

**The Role of the E47-p21 Pathway in Long-Term Hematopoietic Stem Cells During  
Homeostasis and Under Repopulation Stress**

by

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## ABSTRACT

During hematopoiesis the transcription factor E47 plays two important roles. First, E47 promotes lymphoid lineage development. Second, E47 regulates proliferation of LT-HSCs, bone marrow cells uniquely capable of long-term self-renewal and multilineage reconstitution. Because hyperproliferation of LT-HSCs can be detrimental to its function, LT-HSC proliferation must be tightly regulated. We have previously shown that E47 directly activates the cell cycle inhibitor p21 in LT-HSCs. However, the biological relevance of the E47-p21 pathway to LT-HSC function *in vivo* has not yet been examined. Here, we used mice with reduced gene dosage in E47 and p21 individually (E47<sup>het</sup> or p21<sup>het</sup>) versus in tandem (E47<sup>het</sup>p21<sup>het</sup>) and show that E47<sup>het</sup>p21<sup>het</sup> LT-HSCs exhibit hyperproliferation during homeostasis and under transplantation stress. In serial adoptive transfers that rigorously challenge self-renewal, E47<sup>het</sup>p21<sup>het</sup> LT-HSCs dramatically and progressively decline, indicating the importance of cell-intrinsic E47-p21 pathway in preserving LT-HSC self-renewal under repopulation stress. Transient numeric recovery of downstream progenitors enabled production of functionally competent myeloid cells but not lymphoid cells as common lymphoid progenitors (CLPs) were decreased and peripheral lymphocytes virtually ablated. Thus, we demonstrate a developmental compartment-specific and lineage-specific requirement for the E47-p21 pathway in maintaining LT-HSC and lymphoid lineage cells under hematopoietic repopulation stress *in vivo*. It is likely that other mechanisms exist by which E47 regulate LT-HSC proliferation. Therefore, we also examined if p15<sup>PAF</sup> is a

direct E47 target gene since E47-deficient and p15<sup>PAF</sup>-deficient mice share very similar early hematopoietic defects during homeostasis. We show that E47 can activate p15<sup>PAF</sup> promoter-mediated transcription in a model cell line. Unexpectedly, analysis of transcript levels show a 2-fold increase in E47-deficient progenitors compared to WT, while protein levels were comparable. These findings suggest that E47 is a transcriptional regulator of p15<sup>PAF</sup> expression that is part of a broader network of transcriptional and post-translational mechanisms that regulate p15<sup>PAF</sup> expression in primary hematopoietic progenitors. Collectively, these studies demonstrate the importance of the E47-p21 pathway in LT-HSC self-renewal and lymphoid lineage development under transplantation stress and identify p15<sup>PAF</sup> as a novel E47 target gene. These findings might provide mechanistic insights into preserving and enhancing LT-HSC function for improved therapeutic hematopoietic stem cell transplantation.



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## **LIST OF ABBREVIATIONS**

5-FU – 5-Fluorouracil

Ab – Antibody

ALL – Acute Lymphoblastic Leukemia

bHLH – basic Helix-Loop-Helix

BM – Bone Marrow

BrdU – 5-Bromo-2'-deoxyuridine

CLP – Common Lymphoid Progenitor

CMP – Common Myeloid Progenitor

CFU – Colony Forming Unit

ELP – Earliest Lymphoid Progenitor

ETP – Early T progenitor

FACS – Flow Cytometry Cell Sorting

G-CSF – Granulocyte-Colony Stimulating Factor

GMP – Granulocyte/Macrophage Progenitor

HET – Heterozygous

HSC – Hematopoietic Stem Cell

KO – Knockout

LMPP – Lymphoid Myeloid Primed Multipotent Progenitor

LSK – Lineage<sup>-</sup> Sca<sup>+</sup> c-kit<sup>+</sup>

LT-HSC – Long-term Hematopoietic Stem Cell

LPS – Lipopolysaccharide

MACS – Magnetic Automated Cell Separation

MEP – Megakaryocyte/Erythrocyte Progenitor

MPP – Multipotent Progenitor

PBS – Phosphate Buffered Saline

RAG1/2 – Recombination Activating Genes 1 and 2

RU – Repopulating Units

SLAM – Signaling Lymphocyte Activation Molecule

SPL – Spleen

ST-HSC – Short-term Hematopoietic Stem Cell

WT – Wild-Type

## PREFACE

I started my Ph.D. a little over six years ago and in those six years I have changed and grown not only as a scientist but as an individual. I am very lucky to meet and interact with a number of people who have contributed and helped me throughout all these years as I traversed the ups and downs of graduate student life, and so I would like to take this opportunity to give my heartfelt thanks and say a **BIG THANK YOU Y'ALL!**

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Love, hugs, and kisses

– Patti

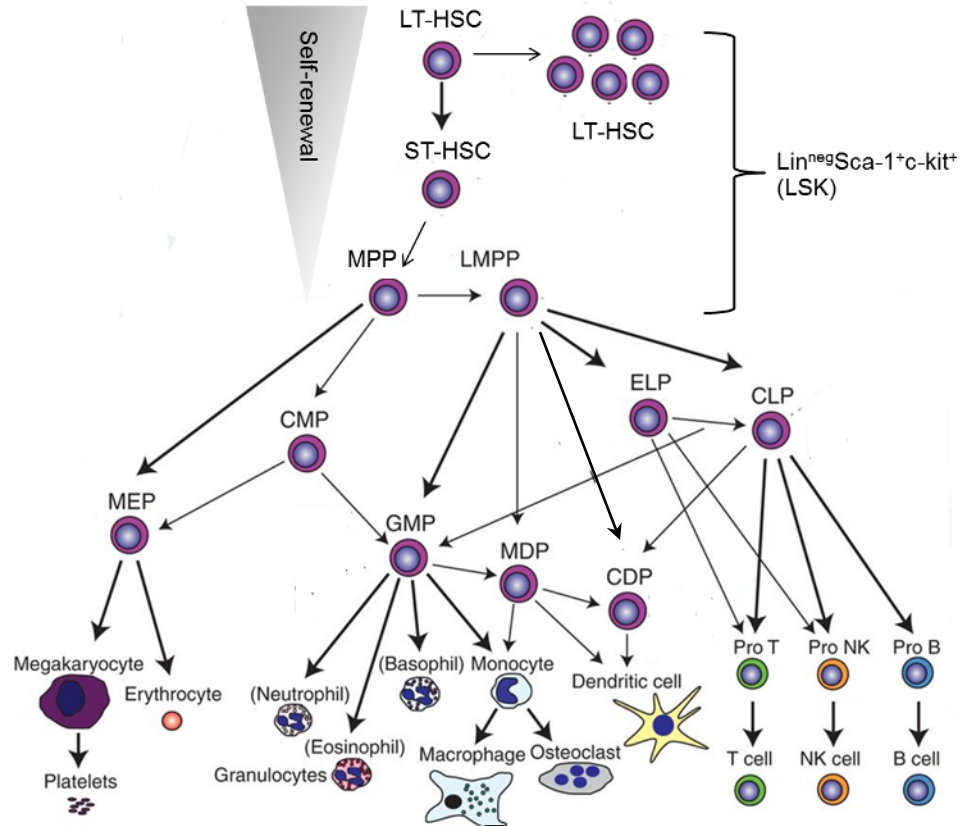


## 1.0 INTRODUCTION

### 1.1 HEMATOPOIESIS

Hematopoiesis (**Figure 1-1**) is the process by which more than ten different mature blood cells with varying and diverse functions is produced (1). The blood is a highly regenerative tissue such that  $\sim 3 \times 10^5$  erythrocytes and  $\sim 3 \times 10^4$  white blood cells are estimated to be produced every second during steady-state conditions (2). While hematopoiesis is capable of sustaining blood cell production during steady-state conditions, it is also a quite dynamic process such that blood cell output can be rapidly increased in response to emergency conditions like unexpected blood loss or during acute and chronic infections (2).

In adults, hematopoiesis occurs in the bone marrow where hematopoietic stem cells (HSCs) undergo a defined differentiation process that is hierarchical in nature. Current models of hematopoiesis indicates a progressive loss of multi-lineage potential as self-renewing, multi-potent HSCs transition to non-self-renewing, lineage-restricted progenitor cells that produce mature, terminally differentiated blood cells (3-5). Long-term repopulating hematopoietic stem cells (LT-HSCs) reside at the apex of the hematopoietic hierarchy as these cells are uniquely capable of self-renewal allowing for long-term reconstitution of the hematopoietic system (6, 7).



**Figure 1-1. Schematic Diagram of Adult Hematopoiesis**

Long-term self-renewing HSCs are at the top of a hierarchy of multiple progenitor cell stages that eventually generates mature cells of all blood lineages. Shown here is murine hematopoiesis as currently defined. Distinct hematopoietic stem and progenitor cell subsets have been described by correlating surface marker expression with functional properties. Abbreviations used are: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lymphoid-myeloid primed MPP; ELP, early lymphoid progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte–macrophage progenitor; MEP, megakaryocyte–erythrocyte progenitor; CDP, common dendritic progenitor; MDP, monocyte–dendritic cell progenitor; NK, natural killer cell. Findings from new studies are continuously refining this model. Figure is modified from original and reprinted with permission from Cold Spring Harbor Laboratory Press, Copyright 2012. Rieger MA and Schroeder T, 2012 *Cold Spring Harb Perspect Biol* 2012;4:a008250.

LT-HSCs differentiate into short-term HSCs (ST-HSCs), which have limited to no self-renewal potential resulting in the ability to reconstitute the immune system short-term. ST-HSCs can differentiate into non-renewing multipotential progenitors (MPP) which still capable of full multi-lineage reconstitution (3, 4). MPPs can generate common myeloid progenitors (CMP) which do not have lymphoid potential, but can generate myeloid, megakaryocytic and erythroid lineage cells through differentiation into lineage-restricted granulocyte/macrophage progenitors (GMP) and megakaryocytic/erythrocytic progenitors (MEP) (4, 8). As MPPs lose megakaryocyte/erythrocyte potential they differentiate into lymphoid-primed multipotent progenitors (LMPP) that retain full lymphoid and myeloid potential characterized by upregulated expression of the flk2/flt3 cytokine receptor (5). LMPPs can generate myeloid precursors via differentiation into GMPs. LMPPs can also generate lymphoid precursors via differentiation into common lymphoid progenitors (CLP) where IL-7R $\alpha$  is expressed on the cell surface indicating lymphoid lineage specification as CLPs have very little to no myeloid potential (3, 5, 9). In addition, MPPs or LMPPs can emigrate from the bone marrow and seed the thymus differentiating into early thymic progenitors (ETP) to generate T lineage cells (10-12). The model of hematopoiesis presented here reflects current state of research where most of the work done is in the murine hematopoietic system. This model will most likely change to incorporate new findings (1). For example, new studies are aimed at identifying factors such as long non-coding RNAs (13-17), microRNAs (18-23), and epigenetic factors (24-28), that are involved in the regulation of hematopoiesis. In addition, new studies are focused on understanding whether all HSCs have equal potential to give rise to all downstream lineages or whether heterogeneity in HSCs exist such that specific HSCs are already primed for commitment towards a specific lineages only (29-31). Another emerging theme is based on findings that LT-HSC can directly

respond to TLR agonists and interferons which are produced during chronic inflammation (32-34). Therefore, new studies are aimed at examining the effects of chronic inflammatory diseases to long-term HSC self-renewal and HSC function *in vivo*. Thus, hematopoiesis is an ideal model system for understanding the molecular mechanisms involved in tissue regeneration, developmental plasticity and lineage fate decisions (35).

## **1.2 LONG-TERM HEMATOPOIETIC STEM CELLS (LT-HSCS)**

### **1.2.1 A Brief Historical Perspective**

The idea of a common precursor or what is now recognized as HSCs, from which all blood cells originate, was first postulated by A. Maximow in 1909 (36). A few decades later, Lorenz et. al discovered that radiation lethality is primarily due to bone marrow failure and this finding was the first tangible evidence for the existence of HSCs. Lorenz et. al showed that lethal effects of radiation-exposure can be prevented upon injection of spleen or bone marrow (BM) cells from unexposed donors in mice and guinea pigs (37). Seminal work by Till and McCulloch established the spleen colony forming assay, a functional assay establishing the concept that HSCs are the only cell population in the BM uniquely capable of multi-lineage differentiation and self-replication (7, 38, 39). The spleen colony forming assay involves transplantation of limiting number of syngeneic bone marrow cells into recipient mice resulting in the formation of colonies, macroscopic clusters of cells deriving from a single progenitor, which can be readily

counted in the recipients' spleen after 7-14 days. Although it has now been shown that the cells that actually form colonies are not HSCs per se (40), the concept of using experimental assays to retrospectively identify HSCs through its function was a major technical advancement. To date, functional assays that establish [1] long term self-renewal and [2] multilineage reconstitution still remain as the gold standard in identifying long-term hematopoietic stem cells from other multipotent progenitor cells. Since then, technical developments especially over the last 30 years in the fields of genetics, molecular biology, mouse transgenics, monoclonal antibody generation, and flow cytometry technology as summarized in (41) and (42), have significantly advanced HSC research.

## **1.2.2 Identification of Long-Term Hematopoietic Stem Cells; Phenotypic and Functional Approaches**

### **1.2.2.1 Phenotypic Identification of Long-Term Hematopoietic Stem Cells**

The use of antigen-specific monoclonal antibodies conjugated to fluorophores, in combination with fluorescence-activated cell sorting (FACS), has become the standard technique used in isolating certain cell populations in the bone marrow prospectively including LT-HSCs (43), multipotent progenitors (5) and various other immune cell populations (44). Using these surface markers, research over the last ten years has seen the field progress from the isolation of cell populations to the isolation of single cells such that mechanistic studies are focused on examining individual HSCs and not on a highly heterogeneous population of cells (31, 45, 46). By extension, the use of a defined set of surface markers to prospectively isolate and/or identify a specific cell population has also been used in other tissues. Specific examples include studies in

gallbladder (47), bronchiolar (48) and cancer stem cells (49), as well as studies involving hepatocyte progenitor cells (50) and liver regeneration studies (51).

In the murine bone marrow, all progenitor cells including LT-HSCs can be found in the LSK (LSK: Lin<sup>-</sup>Sca-1<sup>+</sup> c-kit<sup>+</sup>) fraction. These cells are characterized as negative for expression of all lineage markers (Lin<sup>-</sup>: lacking expression of NK1.1, Ter119, Gr-1/Ly6G, CD11b/Mac1, CD11c, CD3, CD4, CD8, CD19, CD45R/B220) while expressing stem cell antigen-1 (Sca-1) and the c-kit tyrosine kinase receptor for stem cell factor (52, 53). However, the LSK fraction of the bone marrow is a highly heterogeneous population of progenitor cells consisting mostly of MPPs and short-term HSCs; with LT-HSCs representing only a very small fraction (>10%) of cells in the LSK compartment (43, 54). Additional markers have been used to enhance the purity of LT-HSCs within the LSK fraction. The absence of expression of tyrosine kinase receptor flk2/flt3 differentiates total HSCs, containing both LT-HSCs and ST-HSCs (total HSCs: flk2<sup>-</sup> LSK), from MPPs within the LSK compartment (MPP: flk2<sup>+</sup> LSK) (5, 55). More recently, the inclusion of SLAM (signaling lymphocyte activation molecule) family members CD150, CD48, CD229 and CD244 in the antibody panel to identify HSCs via surface marker phenotype has further improved enrichment for LT-HSCs within the LSK fraction (43, 54). LT-HSCs are in the CD150<sup>+</sup>CD48<sup>-</sup> compartment of LSKs and are negative for CD244 expression. Differential expression of CD229 further subdivides LT-HSCs into two fractions, HSC-1 (CD229<sup>-</sup>) and HSC-2 (CD229<sup>+</sup>), wherein HSC-1 cells are more quiescent and have greater self-renewal potential than HSC-2 cells (54). In addition, analysis of CD150 expression together with flk2/flt3 expression has allowed for the phenotypic differentiation of LT-HSCs (LT-HSC: CD150<sup>+</sup> flk2<sup>-</sup> LSK) from ST-HSCs (ST-HSC: CD150<sup>-</sup> flk2<sup>-</sup> LSK) and MPPs (MPP: CD150<sup>-</sup> flk2<sup>+</sup> LSK) within

the LSK compartment (56). Another strategy utilized to enrich for HSCs is via staining of murine bone marrow with fluorescent dyes such as Hoechst 33342 (57, 58). A rare group of bone marrow cells, termed as side population (SP) cells, does not retain Hoechst 33342 due to efflux of the fluorescent dye mediated by the ATP-binding cassette (ABC) family of transporters in these cells (59, 60). SP cells identify a group of cells enriched for HSCs, which is comparable to the LSK fraction in the murine bone marrow (61). Thus, the combinatorial expression of a defined set of surface markers can prospectively identify LT-HSCs amongst other cells in the murine bone marrow.

Similar to mice, enriched population of human HSCs can be found in the lineage negative fraction of cells from adult bone marrow as well as fetal cord blood. However, unlike murine HSCs, human HSCs are identified via the expression of the sialomucin transmembrane protein CD34 (62-64). Differential expression of CD38, a cyclic ADP ribose hydrolase, allows for further enrichment of human HSCs within the Lin<sup>-</sup> CD34<sup>+</sup> population (human HSCs: Lin<sup>-</sup> CD34<sup>+</sup>CD38<sup>-</sup>) (65). The expression of glycoprotein CD133 also serves as a positive and/or alternative marker for selection of human HSCs, that largely overlaps with CD34 expression thereby permitting validation of findings using two independent surface markers (66). Recently, it has been shown that expression of the integrin  $\alpha 6$  protein CD49f, allows phenotypic resolution of human LT-HSCs (CD49f<sup>+</sup>) from ST-HSCs (CD49f<sup>-</sup>), for the first time (67). Therefore, expression of a defined set of surface markers can also be used to prospectively isolate human HSCs and this approach is routinely used in the clinic to isolate and enrich for human HSCs from human bone marrow, peripheral blood or banked cord blood for use in hematopoietic stem cell transplantation (HSCT).

Phenotypic identification of LT-HSCs using surface marker expression does not necessarily correlate to identification of bona fide functional LT-HSCs (43). For example, total HSCs phenotypically identified as flk2<sup>-</sup> LSKs contain only ~1 in 50 cells that have long-term self-renewal capabilities (68). In addition, self-renewal potential is enriched to only ~1 in 3 cells using the CD150<sup>+</sup> CD48<sup>-</sup> LSK (43) or CD150<sup>+</sup> flk2<sup>-</sup> LSK definitions (56). The use of adoptive transfer assays to reconstitute the hematopoietic system of sub-lethally irradiated recipients with donor bone marrow cells remains the gold standard assay for identification of functional LT-HSCs albeit retrospectively.

#### **1.2.2.2 Functional Identification of Long-Term Hematopoietic Stem Cells**

While the use of surface markers are especially useful in the phenotypic identification and prospective isolation of LT-HSCs, only functional tests *in vivo* can identify bona fide LT-HSCs through demonstration of long-term self-renewal and multilineage reconstitution potential, two unique properties of LT-HSCs (1, 41). Similarly, functional assays testing for self-renewal and multipotency have also been used in studying other types of stem cells (69-71). In the case of LT-HSCs, the use of adoptive transfer assay allows for the demonstration of these two key properties of LT-HSCs *in vivo*. This technique involves transfer of donor-derived cells containing LT-HSCs into conditioned hosts irradiated to eradicate the recipients' hematopoietic system. The use of congenic donor and recipient mice, for example CD45.1, CD45.2, or expressing both CD45.1 and CD45.2, has made it possible to track donor and recipient-derived cells within the same host. Thus, LT-HSCs can be retrospectively identified via their ability to fully repopulate the hematopoietic system of irradiated hosts reflecting multi-lineage



reconstitution at least 16 weeks after transplantation (41). The extraordinary potential of HSCs was elegantly demonstrated by Osawa et.al., upon transplantation of a single CD34<sup>+</sup> LSK murine cell able to fully reconstitute the hematopoietic system of recipient mice (72). The most stringent analysis of self-renewal potential is examined using serial transplantation assays (41). In mice, it has been shown that LT-HSCs are capable of self-renewal that lasts beyond the normal lifetime of its original donor as shown by ability of LT-HSCs to reconstitute a recipient's hematopoietic system even after passage through as many as 5 recipients (73, 74).

Research, and therefore knowledge of hematopoiesis and HSC function are based mostly on findings from analyses of specific murine mutants and the use of experimental murine transplantation models. Predominance of murine models over human studies in HSC function is mainly due to technical and ethical reasons (75). In the mouse, easy manipulation of the expression of target genes leads to highly specific mechanistic gain-of-function and loss of function studies. It is also possible to use inbred murine strains for bone marrow transplantation assays resulting in reproducible studies of *in vivo* HSC function. However, there are inherent differences between murine and human HSCs in terms of surface marker expression as discussed previously and in cellular behavior during cell cycling and DNA damage responses (64). For example, mouse HSCs divide once every 30–50 days while human HSCs divide every 175-350 days (42). Therefore to study human HSC function *in vivo*, xenogeneic transplantation models have been developed whereby human HSCs are transplanted in recipient mice which are immune-deficient mice, genetically engineered to be able to sustain human hematopoiesis (42). For example, NOD-Scid mice are able to support human cell engraftment due to absence of functional murine T and B cells and low cytotoxic NK cell activity (76). However, while NOD-

Scid mice can support human B lineage and myeloid lineage development, it is unable to support development of human T cells making it difficult to evaluate the full multi-lineage reconstitution potential of human HSCs. Other immune-deficient mouse models have been developed such as the NSG (NOD-Scid-IL2R $\gamma_c^{-/-}$ ) mice (77, 78), or mice expressing non cross-reactive human cytokines such as thrombopoietin, IL-3, GM-CSF (42, 79, 80). In creating these new mouse models, the goal remains to be improvement of human HSC engraftment and improved human chimerism in order to become more suitable hosts in studying human HSC function *in vivo*.

### **1.3 REGULATION OF LT-HSC PROLIFERATION**

#### **1.3.1 The Biological Importance of Regulating LT-HSC Proliferation**

LT-HSCs must differentiate to replace blood cells lost due to natural turnover or unexpected demand. At the same time LT-HSCs must also self-renew to maintain LT-HSCs numbers allowing hematopoiesis to continue throughout an organism's lifetime (81, 82). Adult LT-HSCs reside in BM niches in a quiescent/G<sub>0</sub> state to preserve LT-HSC longevity and function (83). Excessive proliferation or loss of LT-HSC quiescence can lead to defects in LT-HSC self-renewal resulting in loss of LT-HSCs and eventual hematopoietic failure (81, 84, 85). Hyperproliferation can also cause an accumulation of replication-associated mutations that potentially serves as a starting point for oncogenic transformation (86, 87). Thus, it is important that the balance between proliferation and quiescence in LT-HSCs is tightly regulated.

Numerous studies have elucidated several different factors including cell cycle regulators, transcription factors, growth factors, and even microRNAs that control LT-HSC proliferation in mice (reviewed in (81), (82), and (85)). Table 1 shows a summary of the major identified factors that act as positive or negative regulators of HSC proliferation. These factors can also be subdivided into cell-intrinsic or cell-extrinsic regulators depending on whether these factors are acting within the stem cell itself (cell-intrinsic) or within the microenvironment/BM niche (cell-extrinsic) and are comprehensively reviewed in (88), (85) and (82).

**Table 1. Summary of Several Identified Factors that Regulate HSC Proliferation**

<b>Category</b>	<b>Effect</b>	<b>Name of quiescent regulators</b>
Extrinsic regulators	Positive regulators	Bone-lining osteoblastic cells and stem cell factor/C-kit signaling, Tie2/Ang-1 signaling as well as TPO/MPL signaling Hypoxic environment Other matrix components, N-cadherin, integrins, osteopontin Ca <sup>2+</sup> ions Transforming growth factor $\beta$ Wnt signaling pathway
	Negative regulators	Hedgehog (Hh) signaling pathway
Intrinsic regulators	Positive regulators	Transcription Factors: Gfi-1, Pbx1, p53, Scl, Irf2, TXNIP, Nurr1, GATA-2 Cyclin-dependent kinase inhibitors: p21, p57 Others: Cdc42, Fbw7, pTEN, PML, TSC1, ATM, FoxOs, STAT5, Rb, Lkb1, Mi-2b, and Wnt5
	Negative regulators	MEF/EL4  Lnk c-Myc

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### 1.3.2 Regulation of LT-HSC Proliferation by Cell Cycle Regulators

It is unsurprising that the genetic disruption of factors involved in cell cycle regulation can result in perturbations in LT-HSC proliferation, as these factors are also expressed and functional in these cells. One of the first cell cycle regulators identified to regulate HSC proliferation is the cyclin-dependent kinase (CDK) inhibitor p21, a member of the Cip/Kip family of CDK inhibitors. Cheng et.al, demonstrated that p21 deficient mice had increased frequency of cycling HSCs with a concomitant decrease in the frequency of HSCs in the quiescent phase (84). Further, p21<sup>KO</sup> HSCs were more sensitive to the mitotoxic drug 5-FU, failed to persist long-term in the bone marrow, and were unable to reconstitute recipient mice in a serial adoptive transfer assay (84). Like p21, p27 is also a member of the Cip/Kip family of CDK inhibitors. Unlike p21, absence of p27 in HSCs does not affect HSC proliferation or HSC numbers but rather resulted in enlarged progeny pool size (89). In addition to p21 and p27, the Cip/Kip family also includes p57, which also plays a role in the regulation of HSC proliferation. Conditional deletion of p57 resulted in a reduction in both HSC numbers and frequency of quiescent HSCs. Similar to p21<sup>KO</sup> HSCs, deletion of p57 in HSCs also resulted in poor reconstitution in recipient mice after transplantation (90). In addition, conditional deletion of both p21 and p57 showed a more severe phenotype than deletion of p57 alone (90), indicating that there is functional overlap in the regulation of HSC proliferation, which was also demonstrated by a compensatory role for p27 in the absence of p57 (91). Indeed, the conditional deletion of all three retinoblastoma proteins pRb, p107 and p130, also resulted in hyperproliferation of HSCs and severe defects in HSC self-renewal and reconstitution of recipient mice (92). Lastly, unlike previously discussed cell cycle regulators above, the expression of the cyclin dependent kinase inhibitor p16<sup>INK4a</sup> in HSCs have been shown to significantly increase with age (93) and is associated with onset of senescence

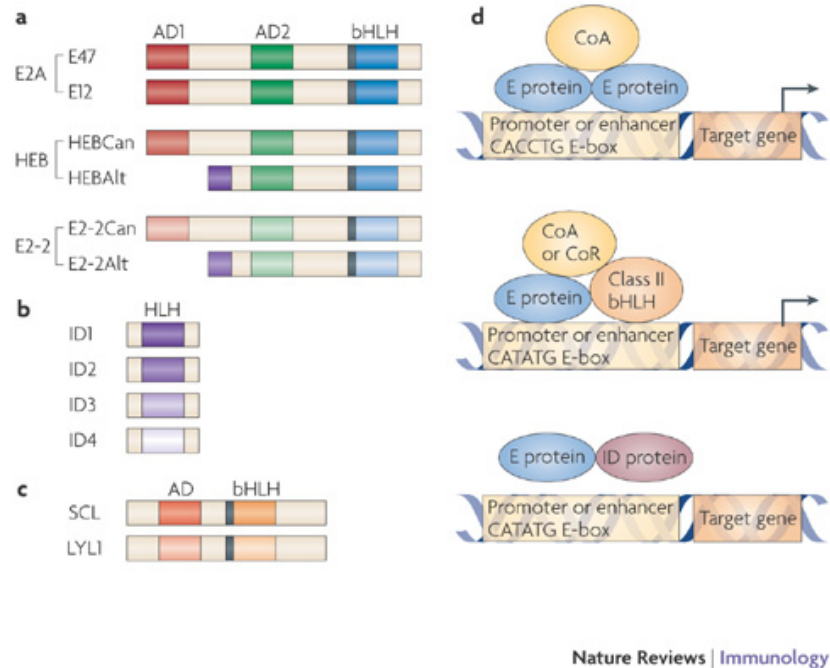
(94, 95). While these studies demonstrate that cell cycle regulators play a role in regulating LT-HSC proliferation, the expression of these cell cycle regulators themselves are controlled by transcription factors, thus providing another level of regulatory control.

### **1.3.3 Regulation of LT-HSC Proliferation by Transcription Factors**

Transcription factors regulate many developmental and homeostatic processes in both vertebrates and invertebrates (96). Thus, transcription factors also represent another group of factors that play an important role in regulating LT-HSC proliferation and maintaining LT-HSC integrity, since an organism's survival and longevity is dependent on a functional and robust hematopoietic system wherein LT-HSCs are the sole source of all hematopoietic cells throughout life. Examples of transcription factors important to LT-HSC proliferation include zinc finger transcriptional repressor *Gfi1*, wherein mice deficient in *Gfi1* had increased HSC proliferation and poor reconstitution of recipients (97). Similarly, haploinsufficiency of the transcription factor *SCL/TAL1* in HSCs also resulted in increased proliferation and impaired long-term reconstitution (98). More recently, studies have shown that genetic deletion of other transcription factors such as *Pbx1*, *p53*, *IRF-2*, *Nurr1*, and *Dmtf1* in mice all resulted in the loss of HSC quiescence and loss of HSC function (99-103). E proteins are a family of transcription factors, and one of its members, E47, play an important role in lineage fate decision. Moreover, the transcription factor E47 is also an important regulator of HSC proliferation and self-renewal.

## 1.4 THE TRANSCRIPTION FACTOR E47

### 1.4.1 E47 is a Member of the Class I bHLH Family of E proteins



**Figure 1-2. E47 is a member of the Class I family of E proteins.**

(A) Schematic diagram of E proteins showing the key protein domains — activation domain 1 (AD1), AD2 and basic helix–loop–helix (bHLH) domain. Alternative transcription start sites give rise to canonical (Can) and alternative (Alt) forms of HEB and E2-2 proteins. (B) The four inhibitor of DNA binding (ID) proteins are shown. (C) The domains of the class II bHLH proteins stem-cell leukemia factor (SCL; also known as TAL1) and lymphoblastic leukemia 1 (LYL1) are shown. Differences in shading signify sequence homology. (D) Schematic diagram of different E protein dimers and their potential functions. *Top*: E protein homodimers that are bound to the CACCTG E-box sequence in target genes function as transcriptional activators through the recruitment of co-activators (CoA) such as p300. *Middle*: E protein–class II bHLH heterodimers that are bound to the CATATG E-box sequence in target genes function as transcriptional activators or repressors depending on the proteins that are recruited (co-activators

or co-repressors (CoR, such as ETO or mSin3A)) and on neighboring transcription factors. *Bottom:* E protein-ID protein heterodimers fail to bind DNA and do not activate gene transcription. Figure reprinted with permission from Nature Publishing Group, Copyright 2009. Kee, B.L. “E and Id proteins branch out”. *Nature Reviews Immunology*. 2009.

E47 is a transcription factor that is a member of the class I basic helix loop helix (bHLH) family of E proteins (**Figure 1-2**). E proteins are characterized by the ability to bind to the E-box consensus sequence CANNTGG in the promoter or enhancer regions of its target genes (104, 105). E47 is transcribed from the E2A (*Tcfe2a*) gene which, upon alternative splicing can produce E47 or E12 proteins, the seminal difference being in the DNA binding domain (106) with E47 binding the E box site with a higher affinity than E12 (107). E47 is expressed in B cells as homodimers but can heterodimerize with other Class I E protein family members such as HEB in T cells through protein-protein interactions between the two helix loop helix domains (105). E47 can also dimerize with antagonistic ID proteins such as ID2 and ID3 that act as dominant negative inhibitors of E47 activity through the formation of a dimer that is unable to bind to DNA due to the absence of the basic DNA binding domain in Id proteins (96, 105). Thus, transcriptional regulation of target genes by E47 is dependent on dimerization partners of E47.

#### **1.4.2 The Role of E47 in Lymphoid Lineage Development**

E47 is a critical transcription factor in lymphoid lineage development and its role in lymphocyte development has been extensively studied in mice. E47-deficient mice have a block in B cell development at the pre-pro-B cell stage prior to B lineage commitment (108-110) and residual progenitors are developmentally plastic (111). Even in mature B cells, conditional disruption of

E47 prompts lineage reprogramming and Hodgkin lymphomagenesis (112), indicating an ongoing requirement for E47 activity in sustaining B cell identity.

In E47-deficient mice, T cell development is partially impaired (113) because within T cell precursors, E47 heterodimerizes with HEB to induce T cell-specific genes and repress genes associated with the natural killer cell fate (114-116). In the absence of E47, E47-deficient mice also develop fatal thymomas of double negative origin (113). By contrast to B and T cells, E47 activity is dispensable in myeloid cells (109). In early myeloid precursors, E47 is antagonized by inhibitor of differentiation (Id) proteins that recruit E47 into functionally inert heterodimers (105). Id levels remain high in mature myeloid cells (117-119).

E47 is an essential factor in B cell development because it initiates the expression of EBF, which in turn activates Pax-5 expression (120-122), while both E47 and EBF bind to regulatory elements in Foxo1 locus (123), all of which results in specification of B lineage fate. This transcriptional cascade leads to the expression of lineage specific genes required for B cell development such as *mb-1*, *λ5*, *VpreB*, *IL7R*, *Rag1*, *Rag2*, and *CD19* (124, 125). E47 is also involved in repression of genes associated with other lineage fates, such as *gata1*, *epo-r* and *c-fms* which are required for myeloid and erythroid development respectively (111). In addition, E47 activity is required to activate *rag1* expression to initiate V(D)J recombination in CLPs (110), and early B (126) and T precursor cells (113, 127). Thus in order to promote lymphoid lineage development, E47 has three main functions within CLPs: [1] initiate a transcriptional cascade to promote B lineage fate, [2] repress activation of genes associated with alternative lineages, and [3] activate *rag* expression to initiate V(D)J recombination of the antigen receptor gene.



In humans, studies involving E47 and ID proteins have led to findings in agreement with mouse models that E47 activity is required in lymphoid development but not for myeloid or NK cell development. For example, in the human thymus where bipotential T/natural killer (NK) progenitors are present, E47 activity is important for progression into T lineage development as dominant negative inhibition of E47 activity through interaction with ID3 led to NK over T cell development (128). Similarly, overexpression of ID3 in multipotent CD34<sup>+</sup> CD38<sup>-</sup> fetal liver cells led to inhibition of B cell development but not myeloid or NK cell development (129). Collectively, these studies underscore the importance of E47 activity in lymphoid lineage development in both mouse and man.

### **1.4.3 E47 and the Development of Specific Blood Cancers In Mouse and Man**

Several studies in mice have shown a role for E47 as a tumor suppressor. Mice lacking functional E47 developed T cell lymphomas as early as 75 days after birth and ectopic expression of E47 in these cells induced apoptosis (130). In addition, enforced expression of inhibitors of E47 such as Id1 and Id2 in T cell precursors also resulted in the development of T lymphomas (131-133).

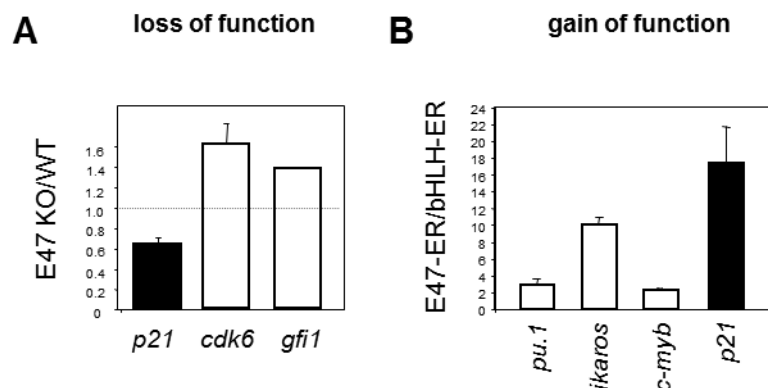
In humans, E47 is linked to certain types of acute lymphoblastic leukemia, as well as the B cell cancer Hodgkin's lymphoma. For example, chromosomal translocation (t17;19) replacing the E47 basic helix loop helix region with HLF, a gene encoding a leucine zipper resulted in pro-B cell leukemia (134). A chimeric E2A-pbx fusion protein resulting from a chromosomal translocation involving E2A gene (t1;19) where the 5' portion of the E2A gene is fused to the

homeodomain-containing region of the *pbx1* gene also resulted in pre-B cell leukemia (135, 136)}. In certain patients with T acute lymphoblastic leukemia, a gain of function mutation resulted in activation of another bHLH protein TAL1 resulting in inhibition of E47 activity (137, 138). Approximately 70% of patients suffering from a T cell lymphoma subtype known as Sezary syndrome had recurrent E2A gene deletion (139). In classical Hodgkin's lymphoma, overexpression of the E47 antagonist Id2 resulting in loss of E47 activity is mechanistically linked to B-cell derived lymphomas (112). Together, these studies suggest a tumor suppressive activity for E47 and indicate that the loss of E47 activity may be an important event in the development of specific lymphoid cancers.

#### **1.4.4 The Role of E47 in LT-HSCs and Multipotent Progenitors**

We have shown that E47 is expressed in common lymphoid progenitors (CLPs) and that E47 deficiency results in reduced CLP numbers (110). This demonstrates that the defects due to E47 deficiency are not only limited to the development of B and T lineage precursors but is also manifested in the defective development of CLPs (110). More recent findings from our laboratory and others have shown that the E47 protein is also expressed in developmental subsets upstream of CLPs, specifically in HSCs, MPPs and LMPPs (12, 56, 140). Furthermore, our data indicates that the E47 protein is present in total LSKs, a heterogeneous population containing both total HSCs and MPPs, and that protein expression increases during developmental progression to B lineage cells (140). This is in agreement with published reports using E2A-GFP knock-in mice to detect E2A expression (141, 142). These findings prompted us to a closer examination of E47-deficient mice focusing on the hematopoietic stem cell and multipotent progenitor compartments, the former of which is the sole source of self-renewing progenitors.

E47-deficiency resulted in LMPPs with poorly primed lymphoid lineage genes indicating a defect in lymphoid lineage progression. More importantly, E47-null total HSCs have increased proliferation and loss of quiescence. Analysis of total HSC function *in vivo* using adoptive transfer assays, showed that E47-null HSCs have poor self-renewal capabilities and as a consequence, recipient mice that received donor BM from E47-deficient mice had defective multilineage reconstitution compared to recipient mice that received WT donor bone marrow (143). Thus, these studies indicate that E47 is required for efficient LT-HSC self-renewal and maintenance.



**Figure 1-3. The transcription factor E47 regulates the expression of cell cycle regulator p21**

(A) p21 expression is downregulated by ~50% in E47-deficient HSC-enriched LSKs. WT or E47 KO LSKs were examined for p21 expression by QPCR. Data were normalized to  $\beta$ -actin and represents the mean of four independent analyses from three independent sorts (B) p21 expression is upregulated in the context of enforced E47 expression. Lineage<sup>neg</sup> cells were transduced with either E47-ER-huCD25 or control bHLH-ER-huCD25 retrovirus lacking the transactivation domain. Cells were incubated with 4-OHT for 6 hours to activate E47 expression. Positively transduced cells (huCD25<sup>+</sup>) were sorted, and cDNA was prepared from extracted mRNA. QPCR was used to measure p21 levels. Data were normalized to  $\beta$ -actin. Results are representative of three independent experiments. Figure reprinted with permission from the

American Association of Immunologists, Copyright 2008. Yang, Q., et.al, “E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors,” published in *The Journal of Immunology*, vol. 181, pp. 5885-5894, 2008 *Journal of Immunology*. 2008.

Data from our studies and others indicate that the cell cycle inhibitor p21 is an E47 target gene (56, 140, 143). We have shown by loss-of-function and gain-of-function assays that E47 directly activates p21 transcription in HSCs (**Figure 1-3**) (140, 143). In addition to HSCs, E47 also regulates p21 expression in both precursor B cell and T cell compartments (115, 144), which is a striking contrast to the developmental stage- and/or lineage specificity of most other E47 regulated genes. Further supporting the role of p21 as an E47 target gene are findings that p21 promoter region contain E-box sites that can physiologically bind to E47 (145). Thus, the recurrent cooperation of E47 and p21 in multiple hematopoietic compartments hints at the fundamental importance of the E47-p21 pathway in LT-HSCs as well as in other hematopoietic compartments.

## 2.0 INTRODUCTION TO THE PROJECT AND STATEMENT OF THE PROBLEM

Several studies have identified multiple different pathways involving various transcription factors and cell cycle regulators that control HSC proliferation. Amongst the factors previously identified are the transcription factor E47 and the cell cycle regulator p21. In the absence of E47, HSCs are functionally exhausted due to a loss of G<sub>0</sub> quiescence, hyperproliferation, and reduced expression of cell cycle regulators including *p21* (56, 140, 143). The p21 cyclin-dependent kinase inhibitor is also a major regulator of LT-HSC self-renewal and integrity under stress conditions (82, 84, 146). Following hematopoietic repopulation challenge or DNA damage, p21-deficient LT-HSCs exhibit loss of quiescence, hyperproliferation, and a 50% reduction in repopulating units (RU) (84, 147). In contrast to a role in stress hematopoiesis, p21 appears to be dispensable for steady-state HSC maintenance (148). The pathways by which p21 is activated in LT-HSCs are not known but appear to be p53-independent in the contexts of radiation damage and serial repopulation stress (147). We have shown by gain-of-function and loss-of-function studies that E47 directly activates p21 in HSCs (140, 143). However, the relevance of the E47-p21 pathway to HSC function *in vivo* has not yet been examined. Whether the E47-p21 pathway is biologically important in maintaining LT-HSC integrity under transplantation stress also remains to be seen. In further establishing the mechanisms of how E47 regulates LT-HSC proliferation to preserve LT-HSC integrity, it is also of great interest to us to determine what other cell cycle-associated factors are regulated by E47 in LT-HSCs. *Our central hypothesis is*

*that E47 acts as a cell-intrinsic factor that preserves LT-HSC self-renewal and prevents loss of LT-HSC integrity associated with hyperproliferation by controlling LT-HSC proliferation through the regulation of the expression of specific cell cycle associated factors in LT-HSCs.*

In Aims 1 and 2, we will establish the biological importance of the E47-p21 pathway in LT-HSC function during homeostasis and under transplantation stress. In Aim 3, we will determine if the nuclear protein p15<sup>PAF</sup> is a novel E47 target gene, whose expression, like p21, is also regulated by E47.

## 2.1 SPECIFIC AIMS

**Specific Aim 1:** Examine the effect of E47-p21 pathway in LT-HSCs in intact animals during homeostasis by using mice with reduced gene dosage for both E47 and p21 in tandem (E47<sup>het</sup>p21<sup>het</sup>) compared to wild-type (WT) and single heterozygous controls (E47<sup>het</sup> and p21<sup>het</sup>). Using these mice, we will examine the expression of E47 and p21 in total HSCs to look at relative contribution of E47 in the regulation of p21 expression. To investigate the specific effect of compound E47 and p21 heterozygosity in LT-HSCs in intact animals, we will determine the phenotypic numbers of HSCs and multipotent progenitors in the bone marrow. We will also examine the proliferation status of LT-HSCs in E47<sup>het</sup>p21<sup>het</sup> mice during steady-state conditions and determine the effect of mitotoxic drug 5-FU in E47<sup>het</sup>p21<sup>het</sup> total HSCs.

**Specific Aim 2:** Examine the biological relevance of the E47-p21 pathway to LT-HSC function *in vivo*. We will examine the effects of combined heterozygosity in E47 and p21 on self-renewal

and multi-lineage reconstitution potentials of LT-HSCs using serial transplantation assay. This is a rigorous test of LT-HSC function *in vivo* where donor LT-HSCs are forced to reconstitute the hematopoietic system of irradiated recipient mice through three rounds of serial transfer. We will also examine the relative contribution of E47-p21 pathway in the development of BM progenitors upstream versus downstream of lympho-myeloid commitment.

**Specific Aim 3:** Determine if p15<sup>PAF</sup> is a novel E47 target gene. Early hematopoietic defects observed during homeostasis in HSCs derived from E47<sup>KO</sup> mice are very similar to defects observed in HSCs from p15<sup>PAF</sup> KO mice, suggesting a possible relationship between the transcription factor E47 and p15<sup>PAF</sup>, a 15 Kda nuclear protein. In addition, analysis of the p15<sup>PAF</sup> promoter region revealed several consensus CAANTG E-box sites which may serve as potential binding sites for E47 in regulating p15<sup>PAF</sup> expression. To determine if p15<sup>PAF</sup> is a novel E47 target gene, we will first determine if p15<sup>PAF</sup> promoter mediated transcription is activated by E47 using a luciferase reporter assay. We will also examine p15<sup>PAF</sup> mRNA and protein levels of p15<sup>PAF</sup> in E47-deficient HSCs.

## 2.2 SIGNIFICANCE AND RELEVANCE

Impaired LT-HSC function has severe consequences for human health since LT-HSCs are the sole source of all blood cells throughout life. First, loss of LT-HSC self-renewal and/or multilineage reconstitution capabilities can result in eventual BM failure and lethality (42). Second, the acquisition of hyperproliferation or disruption of mechanisms controlling HSC proliferation can be a starting point for additional mutations transforming HSCs or its downstream progeny into cancer stem cells (149). Studies in acute myeloid leukemia (AML) have characterized and shown the existence of leukemic stem cells, which are suggested to be the causative agent of the disease (150, 151). Also, loss of function studies of several genes associated with leukemia such as *runx1*, *pten* and *cdc42* have all shown dysregulation in HSC proliferation (152, 153) further emphasizing how perturbations in the mechanisms controlling HSC proliferation can become permissive to cancer (154). Third, loss of LT-HSC functional integrity may also contribute to the overall process of aging. This is supported by findings that aging is associated with decreased marrow cellularity, myeloid lineage skewing of blood cell output, and a blunted response generated by immune effector cells (81, 149, 155, 156). Furthermore, it has been shown that LT-HSC function is impaired with age, as HSCs from old mice have poorer multilineage reconstitution potential than LT-HSCs from young mice in serial transplantation assays (157). Together, these studies indicate that LT-HSC function decreases with age, despite an increase in cellularity, resulting in less robust hematopoietic potential in aged individuals. Thus, it is important to establish the mechanisms controlling LT-HSC proliferation which has important direct implication to LT-HSC integrity. Understanding these mechanisms can lead to



improvement of LT-HSC function and/or reverse the deleterious effects on hematopoiesis due to loss of LT-HSC integrity for future therapeutic application. For example, a major clinical problem is that human HSCs are a very rare population of cells routinely used for BM transplantation. Human HSCs are obtained either from banked cord blood, directly from the bone marrow, or through mobilization of HSCs to the periphery via stimulation with Granulocyte-Colony Stimulating Factor (G-CSF). Therefore, expansion of HSCs without affecting HSC quality can improve the therapeutic effect of BM transplantation by avoiding problems associated with limiting numbers of HSCs and/or loss of high quality HSCs available for transplant use. Lastly, the mechanisms involved in regulation of HSC self-renewal and maintenance of functional integrity may also be applicable to other types of stem cell systems.

### **3.0 CELL-INTRINSIC *IN VIVO* REQUIREMENT FOR THE E47-P21 PATHWAY IN LONG-TERM HEMATOPOIETIC STEM CELLS**

Chapter 3 is adapted from the *Journal of Immunology* manuscript “Cell-Intrinsic *In Vivo* Requirement for the E47-p21 Pathway in Long-Term Hematopoietic Stem Cells” Santos, PM, Ding Y, and Borghesi, L; *published ahead of print November 20, 2013, doi:10.4049/jimmunol.1302502*. Copyright 2013. The American Association of Immunologists, Inc. Copyright permission is kept on file with Patricia M. Santos.

### 3.1 ABSTRACT

Major regulators of long-term hematopoietic stem cell (LT-HSC) self-renewal and proliferation have been identified but knowledge of their *in vivo* interaction in a linear pathway is lacking. Here, we show a direct genetic link between the transcription factor E47 and the major cell cycle regulator p21 in controlling LT-HSC integrity *in vivo* under repopulation stress. Numerous studies have shown that E47 activates p21 transcription in hematopoietic subsets *in vitro* and in this study, we now determine the *in vivo* relevance of the E47-p21 pathway by reducing the gene dose of each factor individually (E47<sup>het</sup> or p21<sup>het</sup>) versus in tandem (E47<sup>het</sup>p21<sup>het</sup>). E47<sup>het</sup>p21<sup>het</sup> LT-HSCs and downstream short-term HSCs (ST-HSCs) exhibit hyperproliferation and preferential susceptibility to mitotoxin compared to wild-type or single haploinsufficient controls. In serial adoptive transfers that rigorously challenge self-renewal, E47<sup>het</sup>p21<sup>het</sup> LT-HSCs dramatically and progressively decline, indicating importance of cell-intrinsic E47-p21 in preserving LT-HSCs under stress. Transient numeric recovery of downstream ST-HSCs enabled the production of functionally competent myeloid but not lymphoid cells as common lymphoid progenitors (CLPs) were decreased and peripheral lymphocytes virtually ablated. Thus, we demonstrate a developmental compartment-specific and lineage-specific requirement for the E47-p21 pathway in maintaining LT-HSC, B and T cells under hematopoietic repopulation stress *in vivo*.

## 3.2 INTRODUCTION

A limited pool of LT-HSCs constantly replenishes downstream lymphoid and myeloid lineages that have been depleted by normal turnover or consumed by infection stress (158). Self-renewing LT-HSCs differentiate to non-renewing multipotent progenitors (MPP) and then lymphoid-myeloid primed progenitors (LMPP), which subsequently produce common lymphoid progenitors (CLP) with B and T lymphoid potential (5, 159). The E47 transcription factor, in conjunction with transcriptional partners, regulates key aspects of hematopoiesis including HSC self-renewal, lymphoid lineage priming, B and T cell fate specification and antigen receptor repertoire formation (35, 96).

Recent progress has defined the E47-dependent genes that coordinate cell activity in both developmental stage-specific and lineage-specific manners (12, 56, 110, 123, 140, 143, 160). In striking contrast to the cohort of E47 target genes that are unique to individual developmental subsets, one recurrent target of E47 binding activity stands out: the major cyclin dependent kinase inhibitor p21 (*cdkn1A/cip1/waf1*). We and others have shown that E47 directly activates p21 transcription in primitive HSCs *in vitro* (56, 140, 143). In addition, E47 levels correlate with p21 expression in B lineage precursors, and loss of a single allele of E47 reduces p21 with concomitant hyperproliferation (144). Similarly, p21 is also a direct target of E47 transcriptional activity in T lineage cells (115). However, while these *in vitro* studies indicate that E47 activates p21 expression in HSCs and lymphoid precursors, the biological relevance of the E47-p21 pathway to HSC function has not yet been examined. Furthermore, in addition to E47, other transcription factors also expressed in HSCs such as *ikaros* and *Notch* are capable of regulating p21 expression (161, 162). Hence, the relative contribution of E47 in regulating p21 expression

in HSCs *in vivo* remains unknown. Also unclear is whether the E47-mediated p21 activity is required in the multipotent stages between HSCs and lympho-myeloid segregation.

Here, we establish the biological relevance of genetic interactions between E47 and p21 in LT-HSCs and downstream compartments *in vivo* using mice with reduced gene dosage of each factor, E47<sup>het</sup>, p21<sup>het</sup>, and E47<sup>het</sup>p21<sup>het</sup>. Defects specific to compound haploinsufficient animals, but not either haploinsufficiency alone, reveal combined effects of two interacting partners (163, 164). Moreover, the use of E47<sup>het</sup> mice permits an analysis of mature B and T cells that is not possible in E47 knockouts due to the severe immune deficiency. We directly track LT-HSC self-renewal integrity in compound heterozygotes and examine cumulative deficiencies in downstream B and T compartments. Since E47 is dispensable after the point of myeloid restriction, myeloid precursors serve as an internal reference population for comparing the magnitude of hematopoietic deficiencies incurred upstream versus downstream of lympho-myeloid specification. Together, this approach enables us to establish the biological importance of genetic interactions between E47 and p21 specifically within LT-HSCs, within developmental compartments upstream versus downstream of lympho-myeloid restriction, and the cumulative impact to B cells and T cells.

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Mice

Mice were bred in accordance with Institutional Animal Care and Use Committee (IACUC) policies at the University of Pittsburgh School of Medicine. E47<sup>het</sup> (C57BL/6) mice (143) were intercrossed with p21<sup>KO</sup> mice (129/Sv; purchased from The Jackson Laboratories), and backcrossed to the C57BL/6 background for 7-8 generations. E47 and p21 genotyping were done as described (84, 106).

#### 3.3.2 Flow Cytometry

BM and spleen were harvested as previously described (110, 126). Cell staining was performed using antibodies from eBioscience, BD Pharmingen or Biolegend. Primary antibodies were AA4.1 APC (clone AA4.1), B220 APC, FITC or biotin (clone RA3-6B2), CD3 FITC or biotin (clone 2C11), CD4 biotin (clone GK1.5), CD11b PE, biotin or FITC (clone M1/70), CD11c FITC (clone N418), CD19 APC, biotin, FITC or Cy5PE or PerCPCy5.5 (clone MB19-1), CD43 PE (clone S7), CD45.2 PacBlue or APC (clone 104), CD48 APC (clone HM48-1), CD117 APCeFluor 780 (clone 2B8), CD127 PeCy5 (clone A7R34), CD135 PE (clone A2F10), CD150 PECy7 (clone 9D1), DX5 biotin (clone DX5), Gr-1 biotin or FITC (clone 8C5), IgM (clone 331) biotin or FITC, Ly6C biotin (clone HK1.4), NK1.1 biotin or FITC (clone PK136), TER-119 biotin or FITC (clone TER-119), and Sca-1 FITC, Cy5PE or PerCPCy5.5 (clone D7). Secondary

reagents were streptavidin-Cy7PE or streptavidin-eFluor 450. Flow cytometry was performed on a four-laser, twelve-detector LSR II and a three-laser, eleven-detector Aria (BD Biosciences). Data were analyzed using FlowJo software Version 9.9.1 (Tree Star).

### **3.3.3 Treatment of Mice with 5-FU or LPS**

Mice were injected i.p or i.v with 150 mg/kg 5-FU (Sigma-Aldrich) (140, 143, 165) and sacrificed either 16 hours or 14 days later as described in figure legends. For LPS treatment, mice were injected i.p. with 15 µg LPS (Sigma-Aldrich) or PBS control once a day for two days and were sacrificed on the third day as described (166).

### **3.3.4 BrdU Incorporation and anti-BrdU Staining**

Mice were injected i.p. with 200 µL of 3 mg/mL BrdU at 12-h intervals for 48 hours as described (140, 143). Two days after the first injection, mice were sacrificed and BM cells stained with antibodies to relevant surface markers. BM cells were fixed and permeabilized followed by staining with anti-BrdU FITC per manufacturer's instructions (BD Pharmingen).

### **3.3.5 Intracellular Staining with g-pH2A.X and Caspase-3**

Mice were injected i.v. with 150 mg/kg 5-FU as previously described (166). Fourteen days after treatment, mice were sacrificed and BM cells were enriched for lineage negative cells via lineage depletion using magnetic separation (AutoMACS, Miltenyi Biotech) (140, Yang, 2011 #25). BM cells were stained with antibodies to relevant surface markers and were fixed and permeabilized (Cytofix/Cytoperm, BD Pharmingen) followed by intracellular staining with phospho-histone H2A.X-Alexa 488 (clone 20E3, Cell Signaling) and caspase-3 PE (clone C92-605, BD Pharmingen).

### **3.3.6 Serial Transplantation Assays**

For serial transplantation assays,  $2 \times 10^6$  BM cells from CD45.2<sup>+</sup> donor mice were injected into the tail veins of sub-lethally irradiated (1000 rads) CD45.1<sup>+</sup> C57BL/6 primary recipients. Sixteen weeks after transplantation,  $2 \times 10^6$  BM cells from primary recipients were transferred into sub-lethally irradiated CD45.1<sup>+</sup> secondary recipients. Eight weeks after transplant,  $2 \times 10^6$  BM cells from secondary recipients were transferred into sub-lethally irradiated CD45.1<sup>+</sup> tertiary recipients. Multi-lineage reconstitution was examined every 4 weeks in the peripheral blood and at 16 weeks post-transplant in spleen and BM of primary, secondary and tertiary recipients.

For homing and niche engraftment analysis,  $2 \times 10^6$  CD45.2<sup>+</sup> donor BM cells were injected into the tail veins of sub-lethally irradiated (1000 rads) CD45.1<sup>+</sup> recipients. Recipient mice were sacrificed 2 weeks post-transplant, and donor-derived precursors enumerated.



### **3.3.7 Gene Expression Analysis**

RNA was extracted using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions and reverse transcribed into cDNA with Superscript III Reverse Transcriptase (Invitrogen) using oligoDT primers (110). Quantitative real-time PCR (qPCR) reactions were performed in triplicates using Taqman probes (Invitrogen) and detected by StepOne Plus System (BioRad). Expression levels were calculated for each gene relative to *actb* and expressed as the fold difference relative to wild type (ddC<sub>T</sub> method).

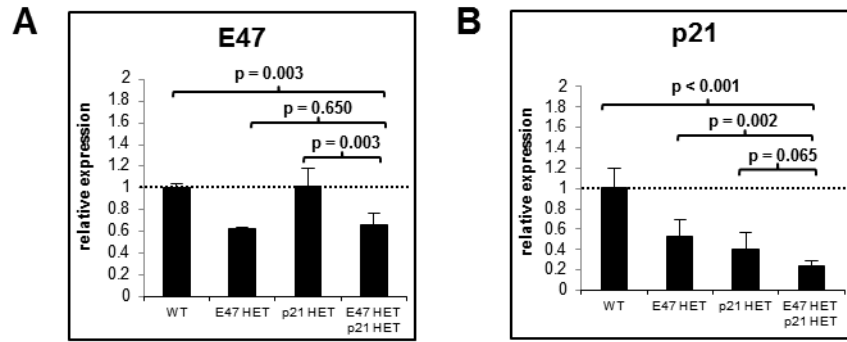
### **3.3.8 Statistics**

Statistical analysis was performed using one-way ANOVA with pairwise comparison. Asterisk (\*) indicates  $p < 0.05$ , ns indicates not statistically significant.

## 3.4 RESULTS

### 3.4.1 E47 and p21 functionally collaborate to regulate homeostatic LT-HSC proliferation *in vivo*

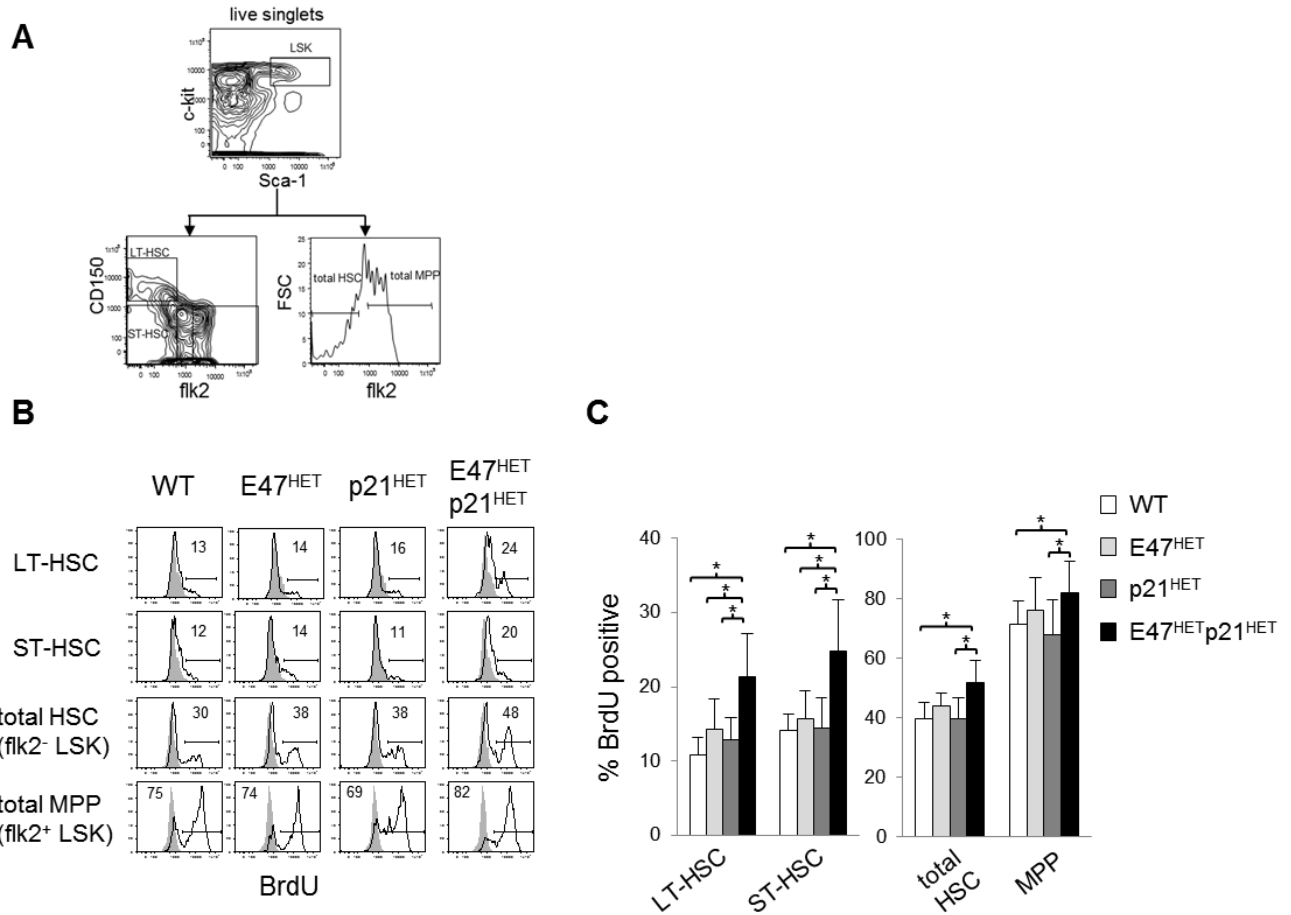
To better understand the biological importance of E47 and p21 collaboration during hematopoiesis, we developed a genetic model in which each gene is haploinsufficient individually, or in combination. We then tracked hematopoiesis during steady-state and under repopulation stress for visualization of defects following functional challenge. The genotypes are wild-type (WT), single haploinsufficiency in E47 or p21 (E47<sup>het</sup>, p21<sup>het</sup>) and compound haploinsufficiency (E47<sup>het</sup>p21<sup>het</sup>). We directly examined E47 and p21 transcript levels in primitive, multipotent lineage negative c-kit<sup>+</sup> Sca-1<sup>+</sup> (LSKs) BM precursors using qPCR. E47 transcript levels were similar in WT and p21<sup>het</sup> LSKs, and were reduced by ~40% in both E47<sup>het</sup> and E47<sup>het</sup>p21<sup>het</sup> LSKs (**Figure 3-1A**). Relative to WT, p21 transcript levels were reduced by 50-60% in both E47<sup>het</sup> LSKs and p21<sup>het</sup> LSKs and by ~75% in E47<sup>het</sup>p21<sup>het</sup> LSKs (**Figure 3-1B**). Thus, E47 activity appears to account for an appreciable proportion of p21 transcript expression. We then compared the impact of single E47 haploinsufficiency versus combined E47-p21 haploinsufficiency to hematopoietic stem and progenitor cell biology.



**Figure 3-1. E47 and p21 expression in WT, E47<sup>HET</sup>, p21<sup>HET</sup> or E47<sup>HET</sup>p21<sup>HET</sup> total LSKs.**

Bone marrow from WT, E47<sup>HET</sup>, p21<sup>HET</sup> or E47<sup>HET</sup> p21<sup>HET</sup> mice was isolated and stained to resolve LSK subset. Total LSKs were sorted and RNA was extracted to examine (A) E47 and (B) p21 transcript levels via QPCR. Gene expression was normalized to  $\beta$ -actin and expression level of each gene is shown relative to WT LSK. Data are shown as mean  $\pm$  SD of triplicates from two independent sorts.

Focusing first on hematopoiesis during homeostasis in intact animals, we examined proliferation of BM progenitors *in vivo*. We pulsed mice with BrdU, a nucleotide analogue that is incorporated during S phase thereby reflecting de novo DNA synthesis, and resolved the sequential developmental subsets long-term renewing LT-HSCs, short-term renewing ST-HSCs, and non-renewing MPPs (**Figure 3-2A**). Bona fide LT-HSCs represent 1% of total LSKs, and complementary phenotypic schemes resolve LT-HSCs to increasing degrees of functional purity. Absence of the flk2 cytokine receptor marks total HSCs, of which 1 in 50 have long-term self-renewal capabilities (68). Self-renewal potential is enriched to  $\sim$ 1 in 3 using the CD150<sup>+</sup>CD48<sup>-</sup> LSK (43) or CD150<sup>+</sup>flk2<sup>-</sup>LSK definitions (56). There is no evidence that E47 transcriptionally regulates the expression of these phenotypic markers, and the mean fluorescence intensity of surface expression of CD150, CD48 and flk2 appears to be preserved in primitive precursors



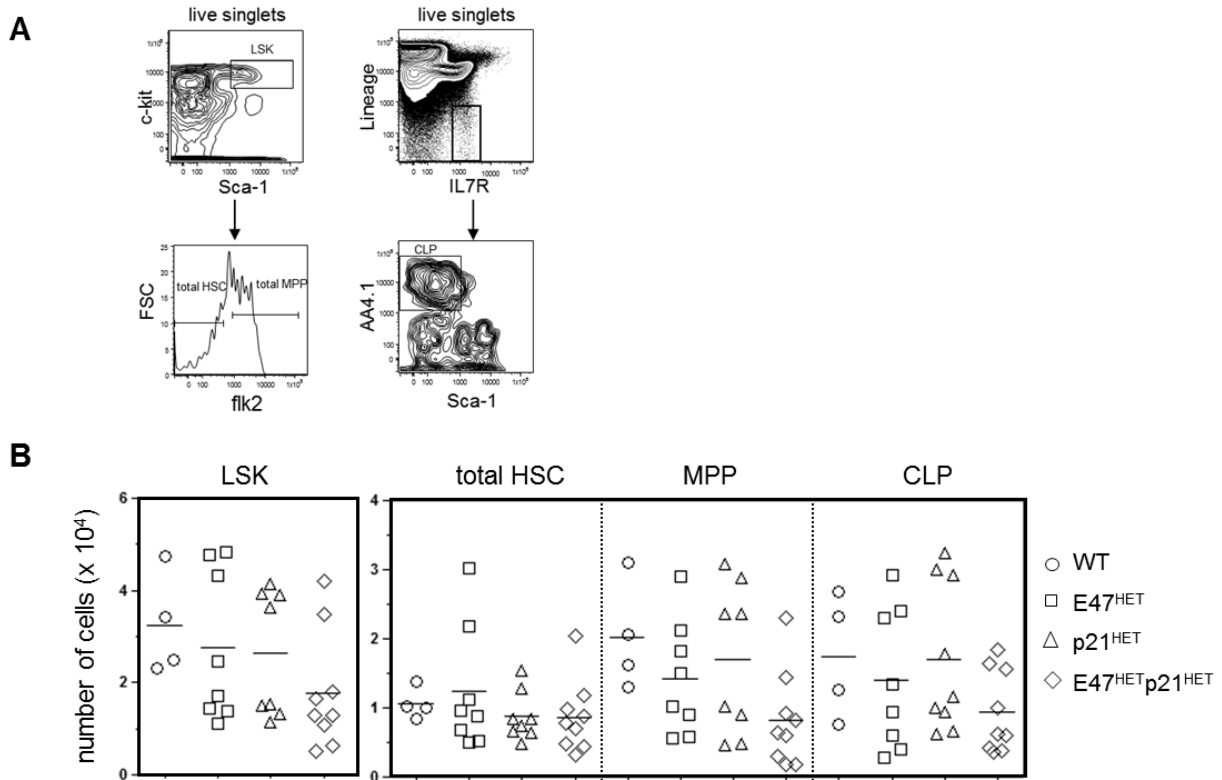
**Figure 3-2. Increased homeostatic proliferation unique to E47<sup>HET</sup> p21<sup>HET</sup> LT-HSCs in vivo.**

Bone marrow from WT, E47<sup>HET</sup>, p21<sup>HET</sup> or E47<sup>HET</sup>p21<sup>HET</sup> mice was isolated and stained to resolve specific subsets of progenitor cells. **(A)** Gating strategy used to identify LSKs (Lineage<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>+</sup>), phenotypic LT-HSCs were identified as CD150<sup>+</sup> flk2<sup>-</sup> LSK, short-term HSCs (ST-HSC) as CD150<sup>-</sup> flk2<sup>-</sup> LSK, total HSCs as flk2<sup>-</sup> LSK and multipotent progenitors (MPP) as flk2<sup>+</sup> LSK. **(B & C)** WT, E47<sup>het</sup>, p21<sup>het</sup> or E47<sup>het</sup>p21<sup>het</sup> mice were injected i.p. with 200  $\mu$ L of 3 mg/mL BrdU twice a day for 2 days then sacrificed to examine proliferation status. **(B)** BM was either stained directly to identify total HSC (flk2<sup>-</sup> LSK) and MPP (flk2<sup>+</sup> LSK); or was enriched for HSCs via depletion of Lineage<sup>+</sup> cells followed by surface marker staining to identify LT-HSC (CD150<sup>+</sup> flk2<sup>-</sup> LSK) and ST-HSC (CD150<sup>-</sup> flk2<sup>-</sup> LSK). Cells were then fixed and permeabilized followed by intracellular staining with anti-BrdU. Flow profiles shown are from one of 4-5

representative experiments. Grey histograms indicate PBS stained control. (C) Bar graph represents mean  $\pm$  SD of data pooled from n=4-6 mice per genotype. \* indicates  $p < 0.05$ .

from E47 knockouts (12, 56, 140, 143). Downstream of LT-HSCs, ST-HSCs are CD150<sup>+</sup>flk2<sup>-</sup> LSKs, and MPPs are flk2<sup>+</sup>LSKs. Here, we explicitly validate our major findings by strategically exploiting all three phenotypic definitions of HSCs rather than relying on any single marker panel.

In intact animals, the percentage of BrdU<sup>+</sup> proliferating E47<sup>het</sup>p21<sup>het</sup> LT-HSCs (CD150<sup>+</sup>flk2<sup>-</sup>LSK;  $21 \pm 5\%$ ), was increased two-fold relative to WT or single E47<sup>het</sup> or p21<sup>het</sup> mice ( $11 \pm 2\%$ ,  $14 \pm 4\%$ , and  $13 \pm 3\%$ , respectively,  $p < 0.05$ , n=4-6 mice/group; **Figure 3-2B&C**). The percentage of BrdU<sup>+</sup> E47<sup>het</sup>p21<sup>het</sup> ST-HSCs (CD150<sup>+</sup>flk2<sup>-</sup>LSK;  $25 \pm 7\%$ ) was also increased relative to controls ( $14 \pm 1\%$ ,  $16 \pm 4\%$ , and  $14 \pm 4\%$ , respectively,  $p < 0.05$ , n=4-6 mice/group; **Figure 3-2B&C**). Proliferation in E47<sup>het</sup>p21<sup>het</sup> MPPs was elevated relative to WT and p21<sup>het</sup>, but was not statistically different than single E47<sup>het</sup> controls. Under these steady-state conditions, absolute numbers of LSKs, total HSC, MPP, and lymphoid-restricted CLP (lineage<sup>-</sup> Sca<sup>lo</sup>IL7R<sup>+</sup>AA4.1<sup>+</sup>) subsets were comparable across all four genotypes (**Figure 3-3**). These data suggest that E47 and p21 interactions may be particularly important within the self-renewing LT-HSC subset.

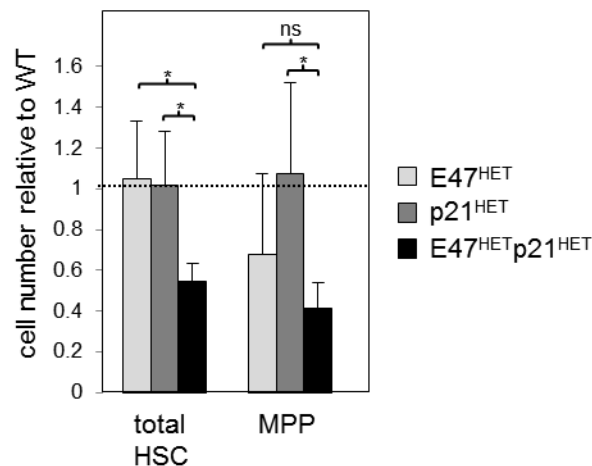


**Figure 3-3. Absolute numbers of different bone marrow multipotent progenitors in E47<sup>het</sup>p21<sup>het</sup> mice are comparable to controls.**

Bone marrow from WT, E47<sup>HET</sup>, p21<sup>HET</sup> or E47<sup>HET</sup>p21<sup>HET</sup> mice was isolated and stained to resolve specific subsets of progenitor cells. (A) Detailed gating strategy used to identify LSKs (Lineage<sup>-</sup>c-kit<sup>+</sup> Sca-1<sup>+</sup>), phenotypic total HSCs were identified as flk2<sup>-</sup> LSK, multipotent progenitors (MPP) as flk2<sup>+</sup> LSK, and common lymphoid progenitors (CLPs) were identified as Lin<sup>-</sup> IL7R<sup>+</sup> AA4.1<sup>+</sup> Sca-1<sup>low</sup>. (B) BM from WT, E47<sup>het</sup>, p21<sup>het</sup> or E47<sup>het</sup>p21<sup>het</sup> mice was stained to enumerate LSK, total HSC, MPP or CLP numbers as indicated in (A). Data represents 4-9 mice per genotype.  $p > 0.05$ , not significant.

### 3.4.2 Preferential susceptibility of E47<sup>het</sup>p21<sup>het</sup> HSCs to a cellular mitotoxin

Hematopoiesis under stress may reveal biological defects not detectable under steady-state conditions in intact animals. Therefore, we examined the biological responses of E47<sup>het</sup>p21<sup>het</sup> mice to three different stresses that test hematopoietic function: challenge with the mitotoxic drug 5-fluorouracil (5-FU), induction of emergency myelopoiesis by lipopolysaccharide (LPS), and long-term serial adoptive transfer. Emergency myelopoiesis is a short-term acute stress while repeated serial transfer is a long-term chronic stress. Serial adoptive transfer studies additionally provide a strategic opportunity to distinguish the biological importance of E47-p21 interactions within hematopoietic tissue (i.e. cell-intrinsic) versus within the cells of the microenvironment (i.e. cell-extrinsic).



**Figure 3-4. E47<sup>het</sup>p21<sup>het</sup> HSCs are more susceptible to a cellular mitotoxin.**

WT, E47<sup>het</sup>, p21<sup>het</sup> or E47<sup>het</sup>p21<sup>het</sup> mice were injected i.p. with 150 mg/kg 5-FU and sacrificed after 16 hours. BM cells were stained to determine total HSC (flk2<sup>-</sup> LSK) or MPP (flk2<sup>+</sup> LSK) numbers. Data is shown as mean  $\pm$  SD of cell number relative to WT, n=4-8 mice per group. \* indicates p<0.05, ns indicates not significant.

The chemotherapeutic 5-FU is selectively toxic to actively dividing cells, providing an independent experimental measure of loss of LT-HSC quiescence *in vivo* (41). Following two rounds of 5-FU treatment, total HSCs from E47<sup>het</sup>p21<sup>het</sup> mice were reduced ~50% compared to any of the control groups (n=4-8 mice/group, p<0.05) (**Figure 3-4**). E47<sup>het</sup>p21<sup>het</sup> MPPs were also reduced by 40-60% relative to WT but were not statistically different than the single haploinsufficient controls. Together, these data reinforce findings in Figure 1 that the E47-p21 pathway regulates LT-HSC proliferation during homeostasis and is important in maintaining HSC numbers under mitotoxic stress.

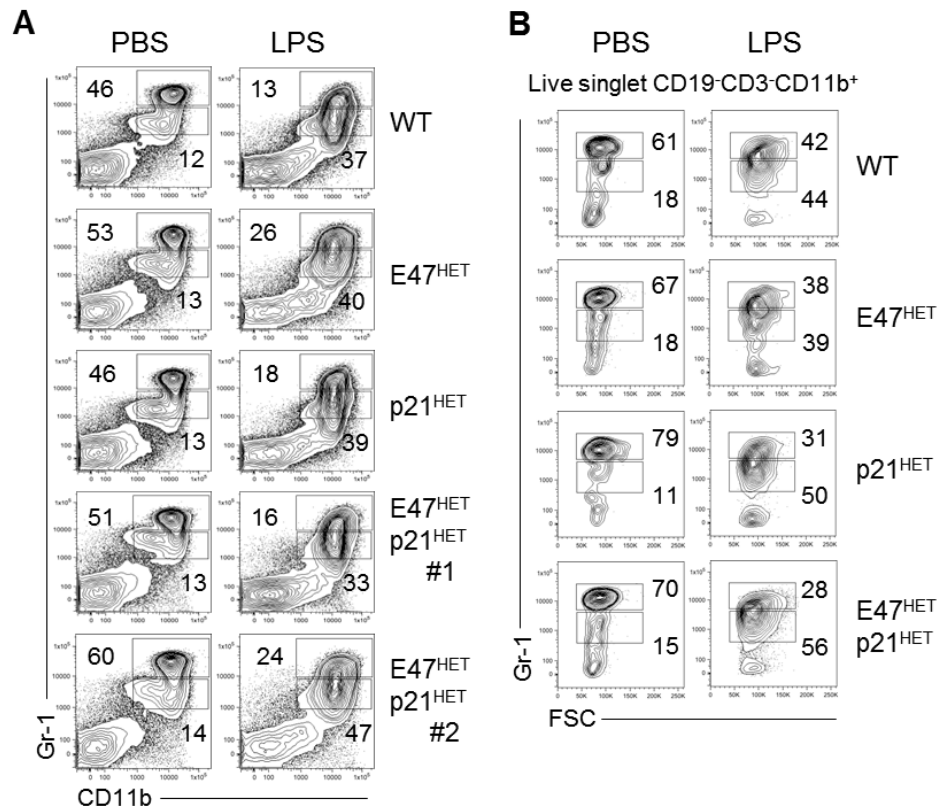
### **3.4.3 E47 and p21 are dispensable for rapid myeloid differentiation under emergency hematopoiesis**

The careful balance between self-renewal and differentiation replenishes HSC numbers and sustains the replacement of mature cells (82). In addition to compromising proliferation and cellularity of LT-HSCs and uncommitted progenitors, we hypothesized that compound haploinsufficiency could impair functional integrity of immediate downstream progeny derived from these primitive subsets during hematopoiesis under stress conditions.

Exposure to bacterial LPS elicits rapid granulopoiesis and concomitant mobilization to peripheral blood. In reactive neutrophilia, multipotent HSCs and MPPs as well as bi-potent granulocyte-monocyte progenitors (GMPs) contribute to accelerated granulopoiesis (166-168). We challenged mice with two rounds of LPS exposure and examined immature (CD11b<sup>+</sup>Gr-1<sup>int</sup>) and mature (CD11b<sup>+</sup>Gr-1<sup>hi</sup>) granulocytes in the BM and peripheral blood (PB), respectively.



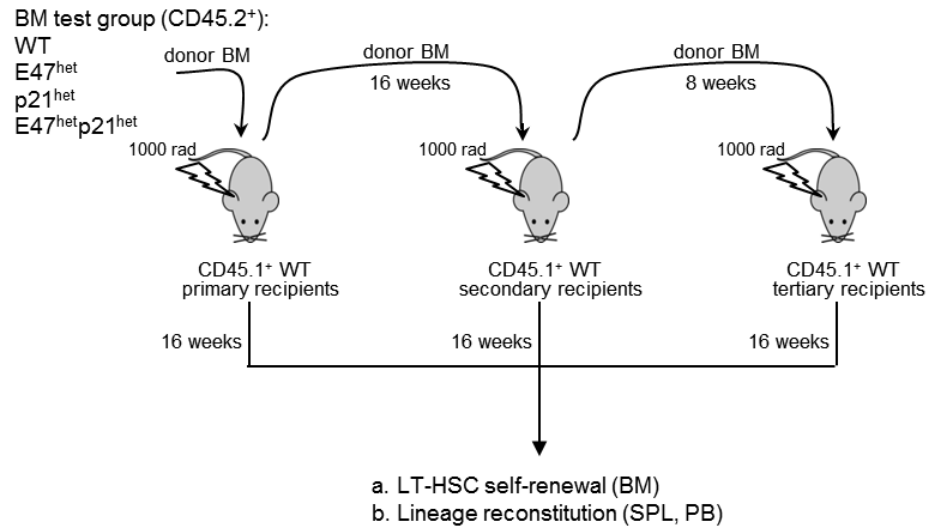
Following LPS exposure, CD11b<sup>+</sup>Gr-1<sup>int</sup> immature BM granulocytes were comparably increased 3-fold (**Figure 3-5A**). Newly differentiated granulocytes also appeared competent to emigrate to PB, regardless of genotype (**Figure 3-5B**). Thus, despite the characteristic hyperproliferation, the immediate progeny of E47<sup>het</sup>p21<sup>het</sup> LT-HSCs and MPPs displayed grossly normal responses to acute LPS challenge.



**Figure 3-5. E47 and p21 are dispensable for rapid myeloid differentiation under emergency hematopoiesis.**

Mice were treated with either PBS or 30  $\mu$ g total LPS over 2 days and sacrificed 24 hours after last treatment. BM (**A**) or PB (**B**) cells were stained to identify mature (CD11b<sup>+</sup> Gr-1<sup>hi</sup>) or immature granulocytes (CD11b<sup>+</sup> Gr-1<sup>int</sup>). Data shown is representative of two independent experiments.  $p > 0.05$ , not significant.

### 3.4.4 Compromised E47<sup>het</sup>p21<sup>het</sup> LT-HSC persistence in long-term serial transfer assays

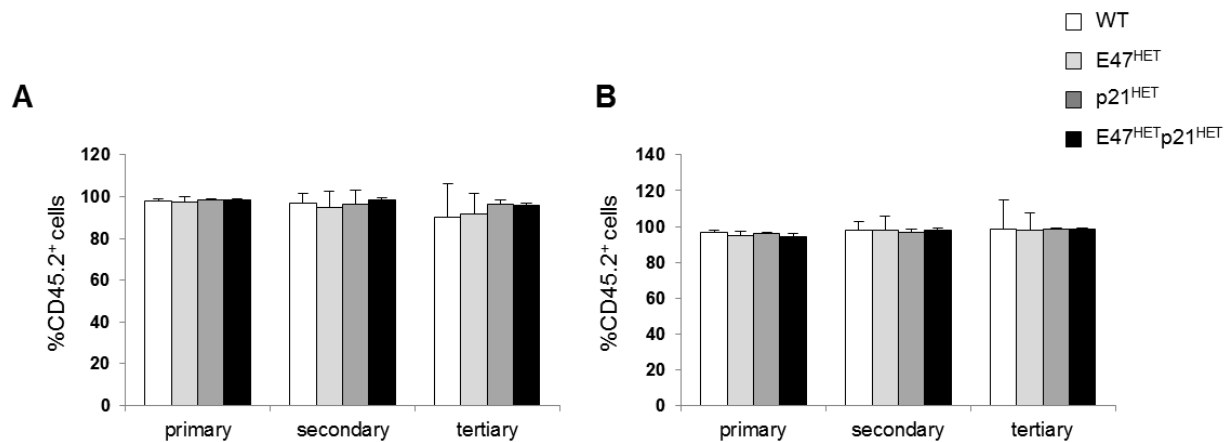


**Figure 3-6. Serial adoptive transfer assay experimental plan.**

Serial transplantation was performed by transferring  $2 \times 10^6$  BM cells from CD45.2<sup>+</sup> WT, E47<sup>het</sup>, p21<sup>het</sup> or E47<sup>het</sup>p21<sup>het</sup> mice into sub-lethally irradiated CD45.1<sup>+</sup> C57BL/6 recipient mice to examine LT-HSC self-renewal and persistence through three rounds of serial transfer. Each set of recipient mice were sacrificed 16 weeks after transplantation.

Serial repopulation remains the gold standard assay for studying LT-HSC function (41). We examined self-renewal and functional integrity of E47<sup>het</sup>p21<sup>het</sup> LT-HSCs in serial transfer through primary, secondary and tertiary WT recipients as illustrated in **Figure 3-6**. CD45 congenic markers permit the tracking of cells of donor origin. Following engraftment, we examined donor-derived leukocytes every 4 weeks in the PB, and at 16 weeks in BM and spleen.

Donor-derived cells were ~95% of total cells in the bone marrow and spleen of primary, secondary and tertiary recipients 16 weeks post-transplant, regardless of donor genotype (**Figure 3-7**). Reconstitution at 16 weeks or longer ensures that hematopoiesis is derived from engrafted LT-HSCs and not by shorter-lived non-renewing progenitors co-transferred during transplantation (41).

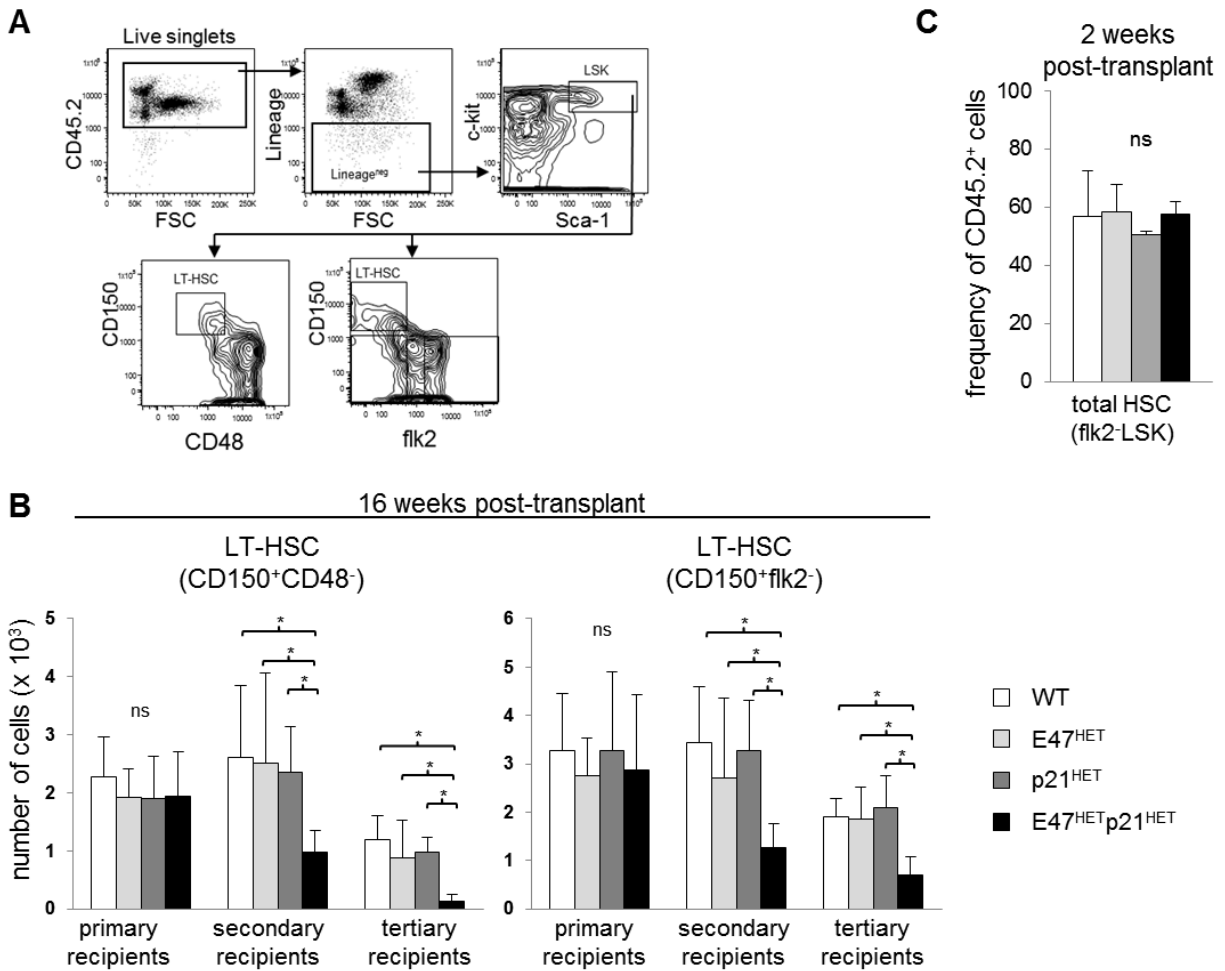


**Figure 3-7. Donor-derived reconstitution of recipient bone marrow and spleen.**

Serial transplantation was performed as described in Figure 3-6. Frequency of donor-derived cells (% CD45.2<sup>+</sup>) in (A) bone marrow or (B) spleen of primary, secondary and tertiary recipients was examined 16 weeks after transplantation. Graphs are shown as mean  $\pm$  SD of data from n= 5-10 mice per recipient for each donor genotype.  $p > 0.05$ , not significant.

In contrast to controls, E47<sup>het</sup>p21<sup>het</sup> LT-HSCs were profoundly depleted by serial transfer. While primary recipients had comparable numbers of LT-HSCs regardless of donor genotype, secondary recipients and tertiary recipients of E47<sup>het</sup>p21<sup>het</sup> donor BM had a striking ~40% and 90% reduction in LT-HSCs, respectively ( $p < 0.05$ , n=4-8 mice/group) (**Figure 3-8B**). By contrast, the progressive loss in LT-HSCs from WT as well as the single heterozygous control

groups in secondary recipients was mild. Further, BM LT-HSCs from tertiary recipients in any of the control groups was reduced to ~50%, consistent with previous findings (84). The magnitude of the dramatic reduction in E47<sup>het</sup>p21<sup>het</sup> donor-derived LT-HSCs was identical using both the



**Figure 3-8. E47<sup>het</sup>p21<sup>het</sup> LT-HSCs exhibit decreased self-renewal and persistence *in vivo*.**

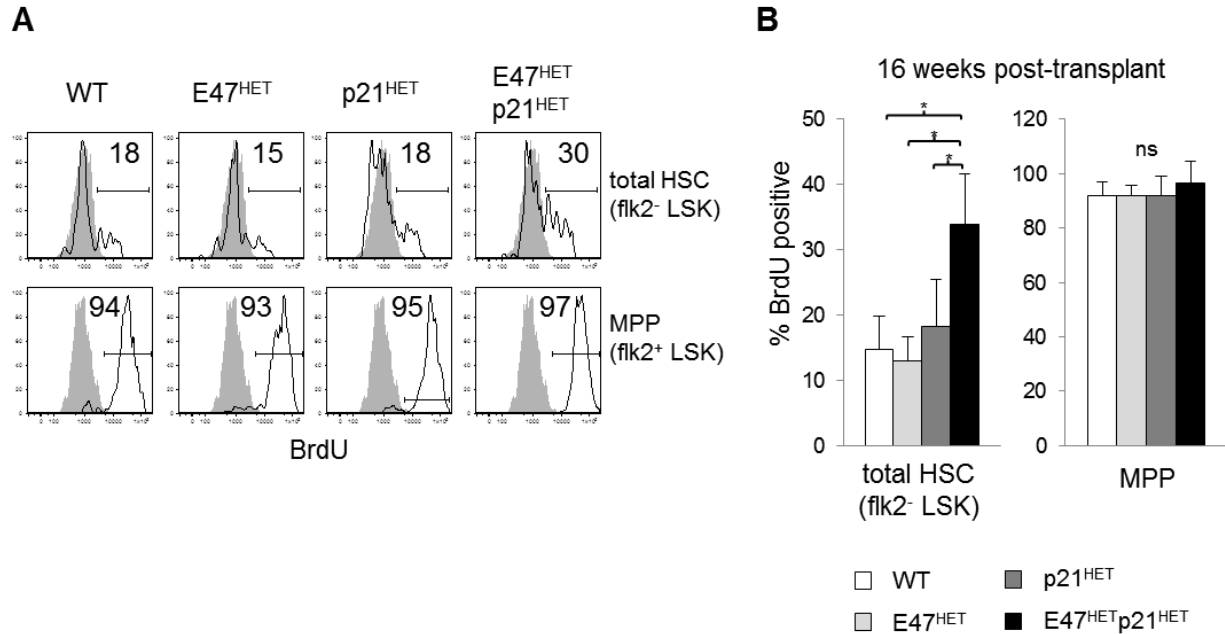
Serial transplantation was performed as described in Figure 3-6. (A) Mice were sacrificed at sixteen weeks post-transplant and the number of donor-derived BM LT-HSCs (CD45.2<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup> LSK or CD45.2<sup>+</sup> CD150<sup>+</sup> flk2<sup>-</sup> LSK) was enumerated in primary, secondary or tertiary recipients. Graph is shown as mean ± SD of data pooled from n= 4-8 recipient mice for each genotype. (B) Mice were sacrificed two weeks after primary transplantation and the frequency of CD45.2<sup>+</sup> total HSCs was examined. Data is shown as mean ± SD from n= 2-3 recipient mice per

group. (C) Gating strategy used to identify donor-derived BM LT-HSCs as CD45.2<sup>+</sup> CD150<sup>+</sup> CD48<sup>-</sup> LSK or CD45.2<sup>+</sup> CD150<sup>+</sup> flk2<sup>-</sup> LSK. \* indicates p<0.05, ns indicates not significant.

stringent CD150<sup>+</sup> CD48<sup>-</sup> LSK and CD150<sup>+</sup> flk2<sup>-</sup> LSK LT-HSC definitions (**Figure 3-8B**) suggesting that the findings were not specific to one particular gating scheme (see **Figure 3-8A** for gating strategy).

The striking reduction of donor-derived E47<sup>het</sup>p21<sup>het</sup> LT-HSCs in serial transfer could not be explained by poor homing/engraftment. Two weeks after transfer, the frequency of donor-derived HSCs (CD45.2<sup>+</sup>flk2<sup>-</sup>LSKs) was similar across all primary recipients regardless of donor genotype, suggesting comparable engraftment (**Figure 3-8C**).

While the frequency of donor-derived E47<sup>het</sup>p21<sup>het</sup> LT-HSC was similar relative to controls, the pattern of proliferation was not. The frequency of BrdU<sup>+</sup> donor-derived LT-HSC from E47<sup>het</sup>p21<sup>het</sup> mice, ~34%, was increased ~2-fold compared to WT, E47<sup>het</sup> or p21<sup>het</sup> in secondary recipients (respectively, p<0.05, n=3-5 mice/group; **Figure 3-9A&B**). As expected under transplantation stress, MPP subsets from all groups were actively cycling (p>0.05, n=3-5 mice/group), and no subtle differences were revealed in this BrdU pulsing regimen.



**Figure 3-9. E47<sup>het</sup>p21<sup>het</sup> total HSCs display hyperproliferation following transplantation stress.**

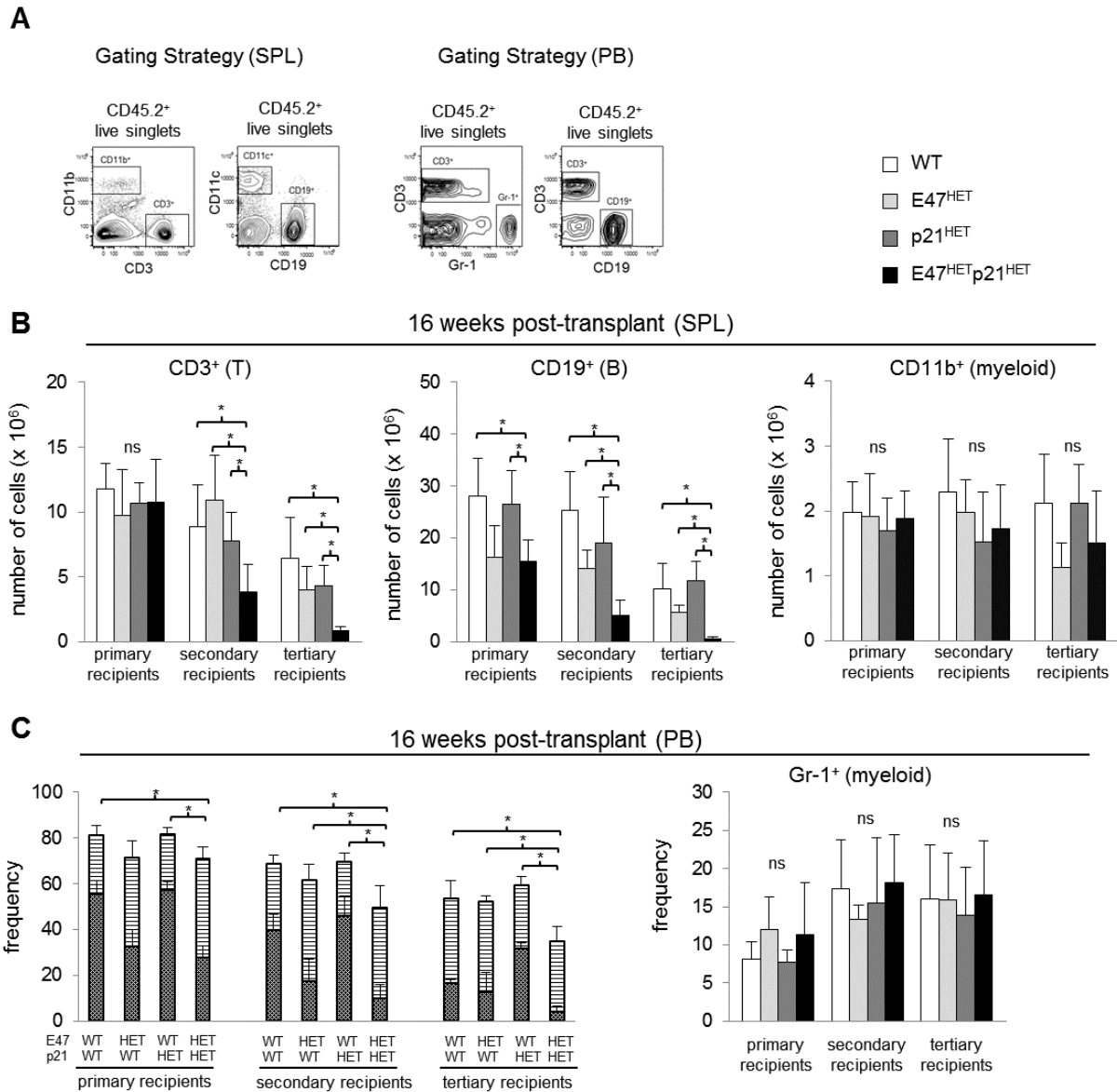
Serial transplantation was performed as described in Figure 3-6. Secondary recipient mice were transplanted with  $2 \times 10^6$  BM cells using primary recipient mice as donors. Sixteen weeks after transplantation, mice were sacrificed and BM cells stained to identify donor-derived total HSCs (CD45.2<sup>+</sup> flk2<sup>-</sup> LSK) and MPPs (CD45.2<sup>+</sup> flk2<sup>+</sup> LSK) followed by anti-BrdU staining to examine proliferation status (A) Flow cytometry profiles from one experiment representative of 3 experiments are shown. Shaded grey histograms indicate PBS stained control. (B) Bar graph represents mean  $\pm$  SD of data from n=3-5 recipient mice per donor genotype. \* indicates p<0.05, ns indicates not significant.

Taken together, E47 and p21 collaborate to restrain LT-HSC proliferation, thereby preserving LT-HSC numbers in the context of long-term repopulation stress. Moreover, the requirement for E47-p21 interactions is cell-intrinsic, since E47<sup>het</sup>p21<sup>het</sup> LT-HSCs fail to persist when engrafted into WT recipient hosts.

### 3.4.5 Cumulative versus developmental-compartment specific burden of combined E47-p21 haploinsufficiency

Lymphopoiesis and myelopoiesis differ in the requirement for E47, providing a unique experimental opportunity to evaluate the biological impact of E47-p21 collaboration to progenitor compartments upstream versus downstream of lympho-myeloid restriction. E47 is essential for multiple aspects of B and T cell development (106, 108, 110, 114, 123, 169) including p21-mediated proliferation (115, 140, 143, 144). By contrast, once uncommitted precursors have restricted to the myeloid lineage, E47 activity and, by extrapolation, E47-dependent p21 activity becomes dispensable (109). Thus, defects in lymphoid output from E47<sup>het</sup>p21<sup>het</sup> LT-HSCs should reflect the aggregate burden of haploinsufficiency at the multipotent and lymphoid specified stages of development while defects in myeloid output should reflect the burden of haploinsufficiency incurred exclusively at the multipotent stages.

The poor persistence of E47<sup>het</sup>p21<sup>het</sup> LT-HSCs in serial transfer through WT hosts was accompanied by marked divergence in peripheral lymphoid and myeloid reconstitution. Specifically, while the failure of B and T lymphoid production closely tracked the numeric loss of LT-HSCs through serial transfer, myeloid production remained intact in these same animals. At 16 weeks post-transplant, the absolute number and frequency of donor-derived E47<sup>het</sup>p21<sup>het</sup> CD19<sup>+</sup> B and CD3<sup>+</sup> T cells was dramatically decreased by ~60% and ~90% in secondary and tertiary recipients, respectively ( $p < 0.05$ ,  $n = 2-9$  mice/group), (**Figure 3-10B&C**), proportional to reductions in E47<sup>het</sup>p21<sup>het</sup> LT-HSCs (**Figure 3-8A**). By contrast, numbers and frequency of myeloid cells (Gr-1<sup>+</sup> or CD11b<sup>+</sup>) derived from E47<sup>het</sup>p21<sup>het</sup> donor BM were normal across all three serial transfers (**Figure 3-10B&C**). Moreover, that E47<sup>het</sup>p21<sup>het</sup> mice had largely normal



**Figure 3-10. E47<sup>het</sup>p21<sup>het</sup> LT-HSCs display progressive *in vivo* decrease in lymphoid lineage reconstitution accompanied by normal myeloid lineage reconstitution.**

Serial transplantation was performed as described in Figure 3-6. Sixteen weeks after transplantation, donor-derived cells were identified from spleen (SPL) or peripheral blood (PB). (A) Left panel, gating strategy used to identify donor-derived B (CD45.2<sup>+</sup> CD19<sup>+</sup>), T (CD45.2<sup>+</sup> CD3<sup>+</sup>) and myeloid lineage (CD45.2<sup>+</sup>CD11b<sup>+</sup>) cells in spleen. Right panel, gating strategy used to identify donor-derived B (CD45.2<sup>+</sup> CD19<sup>+</sup>), T (CD45.2<sup>+</sup> CD3<sup>+</sup>) and myeloid lineage (CD45.2<sup>+</sup> Gr-1<sup>+</sup>) cells in PB. Donor-derived reconstitution of lymphoid and myeloid lineage was examined

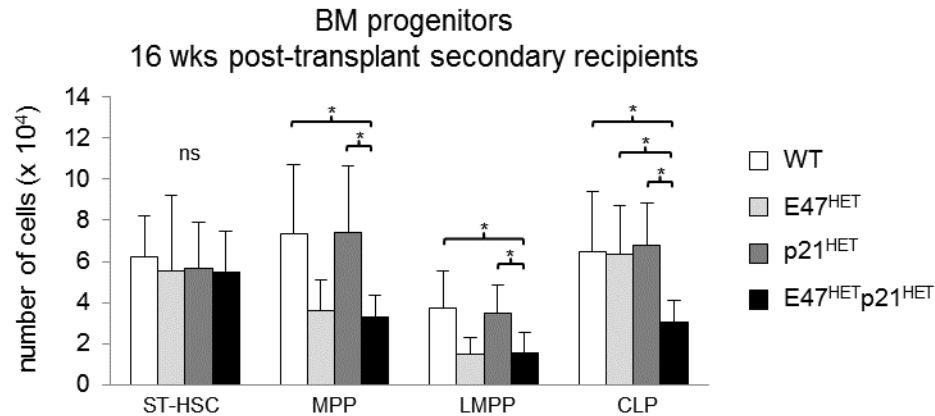


in **(B)** spleen and **(C)** peripheral blood in recipients sixteen weeks after each round of transplantation. Graphs are shown as mean  $\pm$  SD of data from n= 2-9 mice per recipient for each donor genotype. \* indicates  $p < 0.05$ , ns indicates not significant.

neutrophil responsiveness to LPS challenge (**Figures 3-5**), suggests that myeloid lineage cells are grossly intact both phenotypically and functionally.

A recent study showed that significant reductions in CD150<sup>+</sup> LT-HSCs following deletion of the CXCL12 chemokine from BM endothelial niches could be counterbalanced by homeostatic mechanisms that restore downstream MPPs and LMPPs to normal frequencies (170). We examined hematopoietic subsets derived from LT-HSCs to establish the consequences of the E47<sup>het</sup>p21<sup>het</sup> defect to immediate downstream progenitors, 16 weeks post-transfer. Despite a 50% reduction in LT-HSCs, secondary recipients of E47<sup>het</sup>p21<sup>het</sup> donor BM had largely normal numbers of ST-HSCs, suggesting that the requirement for the E47-p21 pathway at the LT-HSC stage could be overcome in downstream compartments. Donor-derived MPPs and LMPPs (using CD150<sup>+</sup>flk2<sup>+</sup>LSK and flk2<sup>high</sup> LSK definitions, respectively (56, 170)) were reduced relative to WT and p21<sup>het</sup> mice, but were identical to that of single E47<sup>het</sup> mice (**Figure 3-11**). By contrast, E47<sup>het</sup>p21<sup>het</sup> CLPs were reduced 2-fold compared to WT or single heterozygous controls, and downstream B and T cells were severely depleted (**Figure 3-10B&C**). Thus, while the striking numerical depletion of E47<sup>het</sup>p21<sup>het</sup> LT-HSCs can be transiently compensated by expansion of downstream multipotent subsets competent to produce a functional myeloid compartment, the lymphoid compartment could not be replenished. Together, our findings indicate a cell-intrinsic

role for genetic interactions between E47 and p21 in the selective maintenance of LT-HSC, B cell and T cell compartments under long-term hematopoietic repopulation stress *in vivo*.



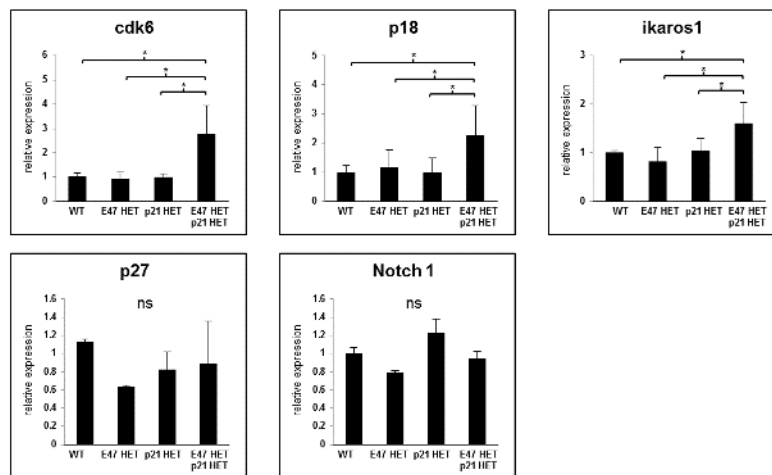
**Figure 3-11. Reduced common lymphoid progenitors in the bone marrow of E47<sup>het</sup>p21<sup>het</sup> secondary recipients.**

Sixteen weeks after transplant, presence of donor-derived short-term HSCs (ST-HSC), multipotent progenitors (MPP), lymphoid myeloid primed progenitors (LMPP) and common lymphoid progenitors (CLP) in BM of secondary recipients was examined. Data are shown as mean  $\pm$  SD from n= 5-9 mice per donor genotype. \* indicates p<0.05, ns indicates not significant.

### 3.4.6 Gene expression analysis in E47<sup>het</sup>p21<sup>het</sup> total HSCs

To obtain a broader perspective on the impact of combined E47 and p21 heterozygosity to the expression profile of other known regulators of HSC pool size, we examined transcript levels of known E47-regulated target genes as well as other cell cycle associated genes (72, 115, 171, 172). Here, we used 5-FU treatment to deplete cycling HSCs and examined the gene expression

profile of residual HSCs subjected to repopulation stress in order to reconstitute the immune system (41, 165). E47<sup>het</sup>p21<sup>het</sup> total HSCs undergoing repopulation stress exhibit a ~1.5-3-fold increase in *cdk6*, *p18*, and *ikaros* transcripts as assessed by QPCR (**Figure 3-12**). There were no detectable changes in *p27* and *Notch1* transcript levels, two factors that regulate the precursor pool size but

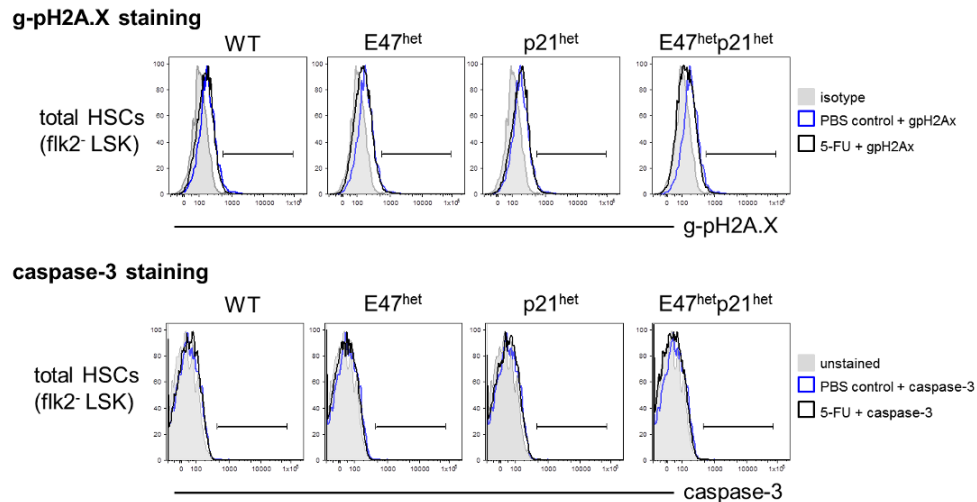


**Figure 3-12. Gene expression analysis in E47<sup>het</sup>p21<sup>het</sup> HSCs.**

At day 14 after a single 5-FU i.v. injection, total HSCs (flk2<sup>-</sup> LSK) were sorted from WT, E47<sup>het</sup>, p21<sup>het</sup> or E47<sup>het</sup>p21<sup>het</sup> mice. RNA was extracted and cDNA was generated via RT-PCR and used to examine expression levels of *cdk6*, *p18*, *ikaros1*, *p27* and *Notch1* using quantitative real-time PCR (qPCR). Gene expression was normalized to  $\beta$ -actin. Data are shown as mean  $\pm$  SD of triplicates from at least two independent sorts. \* indicates  $p < 0.05$ , ns not significant.

not HSC cell cycle activity per se (89, 173). While both *ikaros* and *Notch* can activate p21 expression, neither factor was able to compensate for E47 haploinsufficiency. We saw no evidence of differences in  $\gamma$ -ph2AX levels or caspase-3 protein in E47<sup>het</sup>p21<sup>het</sup> total HSCs undergoing repopulation stress (**Figure 3-13**), suggesting that loss of LT-HSCs could not be

readily explained by impaired DNA damage repair response or increased apoptosis, even though rapid clearance of dying cells *in vivo* may slightly underestimate their detection via flow cytometry. Together, these data demonstrate that E47 mechanistically regulates LT-HSC self-renewal by controlling p21-dependent cell cycle activity.



**Figure 3-13. Expression of g-pH2A.X and caspase-3 in E47<sup>het</sup>p21<sup>het</sup> total HSCs undergoing repopulation stress are comparable to controls.**

Mice were treated with a single dose of 150 mg/kg 5-FU or PBS via tail vein injection. Fourteen days after treatment, mice were sacrificed and bone marrow cells enriched for Lineage<sup>neg</sup> cells were stained for surface markers to identify total HSCs (flk2<sup>-</sup> LSKs) followed by intracellular staining for g-pH2A.X (top panel) and caspase-3 expression (bottom panel).

### 3.5 DISCUSSION

E47 is a key component of gene regulatory networks that establish B and T cell fate, and participates in HSC maintenance (56, 115, 123, 140, 143, 174). While our *in vitro* evidence showed that E47 directly regulates p21 in HSCs, establishing the biological contribution of E47-p21 pathway to hematopoiesis has been elusive due to the difficulty of analyzing rare HSCs *in vivo*, the challenge of overcoming early lymphoid arrest in E47-deficient mice, and the knowledge that both factors are broadly expressed in cells other than leukocytes. To determine the extent of functional collaboration by E47 and p21 during hematopoiesis, we engineered mice with reduced gene dosage of each factor and transplanted BM from E47<sup>het</sup>p21<sup>het</sup> mice into WT hosts. Our findings reveal a severe progressive deficiency in E47<sup>het</sup>p21<sup>het</sup> LT-HSC self-renewal following long-term serial repopulation. A transient recovery of cellularity in donor-derived ST-HSC/MPP stages enabled normal myeloid production but lymphoid production was unrecoverable. The gross severity of the lymphoid defect in compound E47<sup>het</sup>p21<sup>het</sup> mice compared to single haploinsufficiency of either gene alone was striking, an important finding given the increasingly frequent linkage of E47 loss of heterozygosity with lymphoid malignancies and immune deficiency.

While several lines of evidence hint at a relationship between E47 and p21 in each HSCs and lymphoid-committed precursors, the biological impact has been unclear (56, 140, 143). Indeed, the severe defect in the earliest stages of hematopoiesis in p21 null mice has precluded analysis of p21 contributions in compartments downstream of lympho-myeloid divergence. In

this study, we exploited the fact that the E47 pathway is dispensable in myeloid lineage cells to establish the biological importance of the E47-p21 pathway within HSCs upstream of lymphomyeloid divergence. Our results demonstrate a role for the E47-p21 pathway in LT-HSC maintenance under activation stress but not steady state, and reveal a stringent requirement for E47-p21 activity in the lymphoid lineages following forced repopulation. In E47<sup>het</sup>p21<sup>het</sup> HSCs, reductions in p21 were accompanied by dysregulation of *cdk6*, *p18* and *ikaros*, all of which directly regulate cell cycling and likely contribute to the overall phenotype. Since none of these genes are altered in HSCs from mice singly haploinsufficient in either E47 or p21, their dysregulation in E47<sup>het</sup>p21<sup>het</sup> HSCs maybe a consequence of observed hyperproliferation rather than the cause. While E47 is known to directly regulate expression of these genes under homeostatic conditions, our observations may reflect the contribution by other cell cycle regulators activated in HSCs undergoing repopulation stress. p21 null mice on either the original 129 background or the pure B6 background exhibit poor HSC persistence under certain repopulation stress paradigms but changes in companion cell cycle regulators have not been examined (84, 148). Our findings reveal a skewing of B/T ratios that emerges in tertiary recipients of p21 haploinsufficient BM, demonstrating a negative biological consequence of even relatively minor reductions in p21. Indeed, a striking feature of our findings is that the combination of two relatively subtle lesions, haploinsufficiency in each E47 and p21, curtails lymphopoiesis to a severe degree not observed in the context of either single lesion alone.

In summary, our results define the differential contribution of cell-intrinsic E47-p21 within discrete developmental hematopoietic compartments *in vivo*. E47<sup>het</sup>p21<sup>het</sup> LT-HSCs exhibit a profound loss of self-renewal in serial repopulation across mice accompanied by

ablation of the lymphoid lineages. The E47-p21 pathway appeared to be dispensable in the ST-HSC/MPP stages of hematopoiesis, and downstream myeloid activity remained intact. That the combination of two subtle lesions, loss of a single allele of a transcription factor and a cell cycle regulator factor, amplifies the magnitude of hematopoietic disruption beyond either defect alone, has important clinical implications. E47 loss of heterozygosity is linked to multiple leukemias, and our findings provide an opportunity screening at-risk individuals for cooperating lesions.

## **4.0 DETERMINATION OF P15PAF AS A DIRECT TARGET GENE OF THE TRANSCRIPTION FACTOR E47**

### **4.1 ABSTRACT**

HSC proliferation is tightly regulated because it can have adverse effects to HSC function. Our new findings demonstrate E47 as a major regulator of HSC cycling under stress. By reducing the gene dose of each factor individually ( $E47^{het}$  or  $p21^{het}$ ) versus in tandem ( $E47^{het}p21^{het}$ ), we show that  $E47^{het}p21^{het}$  LT-HSCs exhibit hyperproliferation during homeostasis and under transplantation stress. In serial adoptive transfers that rigorously challenge self-renewal, we demonstrate the importance of cell-intrinsic E47-p21 in preserving LT-HSCs under stress as  $E47^{het}p21^{het}$  LT-HSCs dramatically and progressively decline upon each round of transfer. Mechanistically, E47 regulates transcription of the cyclin-dependent kinase inhibitor p21, resulting in a role for E47 in the inhibition of HSC proliferation. Recently, a role for the nuclear protein p15<sup>PAF</sup> in inhibiting HSC proliferation has also been described. Moreover, the phenotype observed in p15<sup>PAF</sup>-deficient mimics the hallmark phenotype also observed in E47-deficient mice. During homeostasis, both mouse models present with relatively modest changes in HSC and MPP numbers that becomes progressively worse in downstream progenitors as LMPP and



CLP numbers are severely reduced. In this study, we examined the role of E47 as a direct transcriptional regulator of p15<sup>PAF</sup> expression by using two different approaches to examine the relationship between E47 and p15<sup>PAF</sup>. First, sequence analysis of the p15<sup>PAF</sup> promoter region revealed over 10 different sites containing the E-box consensus sequence. Our luciferase assay findings show that E47 can directly activate p15<sup>PAF</sup> promoter-mediated transcription by ~20-110-fold in a dose-dependent manner using a model cell line. Moreover, E47 activation of p15<sup>PAF</sup> promoter-mediated transcription was ablated in p15<sup>PAF</sup> promoter constructs in the reverse orientation suggesting importance of the 5' promoter region for E47 binding. Second, we examined the direct consequence of E47 deficiency to p15<sup>PAF</sup> mRNA and protein levels in primary hematopoietic precursors. Unexpectedly, our findings indicate that E47-deficiency resulted in a significant 2-fold increase in p15<sup>PAF</sup> expression in primary HSCs and MPPs. The seemingly contradictory findings that E47 activates p15<sup>PAF</sup> expression in a model cell line but suppresses p15<sup>PAF</sup> expression in primary hematopoietic precursors may be due to the cumulative effects of regulatory elements such as enhancers, trans-acting regulatory proteins and epigenetic regulators, uniquely present or active in E47-deficient primary HSCs and MPPs. Analysis of p15<sup>PAF</sup> protein levels in E47-deficient HSCs and MPPs were comparable to WT, despite a 2-fold increase in p15<sup>PAF</sup> transcripts in the E47-deficient subsets. These findings are in agreement with previous studies that demonstrate a significant role for post-translational mechanisms that regulate p15<sup>PAF</sup> expression under homeostatic conditions. Therefore, additional studies are needed to demonstrate direct binding of E47 to p15<sup>PAF</sup> promoter region, to identify biologically important E-boxes, and to determine the biological contribution of E47 and p15<sup>PAF</sup> in tandem to HSC function *in vivo*.

## 4.2 INTRODUCTION

The transcription factor E47 plays a central role in the regulatory network involved in the specification, commitment and differentiation of progenitor cells to the lymphoid lineage (96). Differentiation of multipotent progenitor cells into mature B or T cells involves a progressive loss of developmental potential, the activation of lineage-specific gene expression, and repression of genes associated with alternative lineages. The E47 protein was originally identified due to its DNA binding to E-box sequences (CANNTG) in the enhancer regions of the immunoglobulin heavy chain (IgH) and kappa light chains (Ig $\kappa$ ) (104, 175). Indeed, E47 has been shown to activate the expression of several different genes with very diverse functions. These target genes include EBF an important transcription factor in B lineage development, the B-cell specific transmembrane signaling receptor CD79a (also known as mb-1); pre-B cell receptor surrogate light chain components  $\lambda$ 5 and V-preB; and rag 1/2, which encode enzymes involved in antigen gene receptor rearrangement unique to B cells and T cells. E47 also acts as a functional repressor of c-fms and GATA-1, genes which are associated with alternative myeloid and erythroid lineages respectively (111). In addition, E47 can modulate DNA methylation and chromatin accessibility upon binding to regulatory elements in target genes including CD79a, rag1 and Foxo1 (123). Together, these studies indicate that E47 promotes lymphoid lineage development through regulation of lineage-specific gene expression and modulation of DNA methylation and chromatin accessibility.

In addition to the role of E47 in promoting lymphoid lineage development, E47 also regulates cellular proliferation. Ectopic expression of E47 in fibroblasts resulted in a block in the G1 phase of the cell cycle (176). E47 activity has also been shown to be inhibitory to

proliferation in both progenitor B and T cells (144, 177). Similarly, we have also shown that HSCs lacking functional E47 exhibit increased proliferation and loss of quiescence (140, 143). Together, these studies indicate that E47 activity regulates cellular proliferation in bone marrow progenitor cells. One mechanism by which E47 can control proliferation is through regulation of expression of the cell cycle inhibitor p21. Using gain of function and loss of function studies involving E47 in HSCs, we have shown that p21 is a downstream target of E47 in restraining HSC proliferation (140), consistent with previous findings (144, 145). However, other pathways likely exist by which E47 can regulate HSC proliferation. In this study, we focused on the candidate E47 target p15<sup>PAF</sup> as both E47-deficient and p15<sup>PAF</sup>-deficient mice share a striking phenotypic resemblance in early hematopoietic defects.

Emerging studies hint at a functional relationship between the transcription factor E47 and the nuclear protein p15<sup>PAF</sup> (KIAA0101). A 15 kDa protein that contains a binding motif for association with proliferating cell nuclear antigen (PCNA), p15<sup>PAF</sup> is highly expressed in the nucleus of proliferating cells and is minimally expressed in quiescent cells (178). Increased expression of p15<sup>PAF</sup> has been found in esophageal, breast, uterine, brain, kidney and lung tumors (178) and overexpression of p15<sup>PAF</sup> correlates to poor prognosis in primary lung cancer patients (179). Previous studies also indicate that the function of p15<sup>PAF</sup> may be linked to PCNA activity during DNA replication and DNA repair (180). Involvement of p15<sup>PAF</sup> in DNA repair after UV-induced DNA damage mediated by the activating transcription factor, ATF3, has also been reported (181). Cell line-based studies have also implicated a role for p15<sup>PAF</sup> in cellular proliferation (180, 182-184). Analysis of p15<sup>PAF</sup>-deficient mice indicates that p15<sup>PAF</sup> is important in HSC proliferation and as a consequence, HSC function. Mice deficient in p15<sup>PAF</sup> had HSC

hyperproliferation, decreased HSC persistence and poor multilineage reconstitution of recipient mice under repopulation stress (185). The early hematopoietic defects observed in p15<sup>PAF</sup>-deficient mice have a striking similarity to the phenotype observed in E47-deficient mice exhibiting moderate reductions in HSCs and MPPs and profound reductions in LMPPs and CLPs under homeostasis. Moreover, we have also identified multiple E-box sites in the murine p15<sup>PAF</sup> promoter region

Collectively, these findings led us to hypothesize that p15<sup>PAF</sup> is a novel target gene transcriptionally regulated by E47 and presents another mechanism through which E47 can control HSC proliferation. To examine whether E47 can activate p15<sup>PAF</sup> transcription, we first performed sequence analysis of the murine p15<sup>PAF</sup> promoter region to characterize candidate E box binding sites for E47 and performed luciferase assays. We then examined p15<sup>PAF</sup> mRNA and protein levels in primary hematopoietic precursor subsets from E47-deficient mice. Results from luciferase reporter assays indicate that E47 directly activates p15<sup>PAF</sup> promoter mediated transcription in 293T cells. Unexpectedly, and in contrast to our findings with the reporter cell line, primary HSCs and MPPs from E47-deficient mice had a significant 2-fold increase in p15<sup>PAF</sup> mRNA expression compared to WT. It is possible that transcriptional regulation of p15<sup>PAF</sup> in primary HSCs is controlled by the interplay of several factors in addition to E47. At the protein level, analysis of p15<sup>PAF</sup> expression in E47-deficient HSCs and MPPs did not reveal any significant differences compared to WT, a finding that reflects previous observations of a significant role for post-translational mechanisms in regulating p15<sup>PAF</sup> activity (185). Thus, our findings suggest that E47 is a transcriptional regulator of p15<sup>PAF</sup> gene expression that is a part of

a broader network of transcriptional and post-translational mechanisms that regulate p15<sup>PAF</sup> expression in primary HSCs.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Plasmid Constructs

The murine C57BL/6 p15<sup>PAF</sup> promoter was amplified by PCR from splenic DNA using the following primers: forward 5' AAGAACCACTGTGCAGTCTG 3' and reverse 5' CGCTTGAGAACCTCGATTCT 3', that targeted a region ~2300 bp upstream of the p15<sup>PAF</sup> transcription start site. The PCR amplified DNA fragment was purified using QiaexII gel extraction kit (Qiagen) and cloned directly into pCR2.1 vector using the TOPO TA cloning kit (Invitrogen) per manufacturer's instructions. The p15<sup>PAF</sup> promoter region was subcloned into the pGL4.11 luciferase reporter plasmid (Promega) using the *Kpn1* and *EcoRV* restriction sites in a ligation reaction using Ligafast (Promega). Two constructs were created such that the p15<sup>PAF</sup> promoter region is cloned into the pGL4.11 plasmid in both the forward and reverse orientations (referred to as mPaF-Fwd and mPaF-Rev, respectively). The E47 expression plasmid was a kind gift from Dr. Barbara L. Kee.

### 4.3.2 Luciferase Reporter Assay

Twelve-well culture plates (BD Falcon) were seeded with  $2 \times 10^5$  293T cells per well in RPMI-1640 (Cellgro) supplemented with 5% fetal bovine growth serum (Hyclone), 100U/mL penicillin (Cellgro) and 100  $\mu$ g/mL streptomycin (Cellgro). Two days after seeding, cells were transfected with mPaF-Fwd or mPaF-Rev plasmid with or without the E47 expression plasmid at the indicated concentrations. Transfection was performed using Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. One day after transfection, culture media for each well was changed with fresh 5% RPMI. Two days after transfection, cells were lysed with 100  $\mu$ l/well of 1x Lysis Buffer (Promega) and lysates were kept at  $-20^{\circ}\text{C}$ . An aliquot of 20  $\mu$ L per cell lysate was used to assay for luciferase activity using Luciferase Assay System (Promega) and quantified using a luminometer (Berthold) (186).

### 4.3.3 Mice

Mice were bred in accordance with Institutional Animal Care and Use Committee (IACUC) policies at the University of Pittsburgh School of Medicine. E47<sup>WT</sup> and E47<sup>KO</sup> (C57BL/6) mice (143) were bred in house from E47<sup>HET</sup> intercrossed with E47<sup>HET</sup> matings. E47 genotyping was done as described (84, 106).

#### 4.3.4 Flow Cytometry

Bone marrow from E47<sup>WT</sup> and E47<sup>KO</sup> mice were harvested as previously described (110, 126). Cell staining was performed using antibodies from eBiosciences, BD Pharmingen or Biolegend. Primary antibodies were B220 biotin (clone RA3-6B2), CD3 biotin (clone 2C11), CD11b biotin (clone M1/70), CD19 biotin (clone MB19-1), CD117 APCeFluor 780 or PeCy5 (clone 2B8), CD135 PE (clone A2F10), Gr-1 biotin (clone 8C5), JYLD 488 (clone JYLD 12), NK1.1 biotin (clone PK136), TER-119 biotin (clone TER-119), and Sca-1 FITC, Cy5PE or PerCPCy5.5 (clone D7). Secondary reagents were streptavidin-Cy7PE or streptavidin-eFluor 450. Flow cytometry was performed on a four-laser, twelve-detector LSR II (BD Biosciences). Data were analyzed using FlowJo software Version 9.9.1 (Tree Star).

#### 4.3.5 Cell Sorting

Cell sorting of total HSCs (flk2<sup>-</sup> LSK) and MPPs (flk2<sup>+</sup> LSK) was performed as previously described (140, 143). Briefly, bone marrow cells from E47<sup>WT</sup> and E47<sup>KO</sup> mice were stained with lineage antibodies (NK1.1, Ter-119, Gr-1, CD11b, B220, CD19, and CD3) conjugated to biotin followed by incubation with streptavidin conjugated beads (Miltenyi). Lineage negative cells from the bone marrow were obtained via magnetic separation (AutoMACS, Miltenyi). Collected lineage negative cells were stained with antibodies to Sca-1, CD117 and CD135. Flk2<sup>-</sup> LSK or flk2<sup>+</sup> LSK cells were sorted using a three-laser, eleven-detector cell sorter (Aria, BD Biosciences).

#### **4.3.6 Gene Expression Analysis**

RNA was extracted from sorted cells using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions and reverse transcribed into cDNA with Superscript III Reverse Transcriptase (Invitrogen) using oligoDT primers (110). Quantitative real-time PCR (qPCR) reactions were performed in triplicates using Taqman probe Hs00207134\_m1 (Invitrogen) and detected by StepOne Plus System (BioRad). Expression levels of p15<sup>PAF</sup> were calculated relative to *actb* and expressed as the fold difference relative to wild type (ddC<sub>T</sub> method).

#### **4.3.7 Intracellular Staining with p15<sup>PAF</sup>**

BM cells were stained with antibodies to relevant surface markers and fixed and permeabilized according to manufacturer's instructions using Cytfix/Cytoperm (BD Pharmingen) followed by intracellular staining with JYLD antibody conjugated to AlexaFluor 488 (courtesy of Dr. Lisa Denzin) to detect intracellular p15<sup>PAF</sup> protein expression as previously described (185).

#### **4.3.8 Statistics**

Statistical analysis was performed using student's t-test. Asterisk (\*) indicates p<0.05, ns indicates not statistically significant.



## 4.4 RESULTS

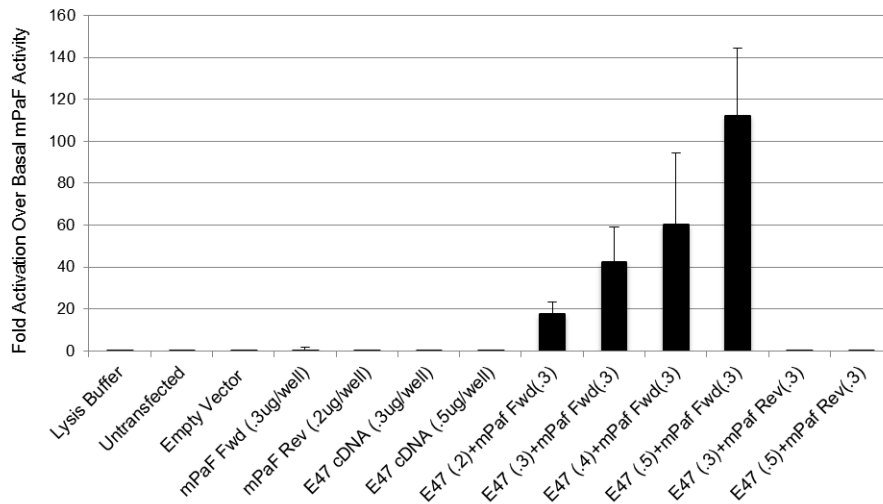
### 4.4.1 E47 Activates Murine p15<sup>PAF</sup> Promoter Mediated Transcription

**Table 2. List of potential E-box sites in the murine p15<sup>PAF</sup> promoter region**

Position Upstream of p15 <sup>PAF</sup> Transcription Start Site	Nucleotide sequence
-68	caattg
-577	catttg
-605	cacgtg
-1118	cacttg
-1344	cagttg
-1497	cacctg
-1602	cacttg
-1658	caattg
-1850	catctg
-2032	cacatg
-2226	cacttg
-2405	catttg

Sequence analysis of the DNA region ~2.5 Kb upstream of the murine p15<sup>PAF</sup> transcription start site revealed 12 different sites containing the E-box consensus sequence motif CANNTG (**Table 2**). We hypothesize that one or more of these E-box sequences can serve as a functional binding site for the transcription factor E47 leading to the activation of p15<sup>PAF</sup> promoter and subsequent gene expression. To determine if E47 can regulate p15<sup>PAF</sup> promoter activity, we cloned the murine p15<sup>PAF</sup> promoter region into the pGL4.11 luciferase reporter plasmid in both the forward and reverse directions (mPaF-Fwd and mPaF-Rev, respectively) and performed luciferase reporter assay in 293T cells (186). Our findings show that E47 can activate the murine p15<sup>PAF</sup> promoter as indicated by the ~20 fold increase in luciferase activity upon transfection of cells

with 0.2  $\mu\text{g}$  of E47 compared to luciferase activity in cells transfected with the p15<sup>PAF</sup> promoter alone (**Figure 4-1**). Increasing concentrations of the E47 expression plasmid were used to co-transfect 293T cells and resulted in a dose-dependent increase in luciferase activity. A 5-fold increase in luciferase activity was observed by using 0.5  $\mu\text{g}$  of E47 compared to 0.2  $\mu\text{g}$  of E47 expression plasmid. Cloning of the murine p15<sup>PAF</sup> promoter into the pGL4.11 reporter plasmid in the reverse orientation did not result in transcriptional activation by E47. Thus, these results suggest that E47 can activate p15<sup>PAF</sup> promoter mediated transcription in 293T cells, a model cell line. In parallel to this gain of E47 function assay, we exploited loss of function assays to examine the *in vivo* consequence of E47 deficiency on p15<sup>PAF</sup> gene and protein expression.



**Figure 4-1. Activation of murine p15<sup>PAF</sup> promoter by E47 in 293T cells.**

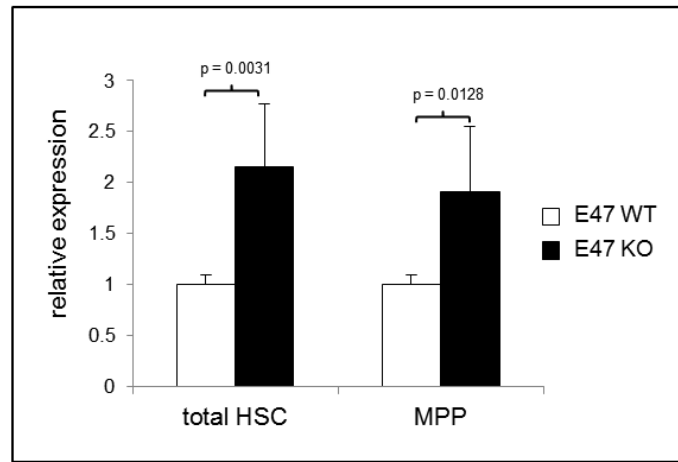
293T cells were transfected with pGL4.11 vector alone, mPaF-Fwd or mPaF-Rev plasmid together with the indicated concentration of E47 expression plasmid. Each transfection condition was performed in triplicates. Two days after transfection, cell lysates were harvested and luciferase activity was measured and normalized to basal luciferase activity of mPaF-Fwd transfected cells alone. Values are expressed as mean  $\pm$  SD and is obtained from n = 5 independent experiments. Numbers in parenthesis indicates DNA concentration of plasmid used per well in each transfection condition. Patricia Santos devised the project, cloned the plasmid

constructs and designed the experiments. Rashmi Kumar performed the experiments. Data courtesy of Rashmi J. Kumar. Both researchers interpreted the data.

#### 4.4.2 Analysis of p15<sup>PAF</sup> Expression in E47-deficient total HSCs

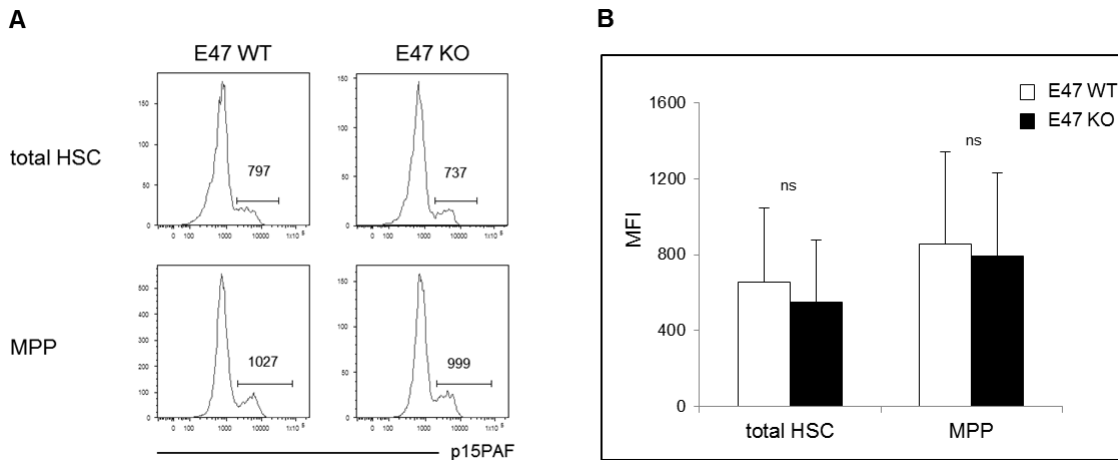
Our luciferase reporter assay results suggest that E47 can activate p15<sup>PAF</sup> promoter-mediated transcription (**Figure 4-1**). To determine if E47 regulates transcription of p15<sup>PAF</sup> in primary hematopoietic precursors, we first examined p15<sup>PAF</sup> mRNA levels in E47<sup>WT</sup> and E47<sup>KO</sup> HSCs and MPPs using QPCR. Unexpectedly, and distinct from our findings in the luciferase reporter assays, results show that HSCs deficient for E47 had a significant 2-fold increase in p15<sup>PAF</sup> mRNA levels (**Figure 4-2**). In addition, E47<sup>KO</sup> MPPs also had a ~2-fold increase in p15<sup>PAF</sup> expression compared to E47<sup>WT</sup> HSCs. These findings suggest that aside from E47, other regulatory factors such as enhancers, trans-acting regulatory proteins and epigenetic regulators, are involved in the transcriptional regulation of p15<sup>PAF</sup> expression in primary HSCs and MPPs. For example, previous studies show that p15<sup>PAF</sup> gene expression is regulated by NF- $\kappa$ B (187), activating transcription factor 3 (ATF3) (181), and by the Rb/E2F complex (188). Next, we examined whether there are any differences in p15<sup>PAF</sup> protein expression between E47<sup>WT</sup> and E47<sup>KO</sup> HSCs using flow cytometry. We found no significant differences in p15<sup>PAF</sup> protein expression in E47<sup>KO</sup> compared to E47<sup>WT</sup> HSCs (**Figure 4-3**). In addition, no significant differences in p15<sup>PAF</sup> protein expression in E47<sup>KO</sup> MPPs versus E47<sup>WT</sup> MPPs were also observed. These findings are consistent with analysis of p15<sup>PAF</sup> expression in the thymus, where high p15<sup>PAF</sup> mRNA expression did not directly correlate with high levels of p15<sup>PAF</sup> protein expression (185), indicating existence of post-translational regulation of p15<sup>PAF</sup> protein levels during homeostasis. Collectively, our findings suggest that in primary hematopoietic precursors, p15<sup>PAF</sup>

expression is regulated by both transcriptional and post-translational mechanisms independent of E47 activity.



**Figure 4-2. Analysis of p15<sup>PAF</sup> mRNA expression in E47-deficient total HSCs and MPPs**

RNA was extracted from sorted total HSCs (flk2<sup>-</sup> LSK) and MPPs (flk2<sup>-</sup> LSK) from either E47 WT or E47 KO mice. cDNA was generated and used to measure p15<sup>PAF</sup> mRNA expression was by QPCR. Data were normalized relative to beta-actin and represents mean ± SD of n=3 independent sorts, with at least 2 independent analyses for each sort.



**Figure 4-3. Analysis of p15<sup>PAF</sup> protein expression in E47-deficient total HSCs and MPPs**

Total HSCs (flk2<sup>-</sup> LSK) and MPPs (flk2<sup>-</sup> LSK) from E47 WT or E47 KO mice were analyzed for p15<sup>PAF</sup> protein expression using flow cytometry. (A) Flow cytometry profiles representative of one experiment showing p15<sup>PAF</sup> protein expression. Numbers in histograms indicate mean

fluorescence index (MFI) of p15<sup>PAF</sup> expression. **(B)** Bar graph summarizing results in (A) represents mean  $\pm$  SD of MFI from data pooled from n = 3 experiments, ns indicates not significant.

## 4.5 DISCUSSION

Several studies collectively indicate 2 major functions for E47. First it is an important transcription factor required for lymphoid lineage development, and second it serves as a negative regulator of proliferation in bone marrow progenitors including HSCs. Strict regulation of HSC proliferation is required because of its direct relevance to HSC self-renewal and function (81, 83-85). For example, E47-deficient HSCs exhibit diminished self-renewal capability and hyperproliferation associated decreased expression of the p21 cell cycle inhibitor (140, 143). We hypothesized that additional pathways likely exist by which E47 can regulate HSC proliferation. Our interest in p15<sup>PAF</sup> as an E47 target gene was generated by the strikingly similar phenotype observed in p15<sup>PAF</sup>-deficient HSCs compared to E47-deficient HSCs. Both mouse models exhibited similar numerical hematopoietic defects in HSCs, MPPs, LMPPs, and CLP (140, 185). Sequence analysis indicates that 12 E-box sites exist in the p15<sup>PAF</sup> promoter region. We also show that E47 can directly activate p15<sup>PAF</sup> promoter-mediated transcription in 293T cells. However, additional studies are needed to determine whether E47 can also activate p15<sup>PAF</sup> promoter-mediated transcription in HSCs. Furthermore, chromatin immunoprecipitation studies are needed to determine if E47 can physiologically bind to any of the E-box sites in the p15<sup>PAF</sup> promoter region and to determine which specific E-box site/s are important for E47 binding.

Using gain of function and loss of function studies, we have shown that p21 is a downstream target of E47 in restraining HSC proliferation (140). In this study, we also examined the ability of E47 to directly affect p15<sup>PAF</sup> expression at both the mRNA and protein level. Unexpectedly, our results indicate that in the absence of E47 activity, p15<sup>PAF</sup> gene expression is increased two-fold in HSCs and MPPs. Transcriptional regulation of gene expression involves not only transcription factors but also involves the contribution of other regulatory elements including enhancers, trans-acting proteins and epigenetic modifiers (189-193). In addition, microRNAs also play a role as post-transcriptional regulators of gene expression (194). In our luciferase assay experiments, only E47 is present to regulate p15<sup>PAF</sup> promoter mediated transcription in the model 293T cell line. Lacking in this experimental system are regulatory factors, present in primary murine HSCs and MPPs, which could contribute to the regulation of p15<sup>PAF</sup> gene expression. Indeed, p15<sup>PAF</sup> gene expression has been shown to be regulated by NF- $\kappa$ B (187), activating transcription factor 3 (ATF3) (181), and Rb/E2F complex (188), indicating that a broad network of factors are involved in regulation of p15<sup>PAF</sup> gene expression. Therefore, it is important to determine if E47 can directly bind to the p15<sup>PAF</sup> promoter region and to examine whether transduction of primary HSCs with E47 in a gain of function experiment, could result in downregulation of p15<sup>PAF</sup> transcripts in primary HSCs.

Analysis of p15<sup>PAF</sup> protein levels between WT and E47-deficient HSCs and MPPs showed comparable protein expression, despite the differences observed in p15<sup>PAF</sup> mRNA expression. These findings are similar to the analysis of p15<sup>PAF</sup> expression in murine thymus wherein high levels of p15<sup>PAF</sup> mRNA expression did not correlate to high levels of p15<sup>PAF</sup> protein expression due to post-translational regulation of the p15<sup>PAF</sup> protein (185). Previous studies have

identified p15<sup>PAF</sup> to be highly expressed in the nucleus of proliferating cells (178). In our studies we examined only the total p15<sup>PAF</sup> protein expression in primary hematopoietic progenitors. What remains unclear is whether there are differences in protein localization between E47<sup>WT</sup> and E47<sup>KO</sup> HSCs. Taking advantage of most recent advances in flow cytometry technology, analysis of p15<sup>PAF</sup> nuclear localization in primary HSCs can be done using ImageStream, which combines conventional flow cytometry with high resolution microscopy (195-200). Also unknown is whether differences in p15<sup>PAF</sup> expression is observed in proliferating cells (S+G<sub>2</sub>+M) from E47<sup>WT</sup> versus E47<sup>KO</sup> HSCs since previous studies indicate that p15<sup>PAF</sup> is highly expressed in proliferating cells (178, 185) and E47<sup>KO</sup> HSCs are hyperproliferating compared to E47<sup>WT</sup> HSCs (140, 143).

This study represents the first step towards determining the additional mechanisms by which E47 can regulate HSC proliferation. While our results suggest that E47 can directly activate p15<sup>PAF</sup> promoter mediated transcription, additional studies are still needed to demonstrate direct binding of E47 to E-box sites in the p15<sup>PAF</sup> promoter region, and importantly, to determine the biological contribution of E47 and p15<sup>PAF</sup> to HSC function *in vivo*.

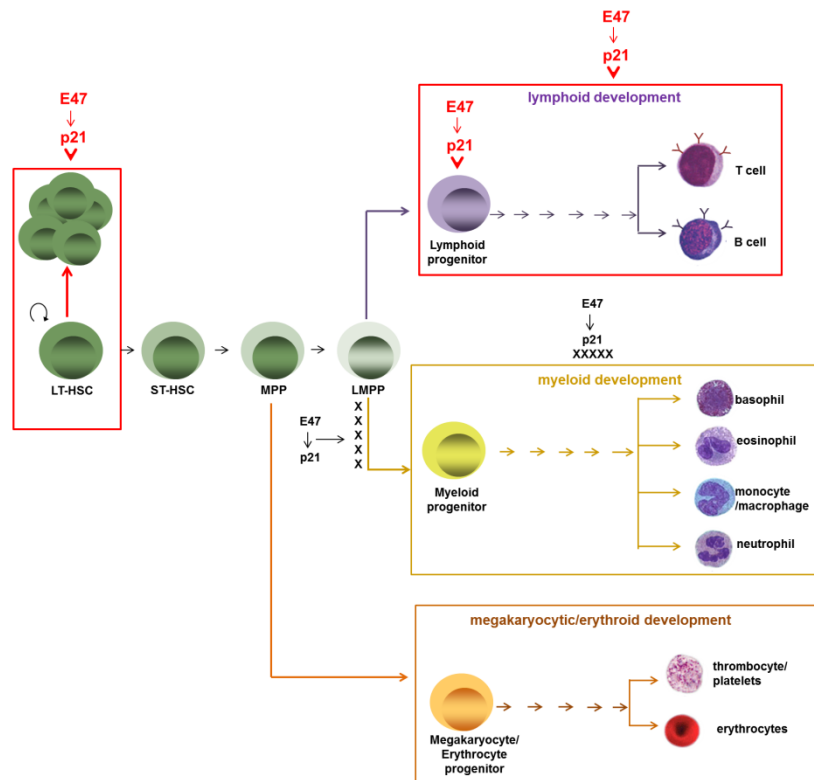
## 5.0 OVERALL SUMMARY AND FUTURE DIRECTIONS

### 5.1 OVERALL SUMMARY

In this study, we specifically show a cell-intrinsic role for the E47-p21 pathway in LT-HSC proliferation and self-renewal. Using mice with combined haploinsufficiency in both E47 and p21, we show a requirement for the E47-p21 pathway in regulation of LT-HSC proliferation under both homeostasis and transplantation stress conditions. Importantly, our findings indicate the biological relevance of the E47-p21 pathway in LT-HSC function *in vivo*. We show that the combination of two subtle lesions, loss of a single allele in a transcription factor and cell cycle regulator factor, resulted not only in LT-HSC hyperproliferation but also in compromised LT-HSC self-renewal during transplantation stress. Deregulation of LT-HSC proliferation can have severe consequences to LT-HSC self-renewal and function (81, 83-85). In our genetic model, transplanted E47<sup>het</sup>p21<sup>het</sup> LT-HSCs failed to persist in WT recipient bone marrow and were unable to self-renew during three rounds of serial adoptive transfer. While immediate downstream multipotent progenitors were able to recover from the decreased number of LT-HSCs and were able to efficiently reconstitute myeloid lineages, lymphoid lineage reconstitution was severely compromised. Common lymphoid progenitors in the bone marrow of E47<sup>het</sup>p21<sup>het</sup>



recipients were dramatically reduced resulting in severe depletion in both B cells and T cells in the periphery. Thus, we not only show a cell-intrinsic role for the E47-p21 pathway in regulation of LT-HSC proliferation, but also a requirement for the E47-p21 pathway in lymphoid lineage progression (**Figure 5-1**).



**Figure 5-1. Cell-intrinsic requirement for the E47-p21 pathway in distinct developmental compartments during hematopoiesis**

The model summarizes major findings and the relevance of E47-p21 pathway in LT-HSC function and hematopoiesis in the context of transplantation stress. [1] Cell-intrinsic role for E47-p21 pathway in the regulation LT-HSC proliferation in both homeostasis and transplantation stress. [2] Requirement for the E47-p21 pathway in lymphoid lineage progression, and [3] E47-p21 pathway is dispensable in myeloid lineage development.

In this study, we took advantage of a genetic model making use of mice that are wild type, haploinsufficient for E47 alone, or haploinsufficient for both E47 and p21 in tandem. Several studies indicate that reduced dosage of E47 predisposes individuals to cancer (112, 133, 139). For example, deletion of E2A, the parent gene of E47, has been reported in a genome-wide mutation study of acute lymphoblastic leukemia (ALL) patients (133). Likewise, ~70% of patients suffering from a T cell lymphoma subtype known as Sezary syndrome also had recurrent E2A gene deletions (139). Overexpression of E47 antagonist Id2 in classical Hodgkin's lymphoma resulted in the loss of E47 activity and formation of B-cell derived lymphomas (112). The sensitive dose requirement for E47 activity in hematopoiesis can exacerbate the impact of additional mutations. Our studies demonstrate that coupled with E47 heterozygosity, loss of a single allele of p21 exacerbates the phenotype associated with simple E47 insufficiency. Specifically, compound heterozygosity of E47 and p21 magnified the defects in hyperproliferation, LT-HSC self-renewal and lymphoid reconstitution observed in E47<sup>het</sup> mice. Thus, this experimental model is an example whereby rather subtle changes in E47 gene dosage in conjunction with modest changes in p21 heterozygosity aggressively exacerbated the effects of E47 haploinsufficiency alone, resulting in immune deficiency. It is possible that a reduction in E47 gene dosage, combined with a second heterozygous mutation in a different gene, can predispose patients afflicted with loss of heterozygosity mutations in E2A to hematologic malignancies.

Because excessive LT-HSC proliferation can have severe consequences to LT-HSC function (81, 83-85), we hypothesized that other mechanisms exist by which E47 can regulate LT-HSC proliferation. Indeed, previous studies have identified that in addition to p21, the

expression of cell cycle regulators such as *cdk6*, *Rb*, *cyclin E1*, *cyclin E2*, and *E2F4* are also regulated by E47 activity (115, 140). During this work, a novel factor p15<sup>PAF</sup>, was identified in which p15<sup>PAF</sup>-deficient mice and E47-deficient mice had a striking phenotypic similarity of early hematopoietic defects. We then focused on examining p15<sup>PAF</sup> as a potential E47 target gene. Our findings demonstrate that E47 directly activates p15<sup>PAF</sup> promoter mediated transcription in 293T cells. However, analysis of p15<sup>PAF</sup> gene and protein expression in E47-deficient primary HSCs and MPPs suggests that the expression of p15<sup>PAF</sup> is regulated at the gene and protein level despite a lack of E47 activity. It remains to be seen whether E47 can also directly activate p15<sup>PAF</sup> promoter mediated transcription and whether forced expression of E47 affects p15<sup>PAF</sup> gene and protein expression in primary HSCs. Therefore, it is possible that E47 is a part of a broader network of factors that regulate p15<sup>PAF</sup> expression at the transcriptional and post-translational level. Finally, it will be interesting to determine the biological relevance of E47-p15<sup>PAF</sup> pathway to HSC function in the context of transplantation stress.

## 5.2 FUTURE DIRECTIONS

In this study we have shown that genetic disruption of one allele each, in both E47 and p21 in LT-HSCs, resulted in loss of lymphoid lineage reconstitution while myeloid lineage reconstitution remained intact in recipient mice. Recent studies indicate that the HSC compartment consists of a very heterogeneous population of stem cells. This heterogeneity is reflected not only in how long these stem cells are able to sustain hematopoiesis (i.e. LT-HSCs versus ST-HSCs) but also in the type of differentiated mature immune cells that these stem cells can produce. Three different types of HSCs are classified based on their respective differentiation potential as tested through serial transplantation. The different HSC types are identified as balanced (Bala-HSC), lymphoid-biased (Ly-bi) and myeloid-biased (My-bi) HSCs (29, 30). Bala-HSCs can reconstitute recipients resulting in donor-derived cells which are ~10% myeloid and ~90% lymphoid cells in the periphery. Ly-bi HSCs can generate only a few myeloid cells, while producing a majority of donor-derived lymphoid cells in recipients. Conversely, adoptive transfer recipients transplanted with My-bi HSCs produce mostly donor-derived myeloid cells and less of donor-derived lymphoid cells (201, 202). Through differential expression of SLAM family member CD229, Oguro et.al. were able to identify myeloid biased HSCs (CD229<sup>-</sup> HSCs) that are more quiescent compared to lymphoid biased HSCs (CD229<sup>-</sup> HSCs) (54). Each type of HSC generates the same type of HSC, such that the differentiation capacities of these HSCs are epigenetically fixed and predetermined (201, 202). Similarly, a recent study using single cell clonal analysis of HSCs have unexpectedly identified myeloid-restricted progenitors with long-term repopulating activity termed as MyRPs, which like My-bi

HSCs, can give rise to megakaryocytic, erythroid and myeloid lineages. However, MyRPs are unlike My-bi HSCs in that MyRP's are already committed to these three lineages and does not produce any lymphoid lineage cells (31). Interestingly, this study also made use of paired daughter cell assay combined with transplantation to show the first experimental evidence of HSCs undergoing symmetrical division (31).

Based on our findings, perturbations in the E47-p21 pathway in LT-HSCs affected only lymphoid lineage reconstitution in recipients. Therefore, it is possible that the E47-p21 pathway is required for the maintenance of self-renewal in lymphoid-biased HSCs only. By contrast, the E47-p21 pathway may not be essential in maintaining self-renewal potential of myeloid-biased HSC as well as that of balanced-HSCs and future studies can be done to address this hypothesis. It will also be interesting to determine if specific epigenetic changes uniquely occurring in Ly-bi HSCs as a result of E47 activity, can mechanistically explain why the E47-p21 pathway would only be relevant to the maintenance of Ly-bi HSC self-renewal potential.

Serial transplantation of E47<sup>het</sup>p21<sup>het</sup> HSCs into WT hosts resulted in recipients characterized by progressive loss of lymphoid lineage reconstitution and normal myeloid lineage reconstitution. This skewing towards myeloid lineage cells over lymphoid lineage cells is highly reminiscent of the hematopoietic composition of aged individuals (cited studies made use of samples from 18-26 month old mice or from 65-85 years old humans unless stated otherwise) (157, 203-206). In addition, our findings of decreased CLP numbers in E47<sup>het</sup>p21<sup>het</sup> secondary recipients were also observed in the bone marrow of mice aged 8 months and older (207, 208). Unexpectedly, analysis of bone marrow from old mice indicates an expansion of the HSC

compartment (209, 210) and similar findings were also observed in humans (204, 211). HSC expansion in elderly individuals seems counter-intuitive to the age-associated changes observed in the hematopoietic system. However, it has been shown that while HSCs are expanded in aged mice, old HSCs have, on a per cell basis, decreased capacity for self-renewal and reconstitution of recipients in serial transplantation assays (212). Furthermore, the use of old HSCs as donors resulted in myeloid-biased reconstitution of recipients, which may be partly explained by the changing composition of HSCs such that My-bi HSCs are selectively expanded in the bone marrow of aged mice (29). At the molecular level, old HSCs are reported to have increased expression of genes linked to oxidative stress, protein aggregation and inflammatory responses and decreased expression of genes involved in DNA repair and chromatin remodeling compared to young HSCs (203). Furthermore, upregulation of myeloid lineage-associated genes and downregulation of lymphoid lineage associated genes were also observed (157, 203, 204). Collectively, these studies demonstrate that part of the changes observed in old HSCs versus young HSCs can be attributed to cell-intrinsic transcriptional changes. In addition, the contribution of epigenetic regulation to changes observed in old HSCs has also been described as a fraction of old HSCs display lower histone H4 lysine acetylation due to the higher levels of Rho GTPase *cdc42* compared to young HSCs (213). Changes in old HSCs due to the cell-extrinsic effects of an aged bone marrow microenvironment or BM niche have also been described. For example, an aged BM niche contributes to myeloid skewing through secretion of high levels of *Ccl5* (also known as RANTES), a pro-inflammatory cytokine (214). The mechanisms that underlie the changes observed in HSCs and HSC function upon aging especially in humans remain poorly understood (215, 216). Thus, it will be interesting to determine if HSCs from aged mice or humans have decreased levels of E47, which in turn can

affect p21 expression. Previous studies have shown that E47 function is highly conserved between mouse and man (108-110, 113-116, 128, 129, 217). Furthermore, it has also been previously shown that E47 levels are downregulated in splenic B cells from aged mice (218) as well as peripheral blood derived B cells from elderly individuals (219, 220). Precursor B cells from the bone marrow of aged mice also had decreased E47 levels due to accelerated Notch-dependent degradation of E47 (221), and decreased E47 activity due to increased levels of ID2, a dominant negative inhibitor of E47 (222). Therefore, it is possible that disruptions in the E47-p21 pathway might also be observed in old HSCs resulting in age-associated changes to HSC function. In summary, additional studies are still needed to determine the mechanisms involved in age associated changes to HSC function which can serve as the basis for future translational applications to improve HSC function and to improve immune protection in the elderly (213, 216, 223).

Beyond the established roles of immune cells in mediating disease pathogenesis, emerging literature demonstrates that chronic autoimmune/inflammatory environments influence hematopoiesis. HSCs in particular are responsive to TLR ligands (32) and interferons (33, 34). Changes in HSC cellularity and lineage-biased output can be influenced by both inflammatory cytokines and genetic risk factors including cell cycle regulators. For example, in a murine model of lupus, the chronic inflammatory environment in these mice favors myeloid over lymphoid lineage development through cytokine-driven expansion of myeloid progenitors and decreased precursor B cell maturation (172). In addition, a SNP in the gene encoding for *p18*, was found to reduce *p18* expression and increase HSC self-renewal in the lupus mice (172). In a KRNxG7 murine model of rheumatoid arthritis, diseased mice exhibited increased tissue-

localized Gr-1<sup>+</sup> and CD11b<sup>+</sup> myeloid cells due in part to enhanced priming of myeloid-specific transcripts in KRNxG7 HSCs and MPPs (224). And, in an IL-23-driven mouse model of colitis, skewing towards myeloid production occurred at the expense of lymphoid and erythroid progenitors, mediated through effects of IFN- $\gamma$  and GM-CSF to HSCs and myeloid progenitors respectively (225). These studies collectively highlight how a chronic inflammatory environment can negatively affect HSC function. E47 levels have been shown to regulate lympho-myeloid fate choice in uncommitted progenitors at the single cell level (119). In an experimental model of chronic inflammation, our laboratory has shown that myeloid bias correlates with reductions in E47 transcript and protein levels (32). Currently unknown is whether the reductions in E47 are also accompanied by reductions in p21 expression, and if it is indeed the case, then the E47-p21 pathway may also be mechanistically relevant to changes in HSC function mediated by a chronic inflammatory environment.

Thus, understanding the molecular mechanisms that regulate HSC self-renewal and function have important implications in the clinical setting for purposes of improved therapeutic bone marrow stem cell transplantation and preservation of HSC integrity. Indeed as emerging literature indicates, clinical diseases such as cancer, aging, and chronic inflammatory conditions, have all been shown to influence HSCs and, in some cases, to negatively affect HSC function. Therefore, knowledge of the molecular mechanisms that regulate self-renewal and hematopoietic potential can also be applied to improving and/or preserving HSC function in the context of these diseases.



## APPENDIX A

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## BIBLIOGRAPHY

1. Rieger, M. A., and T. Schroeder. 2012. Hematopoiesis. *Cold Spring Harb Perspect Biol* 4.
2. Takizawa, H., S. Boettcher, and M. G. Manz. 2012. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* 119: 2991-3002.
3. Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91: 661-672.
4. Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404: 193-197.
5. Adolfsson, J., R. Mansson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O. J. Borge, L. A. Thoren, K. Anderson, E. Sitnicka, Y. Sasaki, M. Sigvardsson, and S. E. Jacobsen. 2005. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* 121: 295-306.
6. Spangrude, G. J., S. Heimfeld, and I. L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. *Science* 241: 58-62.
7. McCulloch, E. A., and J. E. Till. 2005. Perspectives on the properties of stem cells. *Nat Med* 11: 1026-1028.
8. Nakorn, T. N., T. Miyamoto, and I. L. Weissman. 2003. Characterization of mouse clonogenic megakaryocyte progenitors. *Proc Natl Acad Sci U S A* 100: 205-210.
9. Inlay, M. A., D. Bhattacharya, D. Sahoo, T. Serwold, J. Seita, H. Karsunky, S. K. Plevritis, D. L. Dill, and I. L. Weissman. 2009. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev* 23: 2376-2381.



10. Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* 4: 168-174.
11. Lai, A. Y., and M. Kondo. 2007. Identification of a bone marrow precursor of the earliest thymocytes in adult mouse. *Proc Natl Acad Sci U S A* 104: 6311-6316.
12. Dias, S., R. Mansson, S. Gurbuxani, M. Sigvardsson, and B. L. Kee. 2008. E2A Proteins Promote Development of Lymphoid-Primed Multipotent Progenitors. *Immunity* 29: 217-227.
13. Hu, W., B. Yuan, J. Flygare, and H. F. Lodish. 2011. Long noncoding RNA-mediated anti-apoptotic activity in murine erythroid terminal differentiation. *Genes Dev* 25: 2573-2578.
14. Paralkar, V. R., and M. J. Weiss. 2011. A new 'Linc' between noncoding RNAs and blood development. *Genes Dev* 25: 2555-2558.
15. Hu, W., J. R. Alvarez-Dominguez, and H. F. Lodish. 2012. Regulation of mammalian cell differentiation by long non-coding RNAs. *EMBO reports* 13: 971-983.
16. Alvarez-Dominguez, J. R., W. Hu, B. Yuan, J. Shi, S. S. Park, A. A. Gromatzky, A. van Oudenaarden, and H. F. Lodish. 2013. Global discovery of erythroid long non-coding RNAs reveals novel regulators of red cell maturation. *Blood*.
17. Paralkar, V. R., and M. J. Weiss. 2013. Long noncoding RNAs in biology and hematopoiesis. *Blood* 121: 4842-4846.
18. Zhao, J. L., D. S. Rao, R. M. O'Connell, Y. Garcia-Flores, and D. Baltimore. 2013. MicroRNA-146a acts as a guardian of the quality and longevity of hematopoietic stem cells in mice. *eLife* 2: e00537.
19. Caramuta, S., L. Lee, D. M. Ozata, P. Akcakaya, P. Georgii-Hemming, H. Xie, R. M. Amini, C. H. Lawrie, G. Enblad, C. Larsson, M. Berglund, and W. O. Lui. 2013. Role of microRNAs and microRNA machinery in the pathogenesis of diffuse large B-cell lymphoma. *Blood cancer journal* 3: e152.
20. Lawrie, C. H. 2013. MicroRNAs in hematological malignancies. *Blood reviews* 27: 143-154.
21. Wang, X. S., J. N. Gong, J. Yu, F. Wang, X. H. Zhang, X. L. Yin, Z. Q. Tan, Z. M. Luo, G. H. Yang, C. Shen, and J. W. Zhang. 2012. MicroRNA-29a and microRNA-142-3p are regulators of myeloid differentiation and acute myeloid leukemia. *Blood* 119: 4992-5004.
22. Chaudhuri, A. A., A. Y. So, A. Mehta, A. Minisandram, N. Sinha, V. D. Jonsson, D. S. Rao, R. M. O'Connell, and D. Baltimore. 2012. Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A. *Proc Natl Acad Sci U S A* 109: 4233-4238.

23. O'Connell, R. M., and D. Baltimore. 2012. Chapter six - MicroRNAs and Hematopoietic Cell Development. In *Current Topics in Developmental Biology*. H. Eran, ed. Academic Press. 145-174.
24. Park, I. K., D. Qian, M. Kiel, M. W. Becker, M. Pihalja, I. L. Weissman, S. J. Morrison, and M. F. Clarke. 2003. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 423: 302-305.
25. Broske, A. M., L. Vockentanz, S. Kharazi, M. R. Huska, E. Mancini, M. Scheller, C. Kuhl, A. Enns, M. Prinz, R. Jaenisch, C. Nerlov, A. Leutz, M. A. Andrade-Navarro, S. E. Jacobsen, and F. Rosenbauer. 2009. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. *Nature genetics* 41: 1207-1215.
26. Quivoron, C., L. Couronne, V. Della Valle, C. K. Lopez, I. Plo, O. Wagner-Ballon, M. Do Cruzeiro, F. Delhommeau, B. Arnulf, M. H. Stern, L. Godley, P. Opolon, H. Tilly, E. Solary, Y. Duffourd, P. Dessen, H. Merle-Beral, F. Nguyen-Khac, M. Fontenay, W. Vainchenker, C. Bastard, T. Mercher, and O. A. Bernard. 2011. TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell* 20: 25-38.
27. Sashida, G., and A. Iwama. 2012. Epigenetic regulation of hematopoiesis. *International journal of hematology* 96: 405-412.
28. Butler, J. S., and S. Y. Dent. 2013. The role of chromatin modifiers in normal and malignant hematopoiesis. *Blood* 121: 3076-3084.
29. Cho, R. H., H. B. Sieburg, and C. E. Muller-Sieburg. 2008. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. *Blood* 111: 5553-5561.
30. Dykstra, B., D. Kent, M. Bowie, L. McCaffrey, M. Hamilton, K. Lyons, S. J. Lee, R. Brinkman, and C. Eaves. 2007. Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* 1: 218-229.
31. Yamamoto, R., Y. Morita, J. Oebara, S. Hamanaka, M. Onodera, K. L. Rudolph, H. Ema, and H. Nakauchi. 2013. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* 154: 1112-1126.
32. Esplin, B. L., T. Shimazu, R. S. Welner, K. P. Garrett, L. Nie, Q. Zhang, M. B. Humphrey, Q. Yang, L. A. Borghesi, and P. W. Kincade. 2011. Chronic exposure to a TLR ligand injures hematopoietic stem cells. *J Immunol* 186: 5367-5375.
33. Essers, M. A., S. Offner, W. E. Blanco-Bose, Z. Waibler, U. Kalinke, M. A. Duchosal, and A. Trumpp. 2009. IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* 458: 904-908.

34. Baldrige, M. T., K. Y. King, N. C. Boles, D. C. Weksberg, and M. A. Goodell. 2010. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* 465: 793-797.
35. Santos, P. M., and L. Borghesi. 2011. Molecular resolution of the B cell landscape. *Curr Opin Immunol* 23: 163-170.
36. Maximow, A. 1909. Der Lymphozyt als gemeinsame Stammzelle der verschiedenen Blutelemente in der embryonalen Entwicklung und im postfetalen Leben der Säugetiere. *Folia Haematol. (Frankf.)* 8: 125-134.
37. Lorenz, E., D. Uphoff, T. R. Reid, and E. Shelton. 1951. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. *J Natl Cancer Inst* 12: 197-201.
38. Till, J. E., and C. E. Mc. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* 14: 213-222.
39. Seita, J., and I. L. Weissman. 2012. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* 2: 640-653.
40. Ploemacher, R. E., and R. H. Brons. 1989. Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre-CFU-S cell. *Exp Hematol* 17: 263-266.
41. Purton, L. E., and D. T. Scadden. 2007. Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell* 1: 263-270.
42. Doulatov, S., F. Notta, E. Laurenti, and J. E. Dick. 2012. Hematopoiesis: a human perspective. *Cell Stem Cell* 10: 120-136.
43. Kiel, M. J., O. H. Yilmaz, T. Iwashita, C. Terhorst, and S. J. Morrison. 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121: 1109-1121.
44. Lagasse, E., and I. L. Weissman. 1996. Flow cytometric identification of murine neutrophils and monocytes. *Journal of immunological methods* 197: 139-150.
45. Ng, S. Y., T. Yoshida, J. Zhang, and K. Georgopoulos. 2009. Genome-wide lineage-specific transcriptional networks underscore Ikaros-dependent lymphoid priming in hematopoietic stem cells. *Immunity* 30: 493-507.
46. Mansson, R., S. Zandi, E. Welinder, P. Tsapogas, N. Sakaguchi, D. Bryder, and M. Sigvardsson. 2010. Single-cell analysis of the common lymphoid progenitor compartment reveals functional and molecular heterogeneity. *Blood* 115: 2601-2609.

47. Manohar, R., J. Komori, L. Guzik, D. B. Stolz, U. R. Chandran, W. A. LaFramboise, and E. Lagasse. 2011. Identification and expansion of a unique stem cell population from adult mouse gallbladder. *Hepatology* 54: 1830-1841.
48. Teisanu, R. M., E. Lagasse, J. F. Whitesides, and B. R. Stripp. 2009. Prospective isolation of bronchiolar stem cells based upon immunophenotypic and autofluorescence characteristics. *Stem Cells* 27: 612-622.
49. Francipane, M. G., and E. Lagasse. 2013. Selective targeting of human colon cancer stem-like cells by the mTOR inhibitor Torin-1. *Oncotarget*.
50. Xiong, A., T. W. Austin, E. Lagasse, N. Uchida, S. Tamaki, B. B. Bordier, I. L. Weissman, J. S. Glenn, and M. T. Millan. 2008. Isolation of human fetal liver progenitors and their enhanced proliferation by three-dimensional coculture with endothelial cells. *Tissue engineering. Part A* 14: 995-1006.
51. Lagasse, E., H. Connors, M. Al-Dhalimy, M. Reitsma, M. Dohse, L. Osborne, X. Wang, M. Finegold, I. L. Weissman, and M. Grompe. 2000. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 6: 1229-1234.
52. Ikuta, K., and I. L. Weissman. 1992. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci U S A* 89: 1502-1506.
53. Ogawa, M., Y. Matsuzaki, S. Nishikawa, S. Hayashi, T. Kunisada, T. Sudo, T. Kina, and H. Nakauchi. 1991. Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med* 174: 63-71.
54. Oguro, H., L. Ding, and S. J. Morrison. 2013. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell* 13: 102-116.
55. Christensen, J. L., and I. L. Weissman. 2001. Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. *Proc Natl Acad Sci U S A* 98: 14541-14546.
56. Semerad, C. L., E. M. Mercer, M. A. Inlay, I. L. Weissman, and C. Murre. 2009. E2A proteins maintain the hematopoietic stem cell pool and promote the maturation of myelolymphoid and myeloerythroid progenitors. *Proc Natl Acad Sci U S A* 106: 1930-1935.
57. Goodell, M. A., K. Brose, G. Paradis, A. S. Conner, and R. C. Mulligan. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183: 1797-1806.
58. Goodell, M. A., M. Rosenzweig, H. Kim, D. F. Marks, M. DeMaria, G. Paradis, S. A. Grupp, C. A. Sieff, R. C. Mulligan, and R. P. Johnson. 1997. Dye efflux studies suggest

- that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 3: 1337-1345.
59. Zhou, S., J. D. Schuetz, K. D. Bunting, A. M. Colapietro, J. Sampath, J. J. Morris, I. Lagutina, G. C. Grosveld, M. Osawa, H. Nakauchi, and B. P. Sorrentino. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7: 1028-1034.
  60. Scharenberg, C. W., M. A. Harkey, and B. Torok-Storb. 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99: 507-512.
  61. Matsuzaki, Y., K. Kinjo, R. C. Mulligan, and H. Okano. 2004. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* 20: 87-93.
  62. Baum, C. M., I. L. Weissman, A. S. Tsukamoto, A. M. Buckle, and B. Peault. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* 89: 2804-2808.
  63. Civin, C. I., T. Trischmann, N. S. Kadan, J. Davis, S. Noga, K. Cohen, B. Duffy, I. Groenewegen, J. Wiley, P. Law, A. Hardwick, F. Oldham, and A. Gee. 1996. Highly purified CD34-positive cells reconstitute hematopoiesis. *J Clin Oncol* 14: 2224-2233.
  64. Parekh, C., and G. M. Crooks. 2013. Critical differences in hematopoiesis and lymphoid development between humans and mice. *Journal of clinical immunology* 33: 711-715.
  65. Hao, Q. L., F. T. Thiemann, D. Petersen, E. M. Smogorzewska, and G. M. Crooks. 1996. Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* 88: 3306-3313.
  66. de Wynter, E. A., D. Buck, C. Hart, R. Heywood, L. H. Coutinho, A. Clayton, J. A. Rafferty, D. Burt, G. Guenechea, J. A. Bueren, D. Gagen, L. J. Fairbairn, B. I. Lord, and N. G. Testa. 1998. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. *Stem Cells* 16: 387-396.
  67. Notta, F., S. Doulatov, E. Laurenti, A. Poepl, I. Jurisica, and J. E. Dick. 2011. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333: 218-221.
  68. Adolfsson, J., O. J. Borge, D. Bryder, K. Theilgaard-Monch, I. Astrand-Grundstrom, E. Sitnicka, Y. Sasaki, and S. E. Jacobsen. 2001. Upregulation of Flt3 expression within the bone marrow Lin(-)Scal(+)-c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* 15: 659-669.
  69. Wang, X., M. Foster, M. Al-Dhalimy, E. Lagasse, M. Finegold, and M. Grompe. 2003. The origin and liver repopulating capacity of murine oval cells. *Proc Natl Acad Sci U S A* 100 Suppl 1: 11881-11888.

70. Omidvar, N., S. Kogan, S. Beurlet, C. le Pogam, A. Janin, R. West, M. E. Noguera, M. Reboul, A. Soulie, C. Leboeuf, N. Setterblad, D. Felsher, E. Lagasse, A. Mohamedali, N. S. Thomas, P. Fenaux, M. Fontenay, M. Pla, G. J. Mufti, I. Weissman, C. Chomienne, and R. A. Padua. 2007. BCL-2 and mutant NRAS interact physically and functionally in a mouse model of progressive myelodysplasia. *Cancer Res* 67: 11657-11667.
71. Odoux, C., H. Fohrer, T. Hoppe, L. Guzik, D. B. Stolz, D. W. Lewis, S. M. Gollin, T. C. Gamblin, D. A. Geller, and E. Lagasse. 2008. A stochastic model for cancer stem cell origin in metastatic colon cancer. *Cancer Res* 68: 6932-6941.
72. Osawa, M., K. Hanada, H. Hamada, and H. Nakauchi. 1996. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* 273: 242-245.
73. Lemischka, I. R., D. H. Raulet, and R. C. Mulligan. 1986. Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* 45: 917-927.
74. Purton, L. E., S. Dworkin, G. H. Olsen, C. R. Walkley, S. A. Fabb, S. J. Collins, and P. Chambon. 2006. RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. *J Exp Med* 203: 1283-1293.
75. Payne, K. J., and G. M. Crooks. 2007. Immune-cell lineage commitment: translation from mice to humans. *Immunity* 26: 674-677.
76. Shultz, L. D., P. A. Schweitzer, S. W. Christianson, B. Gott, I. B. Schweitzer, B. Tennent, S. McKenna, L. Mobraaten, T. V. Rajan, D. L. Greiner, and et al. 1995. Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* 154: 180-191.
77. Mazurier, F., A. Fontanellas, S. Salesse, L. Taine, S. Landriau, F. Moreau-Gaudry, J. Reiffers, B. Peault, J. P. Di Santo, and H. de Verneuil. 1999. A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res* 19: 533-541.
78. Ito, M., H. Hiramatsu, K. Kobayashi, K. Suzue, M. Kawahata, K. Hioki, Y. Ueyama, Y. Koyanagi, K. Sugamura, K. Tsuji, T. Heike, and T. Nakahata. 2002. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100: 3175-3182.
79. Rongvaux, A., T. Willinger, H. Takizawa, C. Rathinam, W. Auerbach, A. J. Murphy, D. M. Valenzuela, G. D. Yancopoulos, E. E. Eynon, S. Stevens, M. G. Manz, and R. A. Flavell. 2011. Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. *Proc Natl Acad Sci U S A* 108: 2378-2383.
80. Willinger, T., A. Rongvaux, T. Strowig, M. G. Manz, and R. A. Flavell. 2011. Improving human hemato-lymphoid-system mice by cytokine knock-in gene replacement. *Trends Immunol* 32: 321-327.

81. Orford, K. W., and D. T. Scadden. 2008. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet* 9: 115-128.
82. Pietras, E. M., M. R. Warr, and E. Passegue. 2011. Cell cycle regulation in hematopoietic stem cells. *J Cell Biol* 195: 709-720.
83. Wilson, A., and A. Trumpp. 2006. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* 6: 93-106.
84. Cheng, T., N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes, and D. T. Scadden. 2000. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 287: 1804-1808.
85. Li, J. 2011. Quiescence regulators for hematopoietic stem cell. *Exp Hematol* 39: 511-520.
86. Jacob, B., M. Osato, N. Yamashita, C. Q. Wang, I. Taniuchi, D. R. Littman, N. Asou, and Y. Ito. 2010. Stem cell exhaustion due to Runx1 deficiency is prevented by Evi5 activation in leukemogenesis. *Blood* 115: 1610-1620.
87. Lane, S. W., and D. G. Gilliland. 2011. Leukemia stem cells. *Semin Cancer Biol* 20: 71-76.
88. Wilkinson, A. C., and B. Gottgens. 2013. Transcriptional regulation of haematopoietic stem cells. *Advances in experimental medicine and biology* 786: 187-212.
89. Cheng, T., N. Rodrigues, D. Dombkowski, S. Stier, and D. T. Scadden. 2000. Stem cell repopulation efficiency but not pool size is governed by p27(kip1). *Nat Med* 6: 1235-1240.
90. Matsumoto, A., S. Takeishi, T. Kanie, E. Susaki, I. Onoyama, Y. Tateishi, K. Nakayama, and K. I. Nakayama. 2011. p57 is required for quiescence and maintenance of adult hematopoietic stem cells. *Cell Stem Cell* 9: 262-271.
91. Zou, P., H. Yoshihara, K. Hosokawa, I. Tai, K. Shinmyozu, F. Tsukahara, Y. Maru, K. Nakayama, K. I. Nakayama, and T. Suda. 2011. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell* 9: 247-261.
92. Viatour, P., T. C. Somervaille, S. Venkatasubrahmanyam, S. Kogan, M. E. McLaughlin, I. L. Weissman, A. J. Butte, E. Passegue, and J. Sage. 2008. Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. *Cell Stem Cell* 3: 416-428.
93. Janzen, V., R. Forkert, H. E. Fleming, Y. Saito, M. T. Waring, D. M. Dombkowski, T. Cheng, R. A. DePinho, N. E. Sharpless, and D. T. Scadden. 2006. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 443: 421-426.

94. Krishnamurthy, J., C. Torrice, M. R. Ramsey, G. I. Kovalev, K. Al-Regaiey, L. Su, and N. E. Sharpless. 2004. Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* 114: 1299-1307.
95. Zindy, F., D. E. Quelle, M. F. Roussel, and C. J. Sherr. 1997. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* 15: 203-211.
96. Kee, B. L. 2009. E and ID proteins branch out. *Nat Rev Immunol* 9: 175-184.
97. Hock, H., M. J. Hamblen, H. M. Rooke, J. W. Schindler, S. Saleque, Y. Fujiwara, and S. H. Orkin. 2004. Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. *Nature* 431: 1002-1007.
98. Lacombe, J., S. Herblot, S. Rojas-Sutterlin, A. Haman, S. Barakat, N. N. Iscove, G. Sauvageau, and T. Hoang. 2010. Scl regulates the quiescence and the long-term competence of hematopoietic stem cells. *Blood* 115: 792-803.
99. Ficara, F., M. J. Murphy, M. Lin, and M. L. Cleary. 2008. Pbx1 regulates self-renewal of long-term hematopoietic stem cells by maintaining their quiescence. *Cell Stem Cell* 2: 484-496.
100. Kobayashi, M., and E. F. Srour. 2011. Regulation of murine hematopoietic stem cell quiescence by Dmtf1. *Blood* 118: 6562-6571.
101. Liu, Y., S. E. Elf, T. Asai, Y. Miyata, G. Sashida, G. Huang, S. Di Giandomenico, A. Koff, and S. D. Nimer. 2009. The p53 tumor suppressor protein is a critical regulator of hematopoietic stem cell behavior. *Cell Cycle* 8: 3120-3124.
102. Sato, T., N. Onai, H. Yoshihara, F. Arai, T. Suda, and T. Ohteki. 2009. Interferon regulatory factor-2 protects quiescent hematopoietic stem cells from type I interferon-dependent exhaustion. *Nat Med* 15: 696-700.
103. Sirin, O., G. L. Lukov, R. Mao, O. M. Conneely, and M. A. Goodell. 2010. The orphan nuclear receptor Nurr1 restricts the proliferation of haematopoietic stem cells. *Nat Cell Biol* 12: 1213-1219.
104. Henthorn, P., M. Kiledjian, and T. Kadesch. 1990. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/kappa 2 motif. *Science* 247: 467-470.
105. Murre, C. 2005. Helix-loop-helix proteins and lymphocyte development. *Nat Immunol* 6: 1079-1086.
106. Bain, G., E. C. Robanus Maandag, H. P. te Riele, A. J. Feeney, A. Sheehy, M. Schlissel, S. A. Shinton, R. R. Hardy, and C. Murre. 1997. Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 6: 145-154.



107. Sun, X. H., and D. Baltimore. 1991. An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell* 64: 459-470.
108. Bain, G., E. C. Maandag, D. J. Izon, D. Amsen, A. M. Kruisbeek, B. C. Weintraub, I. Krop, M. S. Schlissel, A. J. Feeney, M. van Roon, and et al. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79: 885-892.
109. Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell* 79: 875-884.
110. Borghesi, L., J. Aites, S. Nelson, P. Lefterov, P. James, and R. Gerstein. 2005. E47 is required for V(D)J recombinase activity in common lymphoid progenitors. *J Exp Med* 202: 1669-1677.
111. Ikawa, T., H. Kawamoto, L. Y. Wright, and C. Murre. 2004. Long-term cultured E2A-deficient hematopoietic progenitor cells are pluripotent. *Immunity* 20: 349-360.
112. Mathas, S., M. Janz, F. Hummel, M. Hummel, B. Wollert-Wulf, S. Lusatis, I. Anagnostopoulos, A. Lietz, M. Sigvardsson, F. Jundt, K. Johrens, K. Bommert, H. Stein, and B. Dorken. 2006. Intrinsic inhibition of transcription factor E2A by HLH proteins ABF-1 and Id2 mediates reprogramming of neoplastic B cells in Hodgkin lymphoma. *Nat Immunol* 7: 207-215.
113. Bain, G., I. Engel, E. C. Robanus Maandag, H. P. te Riele, J. R. Volland, L. L. Sharp, J. Chun, B. Huey, D. Pinkel, and C. Murre. 1997. E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. *Mol Cell Biol* 17: 4782-4791.
114. Barndt, R. J., M. Dai, and Y. Zhuang. 2000. Functions of E2A-HEB heterodimers in T-cell development revealed by a dominant negative mutation of HEB. *Mol Cell Biol* 20: 6677-6685.
115. Schwartz, R., I. Engel, M. Fallahi-Sichani, H. T. Petrie, and C. Murre. 2006. Gene expression patterns define novel roles for E47 in cell cycle progression, cytokine-mediated signaling, and T lineage development. *Proc Natl Acad Sci U S A* 103: 9976-9981.
116. Xu, W., and B. L. Kee. 2007. Growth factor independent 1B (Gfi1b) is an E2A target gene that modulates Gata3 in T-cell lymphomas. *Blood* 109: 4406-4414.
117. Leeanansaksiri, W., H. Wang, J. M. Gooya, K. Renn, M. Abshari, S. Tsai, and J. R. Keller. 2005. IL-3 induces inhibitor of DNA-binding protein-1 in hemopoietic progenitor cells and promotes myeloid cell development. *J Immunol* 174: 7014-7021.

118. Jankovic, V., A. Ciarrocchi, P. Boccuni, T. DeBlasio, R. Benezra, and S. D. Nimer. 2007. Id1 restrains myeloid commitment, maintaining the self-renewal capacity of hematopoietic stem cells. *Proc Natl Acad Sci U S A* 104: 1260-1265.
119. Cochrane, S. W., Y. Zhao, R. S. Welner, and X. H. Sun. 2009. Balance between Id and E proteins regulates myeloid-versus-lymphoid lineage decisions. *Blood* 113: 1016-1026.
120. Medina, K. L., J. M. Pongubala, K. L. Reddy, D. W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. *Dev Cell* 7: 607-617.
121. Seet, C. S., R. L. Brumbaugh, and B. L. Kee. 2004. Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. *J Exp Med* 199: 1689-1700.
122. Singh, H., K. L. Medina, and J. M. Pongubala. 2005. Contingent gene regulatory networks and B cell fate specification. *Proc Natl Acad Sci U S A* 102: 4949-4953.
123. Lin, Y. C., S. Jhunjhunwala, C. Benner, S. Heinz, E. Welinder, R. Mansson, M. Sigvardsson, J. Hagman, C. A. Espinoza, J. Dutkowski, T. Ideker, C. K. Glass, and C. Murre. 2010. A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat Immunol* 11: 635-643.
124. Sigvardsson, M., M. O'Riordan, and R. Grosschedl. 1997. EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity* 7: 25-36.
125. Greenbaum, S., and Y. Zhuang. 2002. Identification of E2A target genes in B lymphocyte development by using a gene tagging-based chromatin immunoprecipitation system. *Proc Natl Acad Sci U S A* 99: 15030-15035.
126. Borghesi, L., L. Y. Hsu, J. P. Miller, M. Anderson, L. Herzenberg, M. S. Schlissel, D. Allman, and R. M. Gerstein. 2004. B lineage-specific regulation of V(D)J recombinase activity is established in common lymphoid progenitors. *J Exp Med* 199: 491-502.
127. Bain, G., W. J. Romanow, K. Albers, W. L. Havran, and C. Murre. 1999. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med* 189: 289-300.
128. Heemskerk, M. H., B. Blom, G. Nolan, A. P. Stegmann, A. Q. Bakker, K. Weijer, P. C. Res, and H. Spits. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 186: 1597-1602.
129. Jaleco, A. C., A. P. Stegmann, M. H. Heemskerk, F. Couwenberg, A. Q. Bakker, K. Weijer, and H. Spits. 1999. Genetic modification of human B-cell development: B-cell development is inhibited by the dominant negative helix loop helix factor Id3. *Blood* 94: 2637-2646.

130. Engel, I., and C. Murre. 1999. Ectopic expression of E47 or E12 promotes the death of E2A-deficient lymphomas. *Proc Natl Acad Sci U S A* 96: 996-1001.
131. Kim, D., X. C. Peng, and X. H. Sun. 1999. Massive apoptosis of thymocytes in T-cell-deficient Id1 transgenic mice. *Mol Cell Biol* 19: 8240-8253.
132. Morrow, M. A., E. W. Mayer, C. A. Perez, M. Adlam, and G. Siu. 1999. Overexpression of the Helix-Loop-Helix protein Id2 blocks T cell development at multiple stages. *Mol Immunol* 36: 491-503.
133. Mullighan, C. G., S. Goorha, I. Radtke, C. B. Miller, E. Coustan-Smith, J. D. Dalton, K. Girtman, S. Mathew, J. Ma, S. B. Pounds, X. Su, C. H. Pui, M. V. Relling, W. E. Evans, S. A. Shurtleff, and J. R. Downing. 2007. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446: 758-764.
134. Yoshihara, T., T. Inaba, L. H. Shapiro, J. Y. Kato, and A. T. Look. 1995. E2A-HLF-mediated cell transformation requires both the trans-activation domains of E2A and the leucine zipper dimerization domain of HLF. *Mol Cell Biol* 15: 3247-3255.
135. Kamps, M. P., A. T. Look, and D. Baltimore. 1991. The human t(1;19) translocation in pre-B ALL produces multiple nuclear E2A-Pbx1 fusion proteins with differing transforming potentials. *Genes Dev* 5: 358-368.
136. Aspland, S. E., H. H. Bendall, and C. Murre. 2001. The role of E2A-PBX1 in leukemogenesis. *Oncogene* 20: 5708-5717.
137. Hsu, H. L., I. Wadman, and R. Baer. 1994. Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. *Proc Natl Acad Sci U S A* 91: 3181-3185.
138. O'Neil, J., J. Shank, N. Cusson, C. Murre, and M. Kelliher. 2004. TAL1/SCL induces leukemia by inhibiting the transcriptional activity of E47/HEB. *Cancer Cell* 5: 587-596.
139. Steininger, A., M. Mobs, R. Ullmann, K. Kochert, S. Kreher, B. Lamprecht, I. Anagnostopoulos, M. Hummel, J. Richter, M. Beyer, M. Janz, C. D. Klemke, H. Stein, B. Dorken, W. Sterry, E. Schrock, S. Mathas, and C. Assaf. 2011. Genomic loss of the putative tumor suppressor gene E2A in human lymphoma. *J Exp Med* 208: 1585-1593.
140. Yang, Q., L. Kardava, A. St Leger, K. Martincic, B. Varnum-Finney, I. D. Bernstein, C. Milcarek, and L. Borghesi. 2008. E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors. *J Immunol* 181: 5885-5894.
141. Zhuang, Y., A. Jackson, L. Pan, K. Shen, and M. Dai. 2004. Regulation of E2A gene expression in B-lymphocyte development. *Mol Immunol* 40: 1165-1177.
142. Kwon, K., C. Hutter, Q. Sun, I. Bilic, C. Cobaleda, S. Malin, and M. Busslinger. 2008. Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. *Immunity* 28: 751-762.

143. Yang, Q., B. Esplin, and L. Borghesi. 2011. E47 regulates hematopoietic stem cell proliferation and energetics but not myeloid lineage restriction. *Blood* 117: 3529-3538.
144. Herblot, S., P. D. Aplan, and T. Hoang. 2002. Gradient of E2A activity in B-cell development. *Mol Cell Biol* 22: 886-900.
145. Prabhu, S., A. Ignatova, S. T. Park, and X. H. Sun. 1997. Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. *Mol Cell Biol* 17: 5888-5896.
146. Foudi, A., K. Hochedlinger, D. Van Buren, J. W. Schindler, R. Jaenisch, V. Carey, and H. Hock. 2009. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol* 27: 84-90.
147. Insinga, A., A. Cicalese, M. Faretta, B. Gallo, L. Albano, S. Ronzoni, L. Furia, A. Viale, and P. G. Pelicci. 2013. DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions. *Proc Natl Acad Sci U S A* 110: 3931-3936.
148. van Os, R., L. M. Kamminga, A. Ausema, L. V. Bystrykh, D. P. Draijer, K. van Pelt, B. Dontje, and G. de Haan. 2007. A Limited role for p21Cip1/Waf1 in maintaining normal hematopoietic stem cell functioning. *Stem Cells* 25: 836-843.
149. Bell, D. R., and G. Van Zant. 2004. Stem cells, aging, and cancer: inevitabilities and outcomes. *Oncogene* 23: 7290-7296.
150. Bonnet, D., and J. E. Dick. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 3: 730-737.
151. Jordan, C. T. 2002. Unique molecular and cellular features of acute myelogenous leukemia stem cells. *Leukemia* 16: 559-562.
152. Walkley, C. R., G. A. McArthur, and L. E. Purton. 2005. Cell division and hematopoietic stem cells: not always exhausting. *Cell Cycle* 4: 893-896.
153. Jude, C. D., J. J. Gaudet, N. A. Speck, and P. Ernst. 2008. Leukemia and hematopoietic stem cells: balancing proliferation and quiescence. *Cell Cycle* 7: 586-591.
154. Reya, T., S. J. Morrison, M. F. Clarke, and I. L. Weissman. 2001. Stem cells, cancer, and cancer stem cells. *Nature* 414: 105-111.
155. Dorshkind, K., and S. Swain. 2009. Age-associated declines in immune system development and function: causes, consequences, and reversal. *Curr Opin Immunol* 21: 404-407.
156. Henry, C. J., A. Marusyk, and J. DeGregori. 2011. Aging-associated changes in hematopoiesis and leukemogenesis: what's the connection? *Aging (Albany NY)* 3: 643-656.

157. Rossi, D. J., D. Bryder, J. M. Zahn, H. Ahlenius, R. Sonu, A. J. Wagers, and I. L. Weissman. 2005. Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A* 102: 9194-9199.
158. Weissman, I. L., and J. A. Shizuru. 2008. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 112: 3543-3553.
159. Lai, A. Y., S. M. Lin, and M. Kondo. 2005. Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow. *J Immunol* 175: 5016-5023.
160. Miyazaki, M., R. R. Rivera, K. Miyazaki, Y. C. Lin, Y. Agata, and C. Murre. 2011. The opposing roles of the transcription factor E2A and its antagonist Id3 that orchestrate and enforce the naive fate of T cells. *Nat Immunol* 12: 992-1001.
161. Ferreiros-Vidal, I., T. Carroll, B. Taylor, A. Terry, Z. Liang, L. Bruno, G. Dharmalingam, S. Khadayate, B. S. Cobb, S. T. Smale, M. Spivakov, P. Srivastava, E. Petretto, A. G. Fisher, and M. Merkenschlager. 2013. Genome-wide identification of Ikaros targets elucidates its contribution to mouse B-cell lineage specification and pre-B-cell differentiation. *Blood* 121: 1769-1782.
162. Henning, K., J. Heering, R. Schwanbeck, T. Schroeder, H. Helmbold, H. Schafer, W. Deppert, E. Kim, and U. Just. 2008. Notch1 activation reduces proliferation in the multipotent hematopoietic progenitor cell line FDCP-mix through a p53-dependent pathway but Notch1 effects on myeloid and erythroid differentiation are independent of p53. *Cell Death Differ* 15: 398-407.
163. O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity* 11: 21-31.
164. Lukin, K., S. Fields, D. Lopez, M. Cherrier, K. Ternyak, J. Ramirez, A. J. Feeney, and J. Hagman. 2010. Compound haploinsufficiencies of Ebf1 and Runx1 genes impede B cell lineage progression. *Proc Natl Acad Sci U S A* 107: 7869-7874.
165. Taniguchi Ishikawa, E., D. Gonzalez-Nieto, G. Ghiaur, S. K. Dunn, A. M. Ficker, B. Murali, M. Madhu, D. E. Gutstein, G. I. Fishman, L. C. Barrio, and J. A. Cancelas. 2012. Connexin-43 prevents hematopoietic stem cell senescence through transfer of reactive oxygen species to bone marrow stromal cells. *Proc Natl Acad Sci U S A* 109: 9071-9076.
166. Boettcher, S., P. Ziegler, M. A. Schmid, H. Takizawa, N. van Rooijen, M. Kopf, M. Heikenwalder, and M. G. Manz. 2012. Cutting edge: LPS-induced emergency myelopoiesis depends on TLR4-expressing nonhematopoietic cells. *J Immunol* 188: 5824-5828.
167. Nagai, Y., K. P. Garrett, S. Ohta, U. Bahrn, T. Kouro, S. Akira, K. Takatsu, and P. W. Kincade. 2006. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity* 24: 801-812.

168. Ueda, Y., D. W. Cain, M. Kuraoka, M. Kondo, and G. Kelsoe. 2009. IL-1R type I-dependent hemopoietic stem cell proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia. *J Immunol* 182: 6477-6484.
169. Mansson, R., E. Welinder, J. Ahsberg, Y. C. Lin, C. Benner, C. K. Glass, J. S. Lucas, M. Sigvardsson, and C. Murre. 2012. Positive intergenic feedback circuitry, involving EBF1 and FOXO1, orchestrates B-cell fate. *Proc Natl Acad Sci U S A* 109: 21028-21033.
170. Ding, L., and S. J. Morrison. 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* 495: 231-235.
171. Yuan, Y., H. Shen, D. S. Franklin, D. T. Scadden, and T. Cheng. 2004. In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. *Nat Cell Biol* 6: 436-442.
172. Niu, H., G. Fang, Y. Tang, L. Xie, H. Yang, L. Morel, B. Diamond, and Y. R. Zou. 2013. The function of hematopoietic stem cells is altered by both genetic and inflammatory factors in lupus mice. *Blood* 121: 1986-1994.
173. Duncan, A. W., F. M. Rattis, L. N. DiMascio, K. L. Congdon, G. Pazianos, C. Zhao, K. Yoon, J. M. Cook, K. Willert, N. Gaiano, and T. Reya. 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* 6: 314-322.
174. Georgescu, C., W. J. Longabaugh, D. D. Scripture-Adams, E. S. David-Fung, M. A. Yui, M. A. Zarnegar, H. Bolouri, and E. V. Rothenberg. 2008. A gene regulatory network armature for T lymphocyte specification. *Proc Natl Acad Sci U S A* 105: 20100-20105.
175. Murre, C., P. S. McCaw, and D. Baltimore. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 56: 777-783.
176. Peverali, F. A., T. Ramqvist, R. Saffrich, R. Pepperkok, M. V. Barone, and L. Philipson. 1994. Regulation of G1 progression by E2A and Id helix-loop-helix proteins. *EMBO J* 13: 4291-4301.
177. Engel, I., and C. Murre. 2004. E2A proteins enforce a proliferation checkpoint in developing thymocytes. *EMBO J* 23: 202-211.
178. Yu, P., B. Huang, M. Shen, C. Lau, E. Chan, J. Michel, Y. Xiong, D. G. Payan, and Y. Luo. 2001. p15(PAF), a novel PCNA associated factor with increased expression in tumor tissues. *Oncogene* 20: 484-489.
179. Kato, T., Y. Daigo, M. Aragaki, K. Ishikawa, M. Sato, and M. Kaji. Overexpression of KIAA0101 predicts poor prognosis in primary lung cancer patients. *Lung Cancer* 75: 110-118.
180. Maga, G., and U. Hubscher. 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 116: 3051-3060.

181. Turchi, L., M. Fareh, E. Aberdam, S. Kitajima, F. Simpson, C. Wicking, D. Aberdam, and T. Virolle. 2009. ATF3 and p15PAF are novel gatekeepers of genomic integrity upon UV stress. *Cell Death Differ* 16: 728-737.
182. Guo, M., J. Li, D. Wan, and J. Gu. 2006. KIAA0101 (OEACT-1), an expressionally down-regulated and growth-inhibitory gene in human hepatocellular carcinoma. *BMC Cancer* 6: 109.
183. Simpson, F., K. Lammerts van Bueren, N. Butterfield, J. S. Bennetts, J. Bowles, C. Adolphe, L. A. Simms, J. Young, M. D. Walsh, B. Leggett, L. F. Fowles, and C. Wicking. 2006. The PCNA-associated factor KIAA0101/p15(PAF) binds the potential tumor suppressor product p33ING1b. *Exp Cell Res* 312: 73-85.
184. Hosokawa, M., A. Takehara, K. Matsuda, H. Eguchi, H. Ohigashi, O. Ishikawa, Y. Shinomura, K. Imai, Y. Nakamura, and H. Nakagawa. 2007. Oncogenic role of KIAA0101 interacting with proliferating cell nuclear antigen in pancreatic cancer. *Cancer Res* 67: 2568-2576.
185. Amrani, Y. M., J. Gill, A. Matevossian, E. S. Alonzo, C. Yang, J. H. Shieh, M. A. Moore, C. Y. Park, D. B. Sant'Angelo, and L. K. Denzin. 2011. The Paf oncogene is essential for hematopoietic stem cell function and development. *J Exp Med* 208: 1757-1765.
186. Smale, S. T. 2008. Luciferase assay. *Cold Spring Harb Protoc* 2010: pdb prot5421.
187. Li, K., Q. Ma, L. Shi, C. Dang, Y. Hong, Q. Wang, Y. Li, W. Fan, L. Zhang, and J. Cheng. 2008. NS5ATP9 gene regulated by NF- $\kappa$ B signal pathway. *Archives of Biochemistry and Biophysics* 479: 15-19.
188. Chang, C. N., M. J. Feng, Y. L. Chen, R. H. Yuan, and Y. M. Jeng. 2013. p15(PAF) is an Rb/E2F-regulated S-phase protein essential for DNA synthesis and cell cycle progression. *PLoS one* 8: e61196.
189. Riethoven, J.-J. 2010. Regulatory Regions in DNA: Promoters, Enhancers, Silencers, and Insulators. In *Computational Biology of Transcription Factor Binding*. I. Ladunga, ed. Humana Press. 33-42.
190. Ørom, U. A., and R. Shiekhattar. 2011. Noncoding RNAs and enhancers: complications of a long-distance relationship. *Trends in Genetics* 27: 433-439.
191. Ravasi, T., H. Suzuki, C. V. Cannistraci, S. Katayama, V. B. Bajic, K. Tan, A. Akalin, S. Schmeier, M. Kanamori-Katayama, N. Bertin, P. Carninci, C. O. Daub, A. R. R. Forrest, J. Gough, S. Grimmond, J.-H. Han, T. Hashimoto, W. Hide, O. Hofmann, A. Kamburov, M. Kaur, H. Kawaji, A. Kubosaki, T. Lassmann, E. van Nimwegen, C. R. MacPherson, C. Ogawa, A. Radovanovic, A. Schwartz, R. D. Teasdale, J. Tegnér, B. Lenhard, S. A. Teichmann, T. Arakawa, N. Ninomiya, K. Murakami, M. Tagami, S. Fukuda, K. Imamura, C. Kai, R. Ishihara, Y. Kitazume, J. Kawai, D. A. Hume, T. Ideker, and Y. Hayashizaki. 2010. An Atlas of Combinatorial Transcriptional Regulation in Mouse and Man. *Cell* 140: 744-752.

192. Spitz, F., and E. E. M. Furlong. 2012. Transcription factors: from enhancer binding to developmental control. *Nat Rev Genet* 13: 613-626.
193. Roberts, T. C., K. V. Morris, and M. S. Weinberg. 2013. Perspectives on the mechanism of transcriptional regulation by long non-coding RNAs. *Epigenetics : official journal of the DNA Methylation Society* 9.
194. Filipowicz, W., S. N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 9: 102-114.
195. Beum, P. V., M. A. Lindorfer, B. E. Hall, T. C. George, K. Frost, P. J. Morrissey, and R. P. Taylor. 2006. Quantitative analysis of protein co-localization on B cells opsonized with rituximab and complement using the ImageStream multispectral imaging flow cytometer. *Journal of immunological methods* 317: 90-99.
196. George, T. C., S. L. Fanning, P. Fitzgerald-Bocarsly, R. B. Medeiros, S. Highfill, Y. Shimizu, B. E. Hall, K. Frost, D. Basiji, W. E. Ortyn, P. J. Morrissey, and D. H. Lynch. 2006. Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. *Journal of immunological methods* 311: 117-129.
197. Basiji, D. A., W. E. Ortyn, L. Liang, V. Venkatachalam, and P. Morrissey. 2007. Cellular image analysis and imaging by flow cytometry. *Clinics in laboratory medicine* 27: 653-670, viii.
198. McGrath, K. E., T. P. Bushnell, and J. Palis. 2008. Multispectral imaging of hematopoietic cells: where flow meets morphology. *Journal of immunological methods* 336: 91-97.
199. Khalil, A. M., J. C. Cambier, and M. J. Shlomchik. 2012. B cell receptor signal transduction in the GC is short-circuited by high phosphatase activity. *Science* 336: 1178-1181.
200. McFarlin, B. K., R. R. Williams, A. S. Venable, K. C. Dwyer, and D. L. Haviland. 2013. Image-based cytometry reveals three distinct subsets of activated granulocytes based on phagocytosis and oxidative burst. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 83: 745-751.
201. Muller-Sieburg, C., and H. B. Sieburg. 2008. Stem cell aging: survival of the laziest? *Cell Cycle* 7: 3798-3804.
202. Muller-Sieburg, C. E., H. B. Sieburg, J. M. Bernitz, and G. Cattarossi. 2012. Stem cell heterogeneity: implications for aging and regenerative medicine. *Blood* 119: 3900-3907.
203. Chambers, S. M., C. A. Shaw, C. Gatz, C. J. Fisk, L. A. Donehower, and M. A. Goodell. 2007. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol* 5: e201.



204. Pang, W. W., E. A. Price, D. Sahoo, I. Beerman, W. J. Maloney, D. J. Rossi, S. L. Schrier, and I. L. Weissman. 2011. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci U S A* 108: 20012-20017.
205. Sudo, K., H. Ema, Y. Morita, and H. Nakauchi. 2000. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* 192: 1273-1280.
206. Beerman, I., D. Bhattacharya, S. Zandi, M. Sigvardsson, I. L. Weissman, D. Bryder, and D. J. Rossi. 2010. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proc Natl Acad Sci U S A* 107: 5465-5470.
207. Miller, J. P., and D. Allman. 2003. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. *J Immunol* 171: 2326-2330.
208. Linton, P. J., and K. Dorshkind. 2004. Age-related changes in lymphocyte development and function. *Nat Immunol* 5: 133-139.
209. Harrison, D. E., C. M. Astle, and M. Stone. 1989. Numbers and functions of transplantable primitive immunohematopoietic stem cells. Effects of age. *J Immunol* 142: 3833-3840.
210. Morrison, S. J., A. M. Wandycz, K. Akashi, A. Globerson, and I. L. Weissman. 1996. The aging of hematopoietic stem cells. *Nat Med* 2: 1011-1016.
211. Kuranda, K., J. Vargaftig, P. de la Rochere, C. Dosquet, D. Charron, F. Bardin, C. Tonnelle, D. Bonnet, and M. Goodhardt. 2011. Age-related changes in human hematopoietic stem/progenitor cells. *Aging Cell* 10: 542-546.
212. Dykstra, B., S. Olthof, J. Schreuder, M. Ritsema, and G. de Haan. 2011. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med* 208: 2691-2703.
213. Florian, M. C., K. Dorr, A. Niebel, D. Daria, H. Schrezenmeier, M. Rojewski, M. D. Filippi, A. Hasenberg, M. Gunzer, K. Scharffetter-Kochanek, Y. Zheng, and H. Geiger. 2012. Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. *Cell Stem Cell* 10: 520-530.
214. Ergen, A. V., N. C. Boles, and M. A. Goodell. 2012. Rantes/Ccl5 influences hematopoietic stem cell subtypes and causes myeloid skewing. *Blood* 119: 2500-2509.
215. Geiger, H., G. de Haan, and M. C. Florian. 2013. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol* 13: 376-389.
216. Van Zant, G., and Y. Liang. 2012. Concise review: hematopoietic stem cell aging, life span, and transplantation. *Stem cells translational medicine* 1: 651-657.

217. Blomberg, B. B., and D. Frasca. 2013. Age effects on mouse and human B cells. *Immunologic research*.
218. Frasca, D., E. Van Der Put, R. L. Riley, and B. B. Blomberg. 2004. Age-related differences in the E2A-encoded transcription factor E47 in bone marrow-derived B cell precursors and in splenic B cells. *Exp Gerontol* 39: 481-489.
219. Frasca, D., A. M. Landin, S. C. Lechner, J. G. Ryan, R. Schwartz, R. L. Riley, and B. B. Blomberg. 2008. Aging down-regulates the transcription factor E2A, activation-induced cytidine deaminase, and Ig class switch in human B cells. *J Immunol* 180: 5283-5290.
220. Frasca, D., A. M. Landin, R. L. Riley, and B. B. Blomberg. 2008. Mechanisms for decreased function of B cells in aged mice and humans. *J Immunol* 180: 2741-2746.
221. King, A. M., E. Van der Put, B. B. Blomberg, and R. L. Riley. 2007. Accelerated Notch-dependent degradation of E47 proteins in aged B cell precursors is associated with increased ERK MAPK activation. *J Immunol* 178: 3521-3529.
222. Jensen, K., M. B. Rother, B. S. Brusletto, O. K. Olstad, H. C. Dalsbotten Aass, M. C. van Zelm, P. Kierulf, and K. M. Gautvik. 2013. Increased ID2 levels in adult precursor B cells as compared with children is associated with impaired Ig locus contraction and decreased bone marrow output. *J Immunol* 191: 1210-1219.
223. Vallejo, A. N., J. J. Michel, L. K. Bale, B. H. Lemster, L. Borghesi, and C. A. Conover. 2009. Resistance to age-dependent thymic atrophy in long-lived mice that are deficient in pregnancy-associated plasma protein A. *Proc Natl Acad Sci U S A* 106: 11252-11257.
224. Oduro, K. A., Jr., F. Liu, Q. Tan, C. K. Kim, O. Lubman, D. Fremont, J. C. Mills, and K. Choi. 2012. Myeloid skewing in murine autoimmune arthritis occurs in hematopoietic stem and primitive progenitor cells. *Blood* 120: 2203-2213.
225. Griseri, T., B. S. McKenzie, C. Schiering, and F. Powrie. 2012. Dysregulated hematopoietic stem and progenitor cell activity promotes interleukin-23-driven chronic intestinal inflammation. *Immunity* 37: 1116-1129.