we also analyzed expression of Wnt ligands in bone marrow sections 7 days after damage (Figure 6). This analysis revealed increased expression of the canonical Wnt ligand Wnt10b which is consistent with previous work showing induction of Wnt10b ligands in the bone marrow following administration of the chemotherapeutic agent cyclophosphamide [77]. Recently Wnt10b was also shown to be expressed by tissue resident macrophages during kidney regeneration [103]. Staining for other Wnt ligands such as the canonical Wnt3a ligand and the non-canonical Wnt5a showed no change in expression by radiation at 7 days post injury (data not shown). These data suggest the canonical Wnt pathway is regulated by the microenvironment during regeneration and HSCs activate Wnt signaling as a consequence of increased ligand production.
Figure 5: Regenerating HSCs activate the Wnt signaling pathway

HSCs were sorted from 4.5Gy irradiated animals 7 days after injury and cytospin preparations were stained for activated (non-phosphorylated) β-catenin. Cells were also stained with the nuclear dye DAPI to facilitate co-localization of β-catenin with the nucleus.
Figure 6: Radiation induces expression of Wnt10b in the bone marrow

Bone marrow sections taken from irradiated femurs were stained for expression of the canonical Wnt10b ligand 7 days after 4.5Gy.
Loss of β-catenin impairs stem cell function in vitro after radiation

In order to address whether Wnt signaling has an impact on stem cell self-renewal after irradiation injury we utilized a loss of function mouse model in which β-catenin exons 2 and 6 are flanked by loxP sites [110]. β-catenin<sup>fl/fl</sup> mice were crossed to a mouse strain driving Cre recombinase under control of vav regulatory elements. Vav-Cre has been shown to effectively mediate deletion of floxed sequences in the hematopoietic compartment, including the primitive lin<sup>-</sup>ckit<sup>+</sup> and lin<sup>-</sup>Sca-1<sup>+</sup> fractions [111, 112] We have also specifically confirmed by PCR that β-catenin deletion occurs efficiently with vav-Cre in KLS cells harvested from β-catenin -/- mice [49].

To functionally test for the requirement of β-catenin in HSC regeneration we performed cell proliferation experiments with irradiated cells 90 days after 4.5Gy. KLS34<sup>-</sup> cells from 4.5Gy irradiated control and β-catenin-/ - animals were plated in liquid media and cultured for 5 days and then counted and analyzed by flow cytometry for KLS phenotype. This experiment revealed a defect in β−catenin-/ - HSC proliferation in vitro (average WT 79,000 ± 12,000, β-catenin<sup>-/-</sup> 33,625 ± 1,712  p < 0.01) (Figure 7) and an associated failure to maintain the KLS phenotype. To more rigorously assay self renewal of irradiated β-catenin-/ - HSCs we sorted KLS34<sup>-</sup> cells from control and β-catenin-/ - animals for colony formation in myeloid methylcellulose culture. Colony formation was equivalent in the primary culture but reduced in the secondary
KLS cells were sorted from 4.5Gy irradiated mice 30 days after 4.5Gy and cultured in vitro with 30ng/mL each SCF-1, Flt-3 with 2% serum in X-Vivo media. FACS profile of day 5 cultured cells stained for KLS. Cells are pregated on lineage negative cells (A). Total cells derived from 5000 KLS cells after 5 days in culture (B). p<0.05

**Figure 7:** β-catenin -/- stem cells have a proliferation defect *in vitro*
plating, indicating self renewal dysfunction in β-catenin/- HSCs (average colony WT 45.0 ± 1.44, β-catenin/- 26.4 ± 3.2 p< 0.01) (Figure 8). No specific changes in colony type were evident between colonies derived from control or β-catenin/- cells, suggesting no lineage specific bias caused the reduction in colony number. We examined methylcellulose colony forming potential in serial plating of un-irradiated control or β-catenin/- KLS34 cells to determine if any baseline dysfunction exists in β-catenin/- cells. However, we did not observe any significant differences in colony formation in three rounds of serial plating from un-irradiated KLS34 cells (data not shown). In addition to specifically assaying cell autonomous stem cell function we also analyzed peripheral blood composition by complete blood cell counts after radiation. β-catenin/- animals did not show any significant difference in total white blood cells, lymphocytes, neutrophils, red blood cells or platelets between days 0 and 60 after a single 4.5Gy irradiation dose (data not shown).

We also analyzed apoptosis in control and β-catenin/- KLS cells 24 hours after 4.5Gy by FACS with Annexin-V and propidium iodide (PI) and found no differences in apoptosis (Annexin+ PI) or necrosis (Annexin’ PI+) (Figure 9). We further investigated whether radiation alters the development of double stranded DNA breaks in β-catenin/- HSCs by examining γ-H2A.X foci in 4.5Gy irradiated KLS34 cells 90 minutes after damage (data not shown). Foci development for control and β-catenin/- cells was not
Irradiated KLS cells were sorted from control and β-catenin -/- mice 30 days after 4.5 and 500 cells were plated in methylcellulose media. Colonies were counted on day 7, and cells were replated at 5000 cells per well. Colonies were counted on day 10 of the secondary plating. p<0.05.
Whole bone marrow was harvested from 4.5Gy irradiated control and β-catenin -/- animals 24 hours after injury. Cells were stained for KLS with Annexin-V/ PI to determine frequency of apoptosis in KLS cells by FACs analysis (A). Quantitation of apoptotic cells, (Annexin V + PI -), necrotic cells (Annexin-V - PI +), and dead cells (Annexin-V + PI +) are shown. p =NS for each comparison.

Figure 9: Loss of β-catenin does not alter apoptosis frequency

Whole bone marrow was harvested from 4.5Gy irradiated control and β-catenin -/- animals 24 hours after injury. Cells were stained for KLS with Annexin-V/ PI to determine frequency of apoptosis in KLS cells by FACs analysis (A). Quantitation of apoptotic cells, (Annexin V + PI +), necrotic cells (Annexin-V + PI +), and dead cells (Annexin-V + PI +) are shown. p =NS for each comparison.
statistically different suggesting β–catenin is not required for maintenance of genomic integrity following irradiation injury. In summation, we find functional hematopoietic impairment from β-catenin-/− HSCs when tested in a cell autonomous manner in vitro, but no changes in peripheral blood regeneration in vivo after a single sub-lethal dose of radiation. Therefore, we conclude that stem cell self renewal is impaired in irradiated β-catenin-/− KLS34− cells, but this impairment can be sufficiently compensated for in vivo after a single injury.

**β-catenin is required for hematopoietic regeneration following serial radiation injury**

While β-catenin appears to be required in the hematopoietic stem cell function and not overall peripheral blood regeneration in a single 4.5Gy radiation model, it does not preclude the possibility that β-catenin may have an important role under repeated stress conditions. Therefore we employed a multiple dose irradiation strategy to determine whether β-catenin was required for regeneration following two separate 4.5Gy irradiation injuries on day 0 and day 14. Using this approach we discovered a significant reduction in the total bone marrow cellularity of β-catenin −/− animals compared to controls (Figure 10). Morphologic analysis of bone marrow sections by hematoxylin and eosin at this time showed reduced cellularity throughout (Figure 10). Examination of the KLS34− compartment also revealed a
Cohorts of control and β-catenin -/- mice were irradiated with 4.5Gy on day 0 and day 14 and then sacrificed on day 28. β-catenin -/- mice have reduced total bone marrow cellularity (A) p=0.0069. H&E sections from humorous bones of control and β-catenin -/- mice after serial radiation (B).

**Figure 10: β-catenin -/- mice have impaired bone marrow regeneration**

Cohorts of control and β-catenin -/- mice were irradiated with 4.5Gy on day 0 and day 14 and then sacrificed on day 28. β-catenin -/- mice have reduced total bone marrow cellularity (A) p=0.0069. H&E sections from humorous bones of control and β-catenin -/- mice after serial radiation (B).
KLS34- whole bone marrow analysis from 2x4.5Gy irradiated animals on day 28. FACS analysis of β-catenin -/- stem cells from bone marrow (A). Quantitation of lin-ckit+ cells and KLS cells (B). The frequency of progenitors is not significantly different. KLS34 p= 0.011, n= 6 mice per group.

Figure 11: Regeneration of β-catenin -/- stem cells is impaired
dysfunction in the regeneration of stem cells but not of progenitor lin-ckit+ cells (Figure 11). In conclusion, total bone marrow cellularity as well as progenitor and stem cell absolute numbers are decreased in vivo. Additionally, we find the frequency of KLS34-stem cells is reduced in β-catenin /- animals using a serial radiation model, suggesting a failure to replenish the stem cell pool has occurred.

Whether the bone marrow and stem cell compartment were the only hematopoietic parameters affected by loss of β-catenin was unclear. Therefore we performed histological analysis of β-catenin/- spleens and found a loss of normal follicle architecture/germinal centers and concomitant reduction of red pulp (Figure 12). Additionally, β-catenin/- spleens showed megakaryocyte islands, which are indicative of splenic extramedullary hematopoiesis [113]. No splenomegaly was observed in β-catenin/- spleens. Megakaryocytes were sporadically detected in the spleens of control irradiated mice but overall splenic structure was not disrupted.

Analysis of peripheral blood by automated CBC revealed no changes in any of the peripheral blood parameters from β-catenin/- mice at day 28 (data not shown). At day 75 however, β-catenin /- animals exhibited a latent defect in peripheral blood platelets, p< 0.01 (Figure 12).
β-catenin -/- spleens lack normal splenic architecture, and exhibit signs of extramedullary hematopoiesis such as megakaryocyte islands 28 days after radiation (arrows) (A). Platelet count from peripheral blood of irradiated mice at day 75 (B), p=0.01.

Figure 12: β-catenin -/- spleens lack follicles and show extramedullary hematopoiesis
β-catenin -/- mice fail to maintain a long term KLS150^+48^ stem cell pool after serial treatment with 5-FU.

Our serial radiation injury experiments suggested loss of β-catenin impaired stem cell function and bone marrow regeneration. However, we do not know if β-catenin is required exclusively for radiation mediated repair or whether the Wnt pathway is required for regeneration after other types of injury. To test this hypothesis and to exclude a specific radiation mediated phenotype in β-catenin -/- animals, we examined hematopoietic regeneration after administration of the chemotherapeutic drug 5-Fluorouracil (5-FU). 5-FU is a clinically utilized myelosuppressive agent that effectively ablates proliferating hematopoietic progenitor cells and induces HSC expansion and regeneration [54]. Control and β-catenin -/- animals were treated with a single dose of 120mg/kg 5-FU and peripheral blood regeneration was monitored by CBC at several time points between 7-21 days after injury. Consistent with the single irradiation injury model, there was no evidence of regeneration delay in the peripheral blood, bone marrow, or stem cell compartment at any time point after single 5-FU injury (data not shown). However, methylcellulose colony forming assays from isolated KLS34^- cells harvested from 5-FU treated 7 days after injury showed a significant reduction in colony production from β-catenin -/- cells (Figure 13). Furthermore, consistent with the previous findings
Figure 13: Methylcellulose colony formation from 5-FU treated KLS

500 KLS34- cells were sorted from mice 7 days after injection of 120mg/kg 5-FU. Colonies were counted on day 7 and re-plated at 5000 cells per well. Secondary colonies were counted on day 10. p<0.05, n=3.
from our radiation injury model, we observed Wnt10b ligand expression in the bone marrow of 5-FU treated animals 7 days after injury (data not shown).

To test if stem cell self-renewal dysfunction precedes regeneration delay after 5-FU treatment we expanded the 5-FU injury model to include treatments on day 0 and day 7 and analyzed bone marrow and LT-HSC compartment at day 14. Using this approach we found a significant decrease in total bone marrow cellularity of β-catenin -/- animals (Figure 14). To evaluate the stem cell compartment we stained whole bone marrow for KLS150^48^- LT-HSCs. The combination of conventional KLS staining combined with SLAM family markers highly enriches bone marrow for LT-HSCs [26, 114] The frequency of KLS or KLS150^48^- LT- HSCs is not different control and β-catenin -/- bone marrow (Figure 15). However, the absolute number of KLS150^48^- LT-HSCs, KLS150^48^+ multipotent progenitor 2 and KLS150^48^+ multipotent progenitor 1 populations are significantly decreased in β-catenin -/- bone marrow (Figure 16). [108]. Cumulatively, these data demonstrate bone marrow dysfunction in the absence of β-catenin and show Wnt signaling is required from maintenance of the stem and progenitor cell pools in vivo.

β-catenin is required for hematopoietic regeneration after 5-FU serial treatment
Figure 14: β–catenin is required for bone marrow regeneration after 5-FU

Bone marrow cell counts following serial 5-FU treatments (left). p=0.017, n=4. Representative humorous bone marrow sections stained with H&E (right).
Homeostatic peripheral total white blood cell counts are normal in β-catenin −/− mice and a single 5FU treatment does not impair white cell regeneration. However, serial treatment with 5-FU caused a stem cell pool deficit in β-catenin −/− bone marrow in the LT-HSC and multipotent progenitor compartments. To determine if the stem cell defect caused by two 5-FU treatments impaired peripheral blood regeneration, we also analyzed control and β-catenin −/− mice peripheral blood by CBC after two treatments with 5-FU. We analyzed blood from control and β-catenin−/− mice on day 14 after 5-FU treatment on day 0 and day 7. This serial stress approach revealed a multi-lineage defect in β-catenin −/− mice. Peripheral blood from β-catenin−/− mice showed significant reductions in mature cells, including total white cells and platelets (Figure 17).

We further extended the serial stress model to include 3 consecutive weekly 5-FU treatments on days 0,7 and 14 in accordance with a previously established protocol and monitored survival of control and β-catenin−/− mice [115]. Compared to control animals, a significant number of mice died in β-catenin−/− cohorts after 3 5-FU treatments (Figure 18). In summation, β-catenin mediates the regeneration of all aspects of hematopoiesis including the primitive stem cell compartment, total bone marrow cellularity and peripheral blood following serial 5-FU injury.
Whole bone marrow from 5-FU serially treated mice and stained for KLS and SLAM family markers. The frequency of KLS150^+^48^-^ stem cells or MPPs KLS150^+^48^+^, KLS150^+^48^-^ are not significantly different.

Figure 15: Analysis of KLS150 stem cells in 5-FU treated bone marrow

Whole bone marrow from 5-FU serially treated mice and stained for KLS and SLAM family markers. The frequency of KLS150^+^48^-^ stem cells or MPPs KLS150^+^48^+^, KLS150^+^48^-^ are not significantly different.
Prostaglandin E2 protects from lethal radiation damage

We have demonstrated β-catenin mediated Wnt signaling is required for efficient hematopoietic regeneration following both radiation and 5-FU mediated damage. While the loss of Wnt function has significant impact on regeneration, whether gain of Wnt function can accelerate the repair process is unknown. Recently, prostaglandin E2 (PGE-2) was identified as a mediator of self renewal in zebrafish stem cells by stabilization of β-catenin after radiation damage [116]. Further studies have shown PGE-2 enhances murine HSC homing survival and proliferation in vivo [117]. Other groups demonstrated PGE treatment preferentially expands ST- progenitors and KLS cells but does not alter KSL150+ LT-HSCs [118]. Lastly, administration of PGE-2 can increase the survival of lethally irradiated mice [119]. To explore this phenomenon from a stem cell perspective we repeated these experiments and confirmed PGE-2 treatment does protect mice from lethal radiation damage (Figure 19) Next we analyzed what aspects of hematopoiesis were protected and whether the stem cell compartment was expanded following PGE treatment after 10Gy (Figure 20). Our results indicate there is no protection of the peripheral blood, bone marrow or stem cell compartment after 2 days (data not shown). However, bone marrow cellularity is significantly more in PGE-2 treated animals at 7 days post 10Gy. PGE-2 treatment seems to allow the expansion of lin⁻ ckit⁺ progenitors but there is little expansion of KLS34⁻ cells at this time. Collectively, these data show delivery of a
The absolute cell count for KLS150$^+$ stem cells and MPPs KLS150$^+$, KLS150$^-$ were calculated for each population. $p=0.05$ for MPP1 and MPP2, $p<0.05$ for long term HSCs. $n=4-8$ mice per group.

Figure 16: Absolute cell count for long term HSCs and MPPs
Figure 17: Serial 5-FU peripheral blood analysis

Complete blood count data from serially 5FU treated animals on day 14. Total white blood cell count, p= 0.002, lymphocyte count p=0.002, neutrophils count p= 0.04, platelet count p=0.02.
compound which can modulate the Wnt pathway can accelerate bone marrow progenitor regeneration \textit{in vivo}.

2.4 Discussion

Given the widespread clinical use of radiation for the treatment of malignancies and autoimmune disorders, understanding the molecular pathways which mediate stem cell regeneration may provide new therapeutic options to improve transplant efficiency. Furthermore, because the signaling pathways activated during regeneration might promote radiation resistance, defining the consequences of these signals will allow for additional therapeutic intervention.

Here we have examined hematopoietic regeneration by tracking bone marrow content and stem cell frequency after radiation injury. During the regeneration process we find there is an increase in canonical Wnt ligand production in the bone marrow and this leads to activation of Wnt signaling within the remaining HSC population. Other injury models have also implicated the Wnt pathway in regulating regeneration. For example, regeneration of bone injury is accelerated in Axin 2 null mice, and liposomal delivery of Wnt3a ligand stimulates skeletal stem and progenitor cell proliferation to enhance bone repair \textit{in vivo} [120]. Furthermore parathyroid hormone, a previously known regulator of bone fracture repair, has been identified as enhancing the expression of several Wnt ligands in regenerating bone [121].
To evaluate the requirement for Wnt in hematopoietic regeneration we examined conditional loss of β-catenin and Wnt signaling \textit{in vivo}. This analysis revealed a significant impairment of bone marrow cellularity and decreased KLS34\(^{-}\) stem cell regeneration after serial radiation. Loss of β-catenin significantly impairs both the long-term KLS150\(^{-}\)48\(^{-}\) and short term progenitor HSC compartments after 5-FU treatment. As a consequence of failing to properly regenerate the HSC stem and progenitor cell pool, β-catenin \(-/-\) animals exhibit multi-lineage peripheral blood regeneration defects after 5-FU. Importantly, serial 5-FU treatments preferentially kill β-catenin null animals. We interpret these data to mean Wnt is required for hematopoietic regeneration independent of the damage modality. This data also suggests Wnt mediated self renewal is primarily required for stress hematopoiesis during injury repair, and it may have a less significant role in maintaining homeostatic stem cell function.

Conditional loss of β-catenin impacts the regeneration of other organs \textit{in vivo}. Using a liver specific deletion of β-catenin Apte \textit{et al.} determined that regeneration of acute liver failure is significantly delayed in knock out livers [122]. Interestingly, the mechanism of β-catenin stabilization does not appear to be mediated by increased production of Wnt ligands, and is associated with an increase of inactivated GSK-3β (phospho-Serine-9). This modification prevents GSK-3 mediated phosphorylation of β-catenin, and therefore activates canonical Wnt signaling in a cell autonomous manner.

In the context of skin regeneration, adenovirus mediated β-catenin deletion in skin
Figure 18: Survival proportions following serial 5-FU treatment

5-FU was administered on day 0, 7 and 14 and mice were monitored for survival. p= 0.04 n=10-12 mice per group.
fibroblasts reduces the wound strength after radiation damage, further suggesting a role for this pathway in radiation repair and regeneration [123].

Our initial studies indicate activation of the Wnt pathway with PGE-2 can induce regeneration of the progenitor cell compartment. In zebrafish prostaglandin E2 (PGE2) regulates β-catenin stability by cAMP/PKA mediated inhibition of degradation, and treatment with 16,16-dimethyl-PGE2 enhances regeneration of irradiated hematopoietic cells in the kidney marrow [116]. Other non-Wnt ligands which activate the canonical Wnt signaling pathway enhance regeneration after chemotherapy and radiation damage. Administration of exogenous protein of the R-Spondin (Rspo) family of soluble Wnt activating ligands accelerates the regeneration of intestinal epithelium in a 5-FU mucositis injury model [124]. Recombinant adenovirus expressing Rspo1 also has a protective role in the radiation induced gastrointestinal syndrome in mice [125]. Further evidence suggests Rspo1 protects mice from radiation induced oral mucositis [126]. Collectively, these data suggest the possibility exogenous activation of the Wnt pathway could enhance regeneration of HSCs after injury or in a transplant setting. It will be valuable to learn if direct delivery of Wnt activating ligands alone or in combination with other ligands such as angiopoietins or Notch ligands can enhance stem cell engraftment and regeneration.
Figure 19: PGE-2 treatment protects from lethal irradiation

Cohorts of BA mice were treated with vehicle (4% ethanol) or 20ug prostaglandin E2 15 minutes before (pre) or 15 minutes after (post) a single lethal radiation dose of 10Gy. p=0.013 n=5 per group, one representative experiment of 2 performed.
Mice treated with vehicle or 20 ug PGE-2 were sacrificed at day 7 and bone marrow was analyzed for KLS content. KLS FACs analysis of bone marrow (A). Quantitation of whole bone marrow cellularity (B left panel) and ckit progenitors and stem cells (B right panel). WBM p=0.047, lin´ckit+ p=0.022, KLS34- p=0.016. n=4 mice per group.

Figure 20: PGE-2 protects lin´ckit+ progenitors 7 days after lethal radiation
3. Hematopoietic stem cells activate Notch to alter cell fate during regeneration

3.1 Introduction

The primary function of an adult hematopoietic stem cell (HSC) is to maintain an adequate supply of blood cells throughout the lifetime of an organism. However, hematopoietic stress caused by chemotherapy or radiation treatments commonly used to combat malignancy depletes peripheral blood and bone marrow cells. Consequently, the homeostatic HSC must regenerate the injured hematopoietic compartment. While this regeneration phenomenon exemplifies the endogenous proliferative capacity of HSCs, there is little molecular understanding of how this process is governed. Currently there is only a rudimentary framework for understanding whether regeneration is controlled by the same signaling pathways used to regulate homeostatic HSC proliferation or whether regeneration utilizes a unique set of growth and differentiation pathways. Recent work from our laboratory suggests homeostatic signaling pathways which normally control stem cell fate may be reactivated during regeneration [77]. The Notch pathway is one candidate pathway for controlling both homeostatic and stress hematopoiesis. In homeostatic stem cell biology Notch has a significant role in self renewal by preserving the undifferentiated state. Presently however, Notch has an unappreciated role in HSC radiation injury.

Notch is an evolutionarily conserved cell-cell signal transduction cascade which controls a variety of cell fate decisions in multiple tissues and cancers [127]. There are
four single pass transmembrane Notch receptors in mammals (Notch 1-4) which are activated by binding to ligands of the Drosophila homologs Jagged and Delta [128]. Upon ligand binding the Notch intercellular domain (NICD) is liberated from the transmembrane region of the receptor by a series of proteolytic cleavages requiring a disintegrin and metalloprotease (ADAM) family protease and the \( \gamma \)-secretase complex. These cleavage events permit NICD translocation to the nucleus where it binds to its partner transcription factor of the CSL family RBP-j\( \kappa \) in mammals, and cofactors of the Mastermind-like family to initiate transcription of Notch target genes including Hairy enhancer of split (HES) and Hes-related (Hrt) [129].

The importance of the Notch pathway in hematopoiesis is evident based on analysis of gain and loss of function studies in vivo. Global genetic loss of RBP-j\( \kappa \) reveals a myriad of defects related to impaired Notch signaling throughout development. RBP-j\( \kappa \) null embryos exhibit defects in somitogenesis, microencephaly and growth retardation ultimately resulting in embryonic death for homozygous mutants at E12.5 [130]. Embryonic hematopoiesis in RBP-j\( \kappa \) null mice is also aberrant due to failure of the para-aortic-splanchnopleura/aorta-genital ridge-mesonephros (P-SP/AGM) region to establish or maintain primitive hematopoietic progenitors [131]. Conversely, mutational Notch1 hyper-activation leads to uncontrolled cell growth and is associated with 50% of human T-cell acute lymphoblast leukemia [132].
In adult homeostatic HSC biology activated Notch signaling expands both murine and human hematopoietic stem cells by directing stem cell fate. Notch controls HSC self renewal and constitutive Notch1 expression is sufficient to immortalize murine HSCs [128] [133]. Research from our laboratory suggests Notch is responsible for inhibiting the differentiation of HSCs during division [71]. Consistent with this hypothesis, inhibition of Notch by dominant negative Suppressor of Hairless (dnSu(H)) leads to decreased self renewal in vitro and loss of the stem cell compartment in vivo.

Although it is currently unknown whether the Notch pathway is involved in regulating self renewal during HSC regeneration, Notch already has an established role in the regeneration of other tissues. In murine muscle stem cells (satellite cells) Notch is required to suppress TGFβ/pSmad3 signaling in regenerating young muscle. However, age induced loss of Notch causes increased expression of cyclin dependent kinase inhibitors that can be reversed by Notch restoration [134]. Furthermore, Notch is required for regeneration of the avian retina after injury and Notch pathway impairment with γ-secretase inhibitors prohibit retinal repair [135].

In order to test whether Notch signaling also has a role in regeneration of hematopoietic stems cells, we have analyzed two forms of stem cell injury. Here we demonstrate using a transgenic Notch reporter (TNR) mouse model which drives GFP under the control of a Notch responsive cassette that Notch activation is common to HSC regeneration after radiation injury and 5-flurouracil treatment. Furthermore, using live real-time imaging of regenerating or control HSCs we find regenerating HSCs perform
more symmetric self renewing divisions than control stem cells. To investigate whether Notch activation enhances regeneration after radiation damage we developed an inducible gain of function Notch mouse model and determined constitutive Notch activation enhances self renewal of HSCs after radiation injury.

3.2 Materials and Methods

Mice

CD45.2 Thy1.1 and Transgenic Notch reporter mice were used at 2-4 months of age. Mice were bred and maintained on acidified water in the animal care facility at Duke University Medical Center. 5-Flurouracil was injected inter-peritoneal on day 0 at 200mg/kg. 450cGy of irradiation was delivered using a Shepard Cesium irradiator at a dose rate of 600cGy/min. Rosa 26 knock- in Floxed- NICD-ires-GFP mice were crossed to Mx Cre animals to generate inducible NICD animals. To initiate recombination Poly IC (Sigma) was injected at 20ug/g on day 0,2,4. Recombination was confirmed by peripheral blood analysis for GFP+ cells. All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

Cell isolation and FACS analysis

HSCs were sorted and analyzed from mouse bone marrow based on surface marker expression of c-Kit and Sca-1, low expression and low to negative expression of lineage markers (Lin) using an antibody cocktail CD3,4,5,8, B220, Mac-1, Gr-1 and
Ter119. All antibodies were purchased from Pharmingen or eBioscience. Analysis and cell sorting were carried out on a FACS Vantage (Becton Dickinson) at the Duke Cancer Center FACS facility.

**Live real time imaging analysis**

Stromal layers of OP-9 cells were cultured in glass bottom 35mm dishes overnight to produce a confluent monolayer. KLS34- stem cells were then plated and imaged in a Zeiss axiovert every 10 minutes for 72 hours. Cells were maintained at 37°C and 5% humidified O₂. Cell media was X-vivo and 2%FBS with penicillin. Movies were compiled in MetaMorph software for analysis.

**In vitro colony forming assays**

Freshly purified KLS cells were sorted by FACS and plated into methylcellulose media (Stem Cell Technologies GF 3434) at 500 cells per well and cultured at 37°C in 5% O₂ for 7-10 days. Colonies were counted and cells were harvested and replated at 5000 cells per well.

### 3.3 Results

*Irradiated HSCs activate the Notch pathway and enter the cell cycle in vivo*
Notch signaling had been implicated in the maintenance of HSCs through inhibition of differentiation in homeostatic self renewal [71]. To understand if regenerating HSCs also utilize Notch signaling we irradiated Transgenic Notch Reporter (TNR) mice which express green fluorescent protein (GFP) under control of a CBF responsive element (Figure 21). The homeostatic bone marrow of TNR mice contains a population of GFP+ KLSCD34- HSCs indicating a consistent sub-fraction of stem cells is actively signaling via Notch. To determine if the Notch signal is activated in regenerating HSCs we analyzed total bone marrow KLSCD34- content from control or 450cGy irradiated TNR mice (Figure 22). Although reduced in absolute number, the frequency and intensity of GFP expression in irradiated KLSCD34- cells was significantly increased in regenerating cells (10.9% GFP- vs. 32.0% GFP+). The increased GFP+ population is not strictly a consequence of differential apoptosis of GFP-KLS as the absolute GFP signal is brighter than unirradiated KLS34-. Moreover, GFP+ and GFP- cells show similar viability based on Annexin-V staining and propidium iodide exclusion (data not shown). To correct for auto-fluorescence in the bone marrow of irradiated mice we also examined GFP expression in HSCs lacking the Notch reporter and found no background GFP signal due to irradiation alone (data not shown). To test whether Notch signaling activation within the KLS34- population was specific to irradiation injury we also treated Notch reporter mice with 200mg/Kg of 5-FU and found a similar shift towards an increased GFP+ HSC pool 6 days after injury (Figure 22). Therefore we conclude Notch signaling activation is a common occurrence in regenerating HSC independent of the cause of injury. Whether
A CBF-responsive element drives expression of GFP in cells actively signaling through Notch.

Figure 21: Transgenic Notch Reporter construct
Figure 22: Regenerating HSCs activate the Notch pathway

TNR mice were 4.5Gy irradiated or treated with 200mg/kg 5FU and whole bone marrow was stained for KLS34 on day 7. Histogram of GFP expression from irradiated KLS34-cells are shown in the (A) left panel and KLS34- from 5FU treated mice are in (A) right panel. Quantitation of GFP populations are shown in (B), p <0.05 for irradiated and 5FU treated KLSGFP+ cells.
the Notch positive HSC population has other unique characteristics that distinguish it from the Notch negative pool is unclear. To further investigate how regenerating GFP+ cells may be different from their GFP- counterparts, we examined the S-G2-M cell cycle status of control and irradiated populations. Cell cycle analysis revealed increased cell cycling frequency of irradiated regenerating GFP+ KLS34- cells compared to control GFP+ KLS34- cells (Figure 23). We also examined the cell cycle status of GFP- KLS34-cells from TNR reporter mice and found that while they do enter the cell cycle at a greater frequency than their homeostatic counterparts, they do so to a significantly lesser extent than the GFP+ KLS3CD34- cells after irradiation. Collectively, we interpret this data to mean while both GFP+ and GFP- cells are participating in the regeneration process, the GFP+ Notch signaling cells are entering the cell cycle more frequently to expand the long term HSC pool while GFP- HSCs are cycling to expand the short term and progenitor pools. However, whether the self renewal ability of regenerating HSCs is actually improved by increased Notch signaling is unknown. We therefore wanted to evaluate if these basic changes observed in regenerating HSCs have functional consequences in altering HSC behavior. To address this question we utilized a live imaging strategy to monitor stem cell division patterns for HSC self renewal and commitment.

_HSCs alter division fate choices during regeneration_
GFP+ and GFP- KLS34- cells were sorted from control and 4.5Gy irradiated mice and cell cycle status was analyzed by PI incorporation (A). Quantitation of cycling cells from control mice (B) left panel, and from 4.5Gy mice (B) right panel, control cells are not statistically different, irradiated cells p<0.001, n=3-4 mice per group.

Figure 23: Radiation preferentially induces TNR+ KLS into cycle
Our laboratory has previously used a live real time imaging microscopy technology to examine division patterns of leukemia progenitor cells [85]. Using this approach we determined that HSCs are capable of three distinct types of cell fate choices during division. Using the TNR marker as an indicator of immature cells, we verified the outcome of regenerating stem cell divisions by tracking the GFP expression in each daughter cell. These studies revealed HSCs can perform symmetric renewal divisions, symmetric commitment divisions and asymmetric divisions. In a symmetric renewal division both daughter cells must maintain high GFP expression until the next division occurs, while in a symmetric commitment both daughters lose GFP expression. During an asymmetric division one daughter cell maintains GFP and the other daughter must lose GFP expression. The balance between these cell fate choices determines the outcome of the HSC pool in vivo, with an excess of renewing divisions leading to leukemogenesis and an excess of commitment ultimately causing stem cell exhaustion. To investigate how HSCs may have changed the frequencies of symmetric renewal, symmetric commitment and asymmetric division we used live real time imaging of TNR GFP+ 450cGy irradiated KLS34- cells in a co-culture with OP-9 osteoblast stroma (Figure 3A-H series of symmetric renewals). By scoring the division pattern frequency of each division type from control or irradiated TNR+ HSCs we were able to calculate the ratio of symmetric renewal to symmetric commitment daughter fates (Figure 24). While the ratio of symmetric renewal divisions to symmetric commitment divisions is 4:1 in control cells, irradiated HSCs show approximately a 10:1 ratio of renewal to commitment (Figure
GFP+ HSCs from control or irradiated TNR mice were plated onto a confluent monolayer of OP-9 osteoblastic stromal cells and monitored by live imaging for 72 hours. A example of a GFP+ mother cell dividing into GFP+ daughter cells is shown (A-B GFP, F-G bright field). This GFP renewal continues until there are 8 total GFP+ daughters (C-E GFP and H-J brightfield). GFP cell fates were scored for at least 40 mother cells for each condition. The ratio of symmetric renewal to commitment and the percent of asymmetric division are shown in (K).

Figure 24: Radiation alters TNR+ KLS stem cell division pattern in vitro
HSC cell fate division pattern outcomes diagram. GFP+ mother cells can make 1 of 3 cell fate choices during division. In symmetric renewal divisions both daughter cells remain GFP+, while in a symmetric commitment division both daughter cells lose GFP expression. During an asymmetric division one daughter retains GFP expression and the other daughter loses GFP by making a lineage commitment.
This augmentation in self renewal frequency during regeneration presumably allows the HSC pool to expand much more efficiently than simply maintaining the conventional division pattern frequencies. An example of the division pattern fates is shown (Figure 25). In addition to monitoring the division pattern of control or irradiated HSCs the division rate for these cells was also determined. The division rate for irradiated cells was found to be significantly faster than the control HSCs, with control cells completing a division with an average time of 20 hours, compared to an average division time of 12 hours for regenerating HSCs (Figure 25). This increased division rate in vitro is consistent with the increased frequency of regenerating HSC in cycle in vivo (Figure 23).

Collectively, our data suggest a regenerating HSC is able to replenish the HSC pool and repair the damaged hematopoietic compartment simultaneously through a combination of increased symmetric renewal and an accelerated division rate.

Activation of the Notch pathway enhances self renewal of irradiated HSCs in vitro

While irradiated TNR+ HSCs exhibit an altered division pattern during regeneration it is not clear whether endogenous activation of Notch affects self renewal or proliferation in culture. To test this possibility KLS34 cells were sorted and infected with a control MSCV-IRES-YFP virus or MSCV-ICN-IRES-YFP virus. Lineage negative YFP+ infected cells were sorted and irradiated with 2Gy before plating in methylcellulose media for colony formation (Figure 26). In the primary plating ICN infected cells produced a similar number of total colonies as control cells, but ICN
KLS cells were sorted and infected with either MSCV-IRES-YFP control virus or MSCV-ICN-IRES-YFP and cultured for 48 hours. Lineage-YFP+ cells were sorted and irradiated with 2.0Gy before plating at 500cells/well in methylcellulose. Total cells and colonies were counted on day 7, panel (A). Cells were harvested and replated at 10,000 cells per well (B). A representative photograph of secondary colonies is shown (B) left panel. p= 0.0001 for total cell count from primary plating, n=4 per group. p= 0.002 for total colonies in secondary plating n= 3-4 per group.

Figure 26: Introduction of active Notch alters self renewal of irradiated HSC in vitro
Figure 27: Generation of a conditional mouse model of Notch signaling

An active Notch signaling mouse was created by crossing a Rosa (R 26) knock-in mouse with a loxp-stop-loxp cassette in front of a Notch intercellular domain –IRES-GFP construct with an Mx1 Cre recombinase mouse (A). The NICD mouse strain was a generous gift to the laboratory (Dr. Chay Kuo Duke University Department of Cell Biology). After induction of Cre expression with 3 treatments of polyI:C GFP+ cells are detectable in the peripheral blood of NICD/Mx-1+ mice at 1 month but no GFP signal is found in NICD/Mx-1- mice (B), p=0.0056.
cultures contained significantly more total cells. Cells from the primary plating were replated in a secondary assay to further examine self renewal and stem cell exhaustion. In secondary plating ICN infected cells generated significantly more colonies and total cells (Figure 26). We also confirmed by FACS analysis that all cells generated were myeloid and did not express CD3e (data not shown). These data suggest enhancing Notch signaling prior to radiation can provide an increase in self renewal following radiation damage.

**Development of an inducible Notch signaling mouse model**

Induction of Notch signaling prior to radiation *in vitro* augments self renewal but we do not know if this also occurs to stem cells irradiated *in vivo*. To address whether Notch activation *in vivo* prior to irradiation injury expands self renewal we developed an inducible Notch mouse model by crossing a Rosa 26 knock-in loxp-stop-loxp- NICD-IRES-GFP mouse with a Mx-1 Cre recombinase mouse (Figure 27). Administration of the double stranded RNA compound polyI:C induces production of interferon which leads to Cre induction and recombination [136]. NICD/+ Mx-1+ mice were treated with polyI:C and analyzed for GFP+ cells in the peripheral blood (Figure 27). This analysis confirmed the presence of GFP+ cells (approximately 15%) within the Mx-1 Cre+ polyI:C treated NICD animals. To test self renewal ability of *in vivo* irradiated Notch activated cells we sorted KLS34- cells from NICD/+ MX-1- and NICD/+ MX-1+ 7 days after 4.5Gy for methylcellulose colony forming activity (Figure 28). In summary, these
data show enhanced retention of self renewal ability in vitro after 4.5Gy radiation is delivered in vivo. As an additional confirmation of Notch induction in this model, polyI:C treated NICD/+ Mx-1+ develop a lethal CD4+CD8+ T-ALL disease in the bone marrow and spleen (Figure 29).

3.4 Discussion

Here we have investigated whether Notch activation is involved in hematopoietic regeneration after chemotherapy and radiation damage. However the molecular mechanism of Notch activation during regeneration is still unknown. One possibility is that HSC cell fate determinants are differentially regulated in regenerating HSCs. In Drosophila neuroblasts the protein Numb is asymmetrically localized during division to direct the fate of stem cell daughters by inhibiting Notch. The daughter cell which receives Numb inhibits Notch activation and differentiates into a ganglion mother precursor, while the Numb deficient cell retains Notch signaling and remains a neuroblast [137, 138]. Our lab has previously shown over expression of the fate determinant Numb in hematopoietic progenitor cells accelerates differentiation in a dose dependent manner [85].

One possibility is that Numb proteins are down regulated in irradiated HSCs during regeneration, and because the amount of unphosphorylated Numb protein inherited by daughter cells directly impacts Numb function in those progeny [138]. A
KLS34+ cells were sorted from polyI:C treated NICD+/MX-1- and NICD+/MX-1+ mice 60 days after 4.5Gy and plated in methylcellulose media. Three week old mice were injected with polyI:C on 3 alternating days and irradiated at 9 weeks of age. Bone marrow was sorted one week after radiation and 500KLS cells were plated in methylcellulose media. Colonies were counted on day 8. p=0.002, n=2-3 mice per group.

Figure 28: NICD+ HSCs irradiated in vivo retain more self renewal in vitro
NICD/Mx-1+ and control mice were treated with polyI:C and bone marrow was harvested and stained 60 days after Cre induction. Representative FACS plot showing thymus independent T-cell development occurs in NICD/Mx-1+ bone marrow.

Figure 29: NICD causes ectopic formation of CD4+CD8+ T cells in the bone marrow

NICD/Mx-1+ and control mice were treated with polyI:C and bone marrow was harvested and stained 60 days after Cre induction. Representative FACS plot showing thymus independent T-cell development occurs in NICD/Mx-1+ bone marrow.
decrease in total Numb protein levels could result in daughter cells with an insufficient ability to fully repress Notch signaling. Accordingly, the commitment frequency of regenerating HSCs with reduced Numb should be decreased, as both daughters could maintain Notch activation. This scenario would be consistent with our findings of elevated Notch activity and increased in regenerating divisions following injury.

Recently, a translational repressor of Numb called Musashi 2 (Msi 2) was identified as a protein highly expressed in HSCs. Loss of Msi 2 impairs self renewal in normal and transformed hematopoietic stem cells through a Notch-Numb axis [139, 140]. Msi 2 deficiency also affects the differentiation status of leukemic cells suggesting a cell fate transition towards commitment and loss of self renewal of leukemic progenitors. It will be interesting to pursue this signaling/cell fate paradigm in the context of HSC regeneration. Furthermore, identifying compounds to modulate the Msi2-Numb-Notch cascade could be a valuable approach in accelerating the recovery of transplant patients or myelo-ablated cancer patients.
4: Conclusions and future directions

In the preceding chapters I have outlined the basics of stem cell biology and provided a brief introduction of adult stem cells/iPS cells, described the current understanding of stem cell niches and examined the signaling pathways used to direct stem cell self renewal. Our candidate approach to investigate the molecular signaling basis of hematopoietic regeneration was founded on prior studies of self renewal signals performed in our laboratory. In 2003 Reya et al. and Willert et al. published the discovery of Wnt as an HSC self renewal factor. Since this time the Reya lab has investigated various aspects of Wnt signaling in HSC self renewal in homeostasis, stress hematopoiesis and leukemia development. Each of these projects have contributed new insight into the requirements for Wnt in diverse settings, and have provided valuable new ideas and reagents to further refine our understanding of Wnt signaling in hematopoiesis and disease. To determine the impact of Wnt signaling in a new context I chose to examine hematopoietic regeneration following radiation injury. Radiation is frequently used in clinical settings for conditioning patients prior to bone marrow transplantation as well as for the treatment of solid tumors. Thus it will be valuable to understand signals which can be used to enhance engraftment in a bone marrow transplant and signals that may be activated by tumors to self renew or develop radiation resistance following treatment.

Using a conditional deletion of β-catenin which functions as a critical transcription factor in Wnt signaling we examined hematopoietic regeneration after single
or serial radiation injury. The results of our experiments were interesting in several ways, and have potentially changed our understanding of the role the Wnt pathway has in HSC self renewal. Our data consistently shows loss of β-catenin does not affect the homeostatic peripheral blood, bone marrow or stem cell populations. A single 4.5Gy irradiation injury causes damage to much of the hematopoietic compartment, including the stem cell fraction. However, in the absence of Wnt signaling only the stem cell itself is functionally impaired and regeneration of the bone marrow and peripheral blood proceed normally. Following a second 4.5Gy injury we observe a significant defect in the regeneration of total bone marrow cells and the KLS34^- stem cell compartment.

The interpretation of this combined data leads to a simple model where radiation damage impairs the self renewal of β-catenin stem cells after the first injury but bone marrow and peripheral blood regeneration proceed normally due to progenitor pool mediated regeneration. However, when a second injury is inflicted progenitors are not replaced rapidly enough by the damaged HSC compartment and overall regeneration is delayed. To further our understanding of the role for Wnt we also examined how regeneration occurs after chemotherapeutic damage by 5-FU. We observed a similar pattern of HSC impairment after 1 injury but overall a normal regeneration process occurs. Like in the radiation model, a second 5-FU injury is needed to detect impaired bone marrow and stem cell regeneration. In the case of 5-FU 3 weekly injuries are sufficient to kill β-catenin -/- mice. Collectively this data suggests loss of Wnt signaling delays the hematopoietic repair process.
Loss of Wnt signaling impairs the regeneration process independent of injury method in a serial stress model, but whether gain of Wnt function accelerates regeneration is unclear in the hematopoietic system. Wnt activators have been shown to protect from injury and enhance regeneration in several situations. For example delivery of R-spondin, a canonical Wnt signaling activator, protects mice from radiation injury in the gut [124]. We have attempted to activate the Wnt pathway with prostaglandin E-2, a recently identified molecule that stabilizes β-catenin [116]. These preliminary studies have confirmed PGE-2 treatment prevents the death of animals after lethal radiation but the mechanism of this protection is unclear.

Our future plans for this project are to further explore how PGE-2 protects the progenitor compartment and allows regeneration to begin. We have obtained a variety of other PGE-2 family analogs that activate specific EP receptors to determine whether a specific EP receptor is required for the protection effects observed. To this end, we have obtained an FDA approved drug misoprostol which activates EP 2,3,4 receptors but does not activate EP-1 receptors, sulprostone an EP3 specific analog and butaprost an EP2 selective agonist. We intend to use these pharmacological agents to evaluate the level of radiation resistance conferred by each treatment to identify receptor specificity. We will also determine if these compounds activate Wnt signaling or if they function through a combination of Wnt dependent and Wnt independent mechanisms.

In order to establish a better understanding of the molecular biology of β-catenin mediated regeneration we will examine gene expression in regenerating control and -/-
KLS34 cells. I propose to use 5FU treated HSCs to study gene expression by qRT-PCR. I am interested in understanding which Wnt target genes are differentially expressed in control and -/- before and after injury. It will also be important to determine if there are cell cycle delays in regenerating HSCs, or if the expression pattern of BMI-1, a critical self renewal gene is reduced.

There are also several experiments to perform in order to complete our understanding of how Notch alters regenerating HSCs. To test whether activation of Notch signaling enhances self renewal in vivo it would be valuable to transplant NICD/+ Mx-1 Cre cells and then induce recombination with polyI:C before irradiation. Using this approach we could track the regeneration of GFP+ cells in the peripheral blood. A more sophisticated approach would be to try and stimulate the Notch pathway using soluble ligands in vivo after radiation injury. This strategy has shown to be successful using in vitro Notch activation with a recombinant Delta-IgG [41].

Beyond finding a clinically useful approach to activate Notch in accelerating regeneration it would also be valuable to interrogate the molecular biology occurring during this process. Recently our lab has identified Msi2 as a key regulator of self renewal and the Notch Numb regulatory axis. There it will be important to know if Msi 2 is also regulated during the regeneration process to control Numb translation and potentiate Notch signaling.

Although our preliminary studies indicate there is no regeneration impairment after a single 4.5Gy injury in RBPjk null mice, it does not rule out the possibility of
Notch requirement under serial stress conditions like those used to elicit a defect in β-catenin -/- mice. Loss of Notch has been shown impair self renewal in myeloid leukemia, however other groups have reported Notch is dispensable for HSC self renewal [86, 139].
Appendix A: A novel role for Notch activation in embryonic hematopoiesis

Figure 30: Generation of a hematopoietic driven Notch

Hematopoietic specific Notch signaling was created by breeding Rosa 26-NICD/+ IRES-GFP and Vav Cre mice. As an alternate approach to the inducible Mx-1 Cre NICD mice were bred onto a hematopoietic specific driver to begin expression Notch expression at E11.5.
We observed that NICD/+ Vav+ pups were not born at a Mendelian ratio and do not survive past P7. Therefore we examined E18.5 fetal livers from pregnant females to try and determine if there was a hematopoietic defect evident. Fetal liver total cell counts were not found to be significantly different between NICD/+ Vav- and NICD/+ Vav+. Fetal liver staining for lin- (excluding Mac-1) AA4.1+ KLS revealed a significant reduction in the lin-AA4.1+ cells (left panel). Quantitation of lin-AA4.1+ cells from FACS analysis, p=0.0001.
Figure 32: Constitutive Notch signaling impairs HSC differentiation

FACS analysis of E18.5 fetal liver KLS gated through lineage- AA4.1+ cells (left panel top). GFP expression of KLS cells (left panel bottom). Quantitation of the stem and progenitor cell compartment frequencies pregated through lin-AA4.1+, ckit+ p = 0.379, KLS p=0.0001, K-S- p= 0.001.
Figure 33: Constitutive Notch signaling depletes myeloid cell production

Analysis of E18.5 fetal liver Mac^Gr^+ cells show decreased myeloid cell frequency (left panel). Quantitation of lineage subsets in the fetal liver, Mac^Gr^+ p=0.001, B220+ p=0.058, CD3 p= 0.07.
Appendix B: List of Abbreviations

AML  acute myeloid leukemia
BrdU  5-bromo-2’-deoxyuridine
BMP  bone morphogenetic protein
CD  cluster of differentiation
CFU  colony forming unit
CML  chronic myeloid leukemia
CLP  common lymphoid progenitor
CMP  common myeloid progenitor
dnMAML  dominant negative mastermind-like
DSL  delta/serrate/lag
Dpc  days post coitus
ESC  embryonic stem cell
FACS  fluorescence activated cell sorting
GMP  granulocyte-macrophage progenitor
Gy  gray
HSC  hematopoietic stem cell
H&E  hematoxylin and eosin
ICN  intercellular notch
IL  interleukin
iPS  induced pluripotent stem cell
ISC  intestinal stem cell
KLS34–  ckit^lin^sca^+CD34^–
MEP  myeloid erythroid progenitor
MPP  multipotent progenitor
MSCV  murine stem cell virus
Msi 2  musashi 2
MMTV  mouse mammary tumor virus
Mx-1  myxovirus 1 promotor
NOD  nonobese diabetic
LT-HSC  long term hematopoietic stem cell
OPN  osteopontin
PCR  polymerase chain reaction
PGE-2  prostaglandin E2
qRT-PCR  quantitative reverse transcription PCR
RBC  red blood cell
SCID  severe combined immune-deficient
SDF-1  stromal derived factor 1
SEC  sinusoidal endothelial cell
Shh  sonic hedgehog
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SMP</td>
<td>satellite muscle progenitor</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short term hematopoietic stem cell</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-cell acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>TBI</td>
<td>total body irradiation</td>
</tr>
<tr>
<td>TNR</td>
<td>transgenic notch reporter</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless/int</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-flurouracil</td>
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</tbody>
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References


Biography

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