THE ROLE OF THE TRANSCRIPTION FACTOR 3 GENE PRODUCT E47 IN MULTIPOTENT HEMATOPOIETIC STEM CELLS AND PROGENITORS

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Submitted to the Graduate Faculty of

Department of Human Genetics

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

This dissertation was presented

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It was defended on

Jan 11th, 2010

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E47, an alternative splice product of the transcription factor 3 (TCF3) gene, has been mechanistically linked with multiple leukemias and lymphomas, and thus it is of great **public health importance** to study the mechanisms by which E47 influences the development of the hematopoietic system.

Throughout life, all mature blood cells are constantly replenished from rare, self-renewing bone marrow hematopoietic stem cells (HSCs) and downstream non-renewing multipotent progenitors (MPPs). Little is known about the gene regulatory network that controls the integrity of these essential bone marrow subsets. Previous evidence has suggested a crucial role for the transcription factor E47 in lymphocyte lineage commitment. However, the specific stages of hematopoiesis that require E47 and the underlying mechanisms through which it acts on remain unclear. Our study aims to elucidate the role of the transcription factor E47 in the earliest, multipotent stages of hematopoiesis.

Using E47 deficient mice, we found that E47 is required for the development and functional integrity of uncommitted hematopoietic progenitors. Our results showed that E47 deficient mice had a 50–70% reduction in non-renewing MPPs, and the residual MPPs failed to initiate V(D)J recombination, a hallmark of lymphoid lineage progression. The long-term lineage repopulation and self-renewal activities of the primitive HSCs are also compromised in the

absence of E47. Not only were the *in vivo* long-term repopulating HSCs reduced by 3 fold in the bone marrow of E47 deficient mice, but also these HSCs displayed poor self-renewal efficiency by serial transplantation. The compromised self-renewal of E47 null HSCs appears to be associated with premature exhaustion due to over-proliferation. The multipotent hematopoietic stem/progenitor cells from E47 deficient mice displayed a striking hyperproliferation following transplantation stress, and they exhibited increased susceptibility to *in vivo* challenge with a mitotoxic drug. Finally, loss of function and gain of function assays identified the cell cycle inhibitor p21 as a target gene of E47. Together, these observations suggested that E47 regulates the development and functional potential of multipotent hematopoietic subsets, probably through effects on p21-mediated cell cycle quiescence. These findings might provide novel mechanistic insights into hematopoietic damage repair and malignant transformation.

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ABBREVIATIONS

Ab - Antibody

ALL - Acute Lymphoblastic Leukemia

ATM - Ataxia Telangiectasia Mutated

bHLH - basic Helix-Loop-Helix

BM - Bone Marrow

CI - Confidence Interval

CLP - Common Lymphoid Progenitor

CMP - Common Myeloid Progenitor

CFU - Colony Forming Unit

CSF - Colony Stimulating Factors

E2A - Immunoglobulin Enhancer Binding Factors E47/E12

E47 - Immunoglobulin Enhancer Binding Factor E47

ELP - Early Lymphoid Progenitor

ETP - Early thymic progenitor

FACS - Flow Cytometric Cell Sorting

5-FU - 5-Fluorouracil

CaR - Calcium-sensing Receptor

Gαs - G protein Coupled Receptors

GMP - Granulocyte/Macrophage Progenitor

HET - Heterozygous

HSC - Hematopoietic Stem Cell

LSK - Lineage^{-}Sca^{hi} c-kit^{hi}

LMPP - Lymphoid Primed MPP

LTC-IC - Long-Term Culture-Initiating Cell

LPS - Lipopolysaccharide

LT - Long-term

MACS - Magnetic Automated Cell Separation

MEP - Megakaryocytic/Erythrocyte Progenitor

MPPs - Multipotent Progenitor

KO - Knockout

PBX1 - pre-B-cell Leukemia Transcription Factor 1

PIM – Protease Inhibitor Mixture

RAG - Recombination Activation Gene

ROS - Reactive Oxygen Species

TCF3 –Transcription Factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)

WT - Wild Type

PREFACE

I would like to acknowledge many people for their help and support throughout this project.

First and foremost, I would like to thank my advisor, Dr. Lisa Borghesi, for her great guidance. Not only does her wonderful mentoring allow me to enjoy my graduate study with so much excitement and pleasure, but also she has offered me a lot of valuable help in my stepping into the next stage of my academic career. I am feeling so lucky to have such a great mentor!

I would also like to thank the helpful advice from many other scientists. All the other committee members--- Dr. Robert E. Ferrell, Dr. Abbe N. de Vallejo, and Dr. Susanne M. Gollin, contributed significantly to the development of the key ideas in this project. I also greatly appreciate the thoughtful advice from Dr. Christine Milcarek, Dr. Olivera Finn and Dr. Bingfu Lu in University of Pittsburgh; Dr. Paul Kincade, Dr. Xiaohong Sun, and Dr. Brandt Esplin in Oklahoma Medical Research Foundation; and Dr. Irwin D. Bernstein in Fred Hutchinson Cancer Research Center.

I am very grateful to the amazing flow cytometry cell sorting from Dewayne Falkner, and the friendship from the fellow students, postdocs and technicians in our lab and in our neighboring labs, especially Patricia Santos, Lela Kardava and Kathleen Martincic.

Lastly but not least importantly, my husband, Jiangbo, has been extremely supportive throughout my academic pursuit. Without him, nothing could have been possible. With graduation around the corner, I am looking forward to sharing my happiness and pride with him and with our baby daughter.

1.0 STATEMENT OF THE PROBLEM

The mechanisms that regulate hematopoietic self-renewal and differentiation are of great importance from both public health and clinical prospects. A rare population of multipotent hematopoietic stem/progenitor cells residing in the bone marrow of adult organisms constantly replenishes the entire immune system in steady-state circumstances and regenerate long-term hematopoietic functioning following hematopoietic damage. Given the key role of these uncommitted bone marrow progenitors in blood maintenance and hematopoietic regeneration, it is of great interest to study the mechanisms that control their development and functional integrity.

Despite recent progress that has been made to decipher several cellular and environmental pathways that regulate the replication of bone marrow stem cells, little is known about the transcriptional gene regulatory network controlling the maintenance and long-term activity of multipotent stem/progenitor cells. The transcription factor E47, an essential basic Helix-Loop-Helix (bHLH) transcription factor in lymphocyte development, has recently been implicated in early hematopoiesis. However, the precise roles and mechanisms for E47 in regulating the development and the functional potential of the primitive multipotent hematopoietic subsets remain unclear.

The central hypothesis of this study is that **E47 is an important regulator that controls the development and functional integrity of multipotent hematopoietic subsets, including self-renewing HSCs and downstream non-renewing MPPs.** Here we have assessed the

requirement of cell intrinsic E47 in the development of MPPs, and in the long-term lineage repopulation and self-renewal capabilities of HSCs. We have also investigated the molecular and cellular mechanisms underlying the HSC and MPP defects in E47 deficient mice.

2.0 SPECIFIC AIMS

Specific Aim 1: Test the hypothesis that E47 is required for the developmental integrity of multipotent hematopoietic stem/progenitor cells. The earliest uncommitted hematopoietic progenitors are contained within the LSK (Lineage^{-} Sca^{hi} c-kit^{hi}) subset of bone marrow cells, which are enriched for the self-renewing HSCs and the downstream non-renewing MPPs. Little is known about the molecular pathways that regulate the development and maintenance of these multipotent hematopoietic subsets. E47, an alternative splice product of the TCF3 gene, is an essential transcription factor for both T and B lymphocyte differentiation and has been recently implicated in early hematopoiesis. But the precise role for E47 in the uncommitted hematopoietic stem cells and progenitors remains unknown. Here, we hypothesize that E47 is required for the proper development of HSCs and MPPs. To investigate the requirement of E47 in these multipotent stages of hematopoiesis, we will compare the number of HSCs and MPPs in E47 deficient versus wild type (WT) mice using multiple independent phenotypic definitions. We will also examine the contribution of E47 to the proliferation and survival of these multipotent bone marrow subsets. A key objective of this aim is to define the precise (and earliest) developmental stage(s) that are sensitive to the deficiency of E47.

Specific Aim 2: Test the hypothesis that E47 regulates the long-term multi-lineage repopulation and self-renewal capabilities of hematopoietic stem cells. Despite recent progress that has been made to understand HSC biology, the transcription regulatory pathways that control the functional integrity of HSCs remain unclear. The development of the entire

immune system is tightly regulated by a network of gene regulatory factors, among which the transcription factor E47 is essential for proper lymphopoiesis. Functional disruption of E47 has been associated with cancers of multiple hematopoietic lineages, suggesting a role for E47 in the multipotent stem cells. Indeed, several inhibitors of E47 have been recently found to regulate the maintenance of HSCs. However, direct evidence for the pivotal role of E47 in controlling the functional activity of HSCs remains lacking. In this study, we will thoroughly evaluate the role of the transcription factor E47 in maintaining the long-term self-renewal and multi-lineage differentiation activity of HSCs, using *in vitro* assays and *in vivo* adoptive transfer approaches. We will also investigate the underlying mechanisms through which E47 regulates the functional potential of HSCs.

3.0 PUBLIC HEALTH SIGNIFICANCE

It is of wide public health significance to study the role of the transcription regulatory factors in controlling the development and functional activity of the primitive multipotent hematopoietic stem cells and other early progenitors.

Hematopoiesis is a highly regulated process that is tightly controlled by a network of gene regulatory factors, and their deregulation can result in various forms of diseases, such as bone marrow failure, hematopoietic insufficiency, leukemias and lymphomas (1, 2). The multipotent hematopoietic stem/progenitor cells play a crucial role not only in normal hematopoietic maintenance under steady state circumstances, but also in long-term hematopoietic regeneration in responses to ageing, injury and transplantation stress (3-6). By decoding the transcriptional gene regulatory pathways that control the maintenance and functional potential of multipotent HSCs and MPPs, this project will provide a mechanistic basis for improving our understanding and treatment of hematopoietic failures and malignancies.

Furthermore, functional disruptions of the transcription factor E47 or its parent gene TCF3 have been involved in leukemias and lymphomas of multiple lineages, including B cells, T cells and myeloid cells (7-12). For example, chromosomal translocations involving the TCF3 gene have been detected in more than one quarter of pre-B acute lymphoblastic leukemia (11). Nevertheless, the precise mechanisms underlying the E47 associated hematopoietic cancers remain unclear. Defects in the multipotent bone marrow progenitors may serve as the first lesion

promoting malignancy transformation by secondary injuries during hematopoietic development. Thus, by elucidating the specific roles and mechanisms of E47 in early hematopoiesis, our study might provide new insights into E47 related leukemogenesis.

Finally, hematopoietic stem cells share many important functional properties with stem cells from other tissues. For example, all adult stem cells possess potent long-term self-renewal capability and multipotent differentiation potential. E47 is a ubiquitous bHLH transcription factor that is commonly expressed in almost all tissues. Therefore, our observations on the role of E47 in early hematopoiesis might yield insights into the development of other tissues.

4.0 INTRODUCTION

4.1 HEMATOPOIESIS

Hematopoiesis is a tightly regulated process. In the hematopoietic hierarchy, mature blood cells are derived from lineage committed precursors, which in turn are constantly replenished by the primitive multipotent progenitors and ultimately by hematopoietic stem cells.

4.1.1 HSCs and MPPs

HSCs, which reside on top of the hematopoietic hierarchy, renew themselves and maintain long-term multipotency (13). MPPs are believed to be the downstream progeny of HSCs. Unlike HSCs, MPPs have very limited or no self-renewal potential, but retain multi-lineage differentiation capability. Both self-renewing HSCs and downstream non-renewing MPPs reside in a phenotypic population LSK (Lineage^{-} Sca^{hi} c-kit^{hi}) in the bone marrow of adult mice. Recent studies have identified a number of cell surface molecular markers to discriminate HSCs from MPPs within the total bone marrow LSKs (14-16). The commonly used markers include flk2, CD27, CD34, Thy1.1 and SLAM (signaling lymphocyte activation molecule) family members. These phenotypic markers provide great convenience in decoding the molecular pathways that regulate the integrity of each HSC and MPP subset. However, none of these phenotypic schemes defines a homogenous population of pure HSCs. SLAM LSKs (CD150^{+}CD48^{-} LSKs) resolve the purest population of HSCs, yet only 50% of SLAM LSKs are functional long-term HSCs in young mice and the proportion is even less in old mice. Therefore,

in this thesis, we will use both phenotypic and functional definitions of HSCs for a rigorous study of the stem cell properties.

4.1.2 Lineage Differentiation of MPPs

In the traditional model of hematopoiesis suggested by previous studies, MPPs generate two early lineage restricted progenitor subsets – common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) **(Figure 1)** (17, 18). CMPs lack lymphoid potential, but can develop into myeloid, erythroid, and megakaryocytic progenies. CMPs differentiate into progenies that are restricted to more specific lineages, including granulocyte/macrophage progenitors (GMPs) and megakaryocytic/erythrocyte progenitors (MEPs). In contrast with CMPs, CLPs retain lymphoid potential, but cannot develop into myeloid progenies. CLPs efficiently give rise to B lymphoid progenies and NK cells, but whether they are a major source of T lymphocytes remains controversial (19).

T cells are constantly produced from non self-renewing thymus progenitors, which in turn are seeded from undefined extrathymic bone marrow or blood subset(s). Recent studies suggested that T cells might be repopulated from multipotent progenitors upstream of CLPs, since intact thymopoiesis has been found in genetically mutant mice that lack CLPs (20). Yet a more recent study showed that the $flk2^{+}CD27^{+}$ CLP has potent *in vivo* short-term thymus repopulation activity (21). These observations suggested that T cells might be originated from multiple routes. Inside the thymus, a rare population of Early Thymic Progenitors (ETPs) has

been found to efficiently repopulate the thymus *in vivo* under notch signaling (22). However, other thymus seeding subset(s) and the extrathymic progenitors of ETPs remain to be defined.

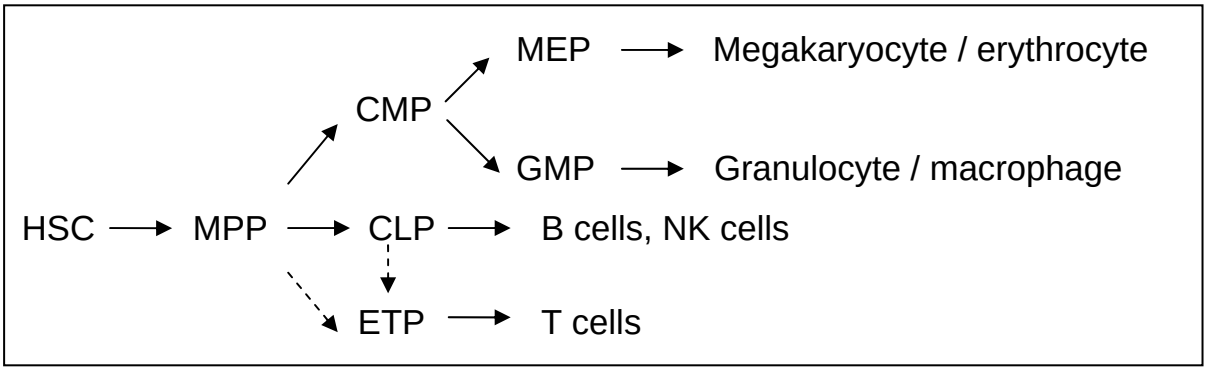

Figure 1: Traditional model of hematopoiesis

Recent findings have also revised the traditional model of hematopoiesis by questioning the specific stage at which the megakaryocytic/erythrocyte potential becomes lost **(Figure 2)** (23). A very recent study demonstrated the presence of lymphoid–primed MPPs (LMPP), a subset of LSKs with the highest expression of the cytokine receptor flk2. The LMPPs have potent lymphoid/myeloid lineage differentiation potential, but have very little potential of progression towards the megakaryocytic/erythroid lineages. LMPPs comprise 25% of total LSKs in wild type mice. Not only do they give rise to GMPs and CLPs, but also they display efficient thymus repopulation activity *in vivo* (23-25). Despite the important role of LMPPs in early hematopoiesis, the molecular pathways that regulate their development from HSCs and their differentiation into downstream subsets remain unclear.

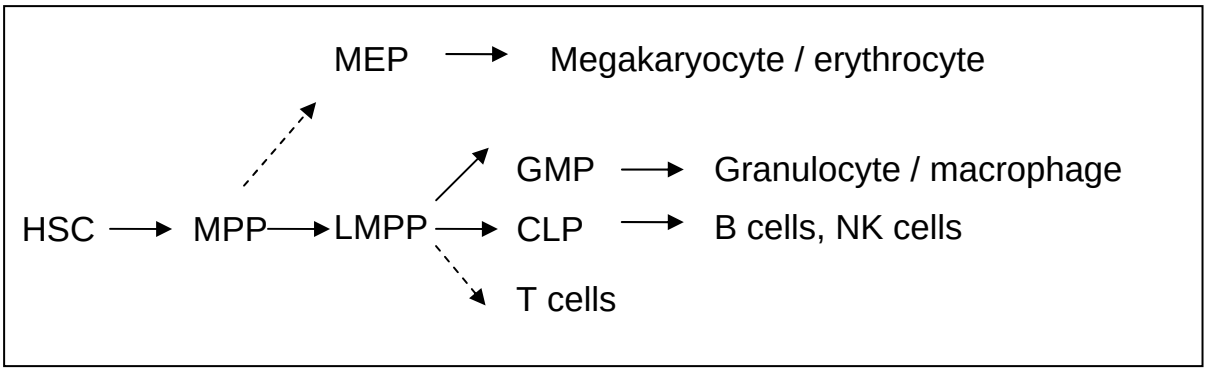

Figure 2: Revised model of hematopoiesis

4.2 Hematopoietic Stem Cells

The entire immune system is constantly seeded by rare hematopoietic stem cells that comprise only about 0.01% of the bone marrow cells in healthy humans and mice (26-29). HSC transplant therapies, in which HSCs are manipulated for tissue and organ repair, have been widely used for a variety of hematopoietic disorders such as multiple myeloma, leukemia, and aplastic anemia, hold promises in treating many other severe diseases originated from the immune system (30-32). Thus, it is of great importance to study the mechanisms that regulate the functional potential of this essential hematopoietic subset.

4.2.1 Functional characteristics of HSCs

Hematopoietic stem cells are defined by their long-term self-renewal capability and their multipotent lineage differentiation potential (13). A functionally robust HSC is able to reconstitute all blood lineages of the entire immune system of a lethally irradiated mouse, and it can renew itself in a very long term even beyond the life span of the donor organism. The ability of HSCs to renew themselves while preserving their “stemness” is essential in maintaining normal hematopoietic turn over under regular steady-state circumstance, and in conferring the

long-term hematopoietic regeneration following hematopoietic damage. Impaired HSC self-renewal may result in inefficient tissue damage repair, bone marrow failure and even hematopoietic malignancies (2, 4). Given the essential role of HSCs in normal hematopoiesis and in the development of hematopoietic diseases, it is thus of great public health and clinical significance to study the mechanisms governing their replication, maintenance and differentiation.

4.2.2 Mechanisms Regulating the Functional Integrity of HSCs

HSCs play an essential role in blood maintenance and hematopoietic damage repair. Multiple cellular and environmental mechanisms are involved in regulating the long-term functional potential of HSCs. The most intensely studied mechanisms include the molecules essential for HSC homing and niche engraftment, the cell autonomous (cell intrinsic) factors regulating HSC long-term self-renewal and maintenance, and the cell nonautonomous (environmental) factors regulating the bone marrow HSC niche.

4.2.2.1 Factors Regulating HSC homing and Niche Engraftment

During organism development or clinical transplantation, HSCs home to the bone marrow of the adult/recipient mice and engraft with the bone marrow niche for hematopoietic development. Recent studies have identified several factors that are required for efficient homing or niche engraftment of HSCs. For example, HSCs lacking the alpha stimulatory subunit of G protein coupled receptors (Gαs) fail to home to the bone marrow of the recipient mice after adoptive transfer, and thus cannot reconstitute lethally irradiated recipient mice (33). For another instance, HSCs in calcium-sensing receptor (CaR) deficient mice home normally to the bone marrow of adult mice but fail to lodge in the endosteal niche, causing depletion of HSC pool in the bone marrow of these mice (33). Together, these observations suggested that molecules that

contribute to HSC homing and niche engraftment are crucial in hematopoietic development and transplantation success.

4.2.2.2 Cell Intrinsic Mechanisms Regulating The Size of Functional HSC Pool

Recent studies suggested that the size of the functional HSC pool is regulated by the coordinated actions of a number of cell intrinsic mechanisms, such as cell cycle regulation, apoptosis and reactive oxygen species metabolism.

First of all, the long-term maintenance of a robust functional HSC pool requires efficient self-renewal. Previous studies indicated that the self-renewal capability of long-term HSCs is tightly controlled by cell cycle regulation (34). The majority of HSCs reside in cell cycle quiescent state in steady state circumstances. About 70% of the HSC highly enriched $CD34^{neg}CD150^{+}48^{-}$ LSK bone marrow cells are in G_0 quiescent phase by Ki67 proliferation antigen staining, and only less than 2% are in actively cycling (S + G2 +M) phases by DNA content staining (35). Hyperproliferation of the slow-cycling HSCs may cause premature exhaustion and diminished long-term self-renewal of HSCs. For example, HSCs in mice lacking the key cell cycle regulator p21 or p16 overproliferated and displayed serious long-term self-renewal defects by serial transplantation (36, 37). HSCs in many other genetically mutant mice, such as those deficient for Gfi1, an important zinc finger transcription factor in hematopoiesis, or c-kit, the cellular receptor for the stem cell factor, showed severe defects in long-term multi-lineage repopulation activity associated with a loss of cell cycle quiescence (38-40). Therefore, cell cycle regulatory factors that keep the HSCs in quiescence play a critical role in maintaining the long-term functional activity of HSCs.

The proper size of HSC pool is controlled not only by self-renewal efficiency, but also by concomitant regulation of the apoptosis pathways. Previous studies suggested that the number of

HSCs is sensitive to the dose of apoptosis regulators (41, 42). For example, anti-apoptotic BCL-2 family members are highly expressed in HSCs, and BCL-2 over-expression increased the number of HSCs by 2.4 fold and enhanced the *in vivo* repopulation capability of the bone marrow progenitors (41). Conversely, conditional deletion of *Mcl-1*, a member of the BCL-2 superfamily, results in a large decrease of murine HSCs associated with a dramatically increased apoptosis rate (42). Evidence from other studies has also linked the caspase apoptosis factors with the size of HSC pool (43, 44). Deletion of caspase 3, an executioner protease in apoptosis, increased the number of phenotypic long-term HSCs in mice (44). In humans, a variant of caspase 8 is upregulated in $CD34^+$ bone marrow progenitors from normal people, and in the stem cell-derived leukemic blasts from acute myeloid leukemia patients (43). Therefore, multiple apoptosis factors are involved in regulating the size of HSC pool, and their deregulation might result in hematopoietic insufficiency or even malignant transformation.

Finally, other cellular and molecular mechanisms, such as DNA damage response related factors, are also involved in regulating the HSC number. For example, reactive oxygen species (ROS), the free radicals accumulated under environmental stresses, are known to cause DNA damage and initiate apoptosis (45). Recent studies suggested that HSCs with potent long-term self-renewal capability preferentially reside in the low-oxygenic bone marrow niches for protection from ROS damage (46). Unchecked ROS accumulation due to deregulation of ROS regulators has been found to compromise the maintenance and functional integrity of HSCs. Loss of ataxia telangiectasia mutated (ATM), a key DNA damage response protein, results in a dramatically increased ROS level in murine HSCs and the depletion of HSC pool in mice (47). The Forkhead Box O transcription factor, Foxo3, is also required for maintaining the HSC pool by preventing ROS accumulation, possibly through effects on ATM (48). Other signaling

pathways that control the ROS metabolism in HSCs are yet to be decoded, which may improve our understanding of the long-term HSC maintenance in responses to hematopoietic damage.

4.2.2.3 Cell Nonautonomous Factors Regulating HSC Niche

Not only do cell intrinsic molecules play an importance role in hematopoietic maintenance and development, but also the functional potential of HSCs is sensitive to the bone marrow microenvironment. HSCs interact with their bone marrow osteoblastic or vascular niche for self-renewal and multi-lineage differentiation activity (49). The osteoblastic niche is required to keep the HSCs in quiescence, whereas the vascular niche promotes the proliferation and differentiation of HSCs (50, 51). Recent studies suggested that stem cell niche is regulated by multiple signaling pathways, such as Notch and WNT pathways. Jag1, a Notch ligand, is expressed on osteoblasts, and enhanced Jag1/Notch signaling activity by PPR activation results in the expansion of both osteoblastic cells and HSCs (52). Dkk1, a Wnt inhibitor, is also expressed in osteoblasts, and regulates the long-term self-renewal of HSCs in a cell nonautonomous manner (53). The transcription factors of the E protein family have also been recently implicated in regulating the integrity of the HSC niche. For example, E protein inhibitor, Id1, has been found to regulate early hematopoietic development through effects on the bone marrow niche (54). However, the precise expressions and functions of E proteins in the osteoblastic or vascular niche are yet to be determined.

4.3 Transcriptional Factor 3 and E47

Despite recent progress that has been made to understand the cellular and environmental mechanisms involved in stem cell regulation, relatively little is known of the transcriptional gene regulatory pathways that control the maintenance and function of HSCs. The development of the hematopoietic system is tightly regulated by a network of transcription factors. Among them, E

proteins, a group of Class I basic helix-loop-helix (bHLH) transcription factors, play a critical role in lymphoid lineage commitment (55), and are linked to the survival and proliferation of both B and T lymphoid progenitors. Recent studies have suggested that E47, an alternative splice product of the TCF3 gene, is required for the proper development of early hematopoietic progenitors including CLPs and ETPs (56, 57). However, the specific roles for E47 in the more upstream multipotent hematopoietic stem cells and progenitors remain unclear.

4.3.1 Gene and Protein Characteristics of TCF3 Gene

TCF3, the human homolog of murine E2A, is located at chromosome19p13.3 (58). TCF3 is one of the three known E protein genes in mammals, with the other two being E2-2 and HEB (59). E proteins are ubiquitously expressed class I bHLH (basic Helix-Loop-Helix) transcription factors that contain two transcription activation domains at the N-terminal, and a bHLH domain at the C-terminal. The bHLH domain is required for the dimerization and DNA binding function of the transcription factors. The TCF3 gene generates three E protein products, E12, E47 and E2-5, by alternative splicing (60). E47 and E12 share identical N-terminal sequences including the two transcription activation domains, and differ in their C-terminal bHLH domains that confer the DNA binding and dimerization activities. Previous studies suggested that E12 and E47 might have overlapping or synergic functions in regulating lymphoid development, whereas E47 has been found to be essential for early B development and E12 relatively dispensable (76). The third alternative splice product of TCF3 gene, E2-5, differs from E47 only in the NH_2 portion and demonstrates no functional difference from E47. Therefore, in this study, we are particularly interested in the function of the E47 product of the TCF3 gene.

By forming homodimers or heterodimers with other HLH proteins, TCF3 proteins bind with the E box motif (CANNTG) in the promoter/enhancer regions and regulate the transcription

of the target genes (59). TCF3 homodimers are restricted in B lymphocytes in the form of E47 homodimers, which have been shown to play a critical role in B lymphocyte differentiation and Immunoglobulin gene rearrangement (61). In T lymphocytes, the predominant E proteins are E47/HEB heterodimers that are required for proper T cell differentiation (62). Functional ablation of the E47/HEB heterodimers by a dominant negative HEB mutant caused an early block of T cell development at the stage before T cell receptor (TCR) beta gene rearrangement. In non-hematopoietic tissues, E47 binds the DNA mainly by forming heterodimers with class II tissue specific HLH proteins, such as the muscle specific regulator MyoD and neuron specific factor NeuroD (63, 64).

The activity of the E47 product of the TCF3 gene is regulated by a variety of factors. The known TCF3 inhibitors include Notch signaling molecules, some Class II HLH factors, and Class III inhibitory HLH factors. Notch signaling, an important signaling pathway in T-lymphocyte development, has been found to induce the ubiquitination and degradation of the E47 protein (65). Phosphoration of E47 by increased ERK/MAP kinases activities is involved in this process. The transcriptional activity of E47 is also regulated by some other HLH factors. In lymphocytes, E47 normally form homodimers or heterodimers with other Class I bHLH genes, but they can also form heterodimers with Class II HLH genes, such as SCL1/Tal1 and LyL1 (66-68). These E47/ Class II HLH heterodimers have distinct transcriptional activation or DNA binding activities, which interfere with the normal function of E47. For example, E47/SCL1 heterodimers are very poor transactivators and their formation inhibits the normal transcriptional function of E47 in leukemic T cells (68). The activity of E47 is also inhibited by a third class of HLH proteins lacking the basic DNA binding region (69). These inhibitory HLH proteins, such as Id1, do not bind DNA, but avidly form heterodimers with other HLH proteins inactivating

their DNA binding functions. Id1 has been found to regulate the development of both B and T lymphocytes by acting as a dominant negative antagonist of the E protein homodimers and heterodimers. Many of these E47 regulators, including SCL1/Tal1, LyL1, Id1 and Notch receptors, have been recently suggested to contribute to the integrity of HSCs or their bone marrow niche, hinting a role for E47 in regulating the functional potential of HSCs (70-72). However, the existing forms of E47 and its precise function in the primitive multipotent hematopoietic stem/progenitors remain unknown.

4.3.2 The E47 Product of TCF3 Gene in Hematopoietic Development

E47 plays an essential role in immune system development. In E47 deficient mice, B cells are arrested at the pre-pro B stage and the residual pre-pro B cells fail to express the recombination activation gene (Rag), the key lymphocyte specific enzyme that initiates V(D)J DNA recombination in lymphoid progenitors (73, 74). In these mice, T cell development is also partially blocked at DN1 ($CD44^{+}CD25^{-}$) to DN2 ($CD44^{+}CD25^{+}$) progenitor cell transition (75). The other main alternative splice product of TCF3, E12, might have overlapping or synergic actions with E47 in regulating lymphoid development, whereas E47 has been found to be essential for early B development and E12 relatively dispensable (76). Recent studies revealed that E47 is also required for the development of early lymphoid progenitors, including the bone marrow CLPs and the thymic ETPs (74). In E47 deficient mice, CLPs are almost abolished and ETPs are significantly reduced. A major unanswered question is whether E47 also contributes to the development of the even more upstream multipotent hematopoietic subsets.

4.3.3 The E47 Product of TCF3 Gene in Non-Hematopoietic System

E47 is a ubiquitously expressed transcription factor. Not only is it essential for hematopoietic development, but also it regulates the differentiation, survival and proliferation of

cells from multiple non-hematopoietic tissues. For example, the formation of MyoD/E47 heterodimers promotes the differentiation of muscle cells by activating the transcription of a number of muscle-specific genes (77). In the nervous system, E47 forms heterodimers with neuron specific NeuroD and regulates the development and survival of the neurons (78). E47 has also been found to contribute to the cell cycle regulation and differentiation of human osteoblastic cell line MG63, suggesting a possible role for E47 in regulating the bone marrow osteoblastic niche of HSCs (79). Indeed, Id1, the inhibitor of E47, has been found to regulate early hematopoiesis through effects on the bone marrow microenvironment (54). However, whether E47 directly regulates the HSC niche is yet to be determined.

4.3.4 TCF3 Gene and Human Diseases

Disruption of TCF3 or its alternative splice product E47 protein is associated with hematopoietic malignancies of both T and B lymphoid lineages in human. Functional ablation of E47 by the activation of its inhibitor TAL1/SCL has been detected in more than 60% of patients with T-cell acute lymphoblastic leukemia (ALL). E47 deregulation has also been linked with cancers of B lymphocyte origins. Halpo-insufficiency of TCF3, the parent gene of E47, has been identified in pre-B ALL patients, and inhibition of the E47 transcriptional activity is mechanistically associated with Hodgkin lymphoma (80, 81). Furthermore, the t(1;19)(q23;pl3.3) chromosomal translocation, in which the transcription activation domain encoding exons of the TCF3 gene are fused with the DNA binding domain encoding exons of pre-B-cell leukemia transcription factor 1 (PBX1) gene, occurs in more than 25% of children with pre-B ALL (11). The TCF3/HLF fusion protein resulting from the t(17;19)(q22;p13) chromosomal translocation is also frequently found in pre-B ALL patients, and is an indicator of poor prognosis (8). Together, these observations suggested that functional deficiency or

disruption of E47 is involved in the development of hematopoietic cancers of both T and B lymphoid lineages.

Not only does the disruption of TCF3 or its alternative splice product E47 contribute to the development of cancers of the lymphoid lineages, but also TCF3 aberrancy is associated with malignant transformations of the myeloid lineages. A previous study suggested that TCF3/PBX1 is not only linked with acute lymphoid leukemia, but also can cause acute myeloid leukemia (82). When TCF3/PBX1 fusion protein expressing bone marrow progenitors were adoptively transferred into lethally irradiated mice, 7 out of the 8 recipient mice developed acute myeloid leukemia. Furthermore, a recent study showed that the inhibition of TCF3 activity by its antagonist Id1 is associated with poor prognosis in patients with acute myeloid leukemia (10). That TCF3 is associated with diseases of both myeloid and lymphoid lineages suggested the possibility that multipotent hematopoietic stem/progenitor cells might serve as the the first lesion that renders the TCF3 deficient cells more susceptitable for secondary damages during development. Indeed, several inhibitors of TCF3, including Id1 and SCL/Tal-1, have recently been implicated in the maintenance and/or functional integrity of multipotent bone marrow progenitors (54, 70, 71). However, the direct roles for TCF3 proteins in regulating the HSC or MPP subsets and the mechanisms through which it acts on remain unclear.

5.0 RESULTS

This dissertation project focuses on dissecting the pivotal role of the E47 product of the TCF3 gene in regulating the development and functional integrity of HSCs and MPPs. The first part of this project tested the hypothesis in Specific Aim 1 that E47 is required for the developmental integrity of multipotent hematopoietic stem/progenitor cells. The second part focuses on Specific Aim 2 that addresses the specific roles and mechanisms for E47 in regulating the functional potential of HSCs.

To investigate the requirement of the E47 product of the TCF3 gene in the earliest, multipotent stages of hematopoiesis, we used the genetically mutant mouse that lacks E47 expression and has only less than 20% expression of E12 of a normal mouse (designated E47 knockout) (73). This model is chosen because of the good survivability of the adult mouse, and the relatively more essential role of E47 in hematopoietic development as compared with the other alternative splice product E12. The other mouse model, the E2A/TCF3 deficient mouse in which both E47 and E12 are totally absent, has very poor postnatal survivability (less than 10%), making it difficult to study their adult HSCs, and is thus not chosen as the model of this project. The genetical background of the mice that we used in this study is C57BL/6 (*B6*) congenic background. This mouse strain is chosen due to their easy availability and robustness.

In the first part of this project, we used E47 deficient mice to assess the requirement of E47 in the development and cell cycle regulation of each HSC and MPP subset. Mutiple phenotypic schemes have been used to define HSCs and MPPs, since E47 might directly regulate

the expression of a specific phenotypic marker rendering it unreliable. Results from all phenotypic schemes uniformally suggested that E47 is required for the developmental transition from HSC to MPP. Furthermore, for the first time, this study demonstrated *in vivo* hyperproliferation of primitive E47 deficient HSCs following challege by a mitotoxic drug. The results from this study have been published in The Journal of Immunology (2008) 181: 5885 - 5894 (83), and bolded for sepcifial emphasis in a review within Nature Reviews Immunology (2009) 9:175 (84).

The second part of this project focuses on investigating the role for E47 in regulating the functional integrity of HSCs. Using quantitative adoptive transfer experiments, we quantified the *in vivo* functional HSC size in E47 deficient versus WT mice. A number of *in vivo* and *in vitro* assays have also revealed that E47 is required for the long-term self-renewal and lineage differentiation of HSCs, possibly by restricting hyperproliferation under hematopoietic stress. Not only may premature exhaustion of HSCs result in compromised long-term hematopoiesis in response to hematopoietic injury, ageing and other replication stresses, but also hyperproliferation of HSCs under these persistent stresses might cause accumulation of mutations and provide the molecular and cellular basis for tumorigenesis. Thus, this study might provide new insights into hematopoietic damage repair, ageing and E47 associated leukemogenesis.

The specific methods and results are detailed in the following two manuscripts that have been published or submitted for publication.

5.1 SPECIFIC AIM 1 AND FIRST MANUSCRIPT

The following manuscript has been published in The Journal of Immunology, 2008, 181: 5885 -5894 (reprinted with permission from the Journal of Immunology) (83). This study found that E47 is required for the proper development and cell cycle quiescence of multipotent hematopoietic progenitors.

This work has been highlighted in "In This Issue" section in The Journal of Immunology as top 10% in the research field (www.jimmunol.org/cgi/content/full/181/9/5811). It has also been bolded for special emphasis by Nature Reviews Immunology as the first demonstration of a functional role for E47 in regulating the proliferation of primary hematopoietic stem cell *in vivo* (Nature Reviews Immunology, 2009, 9:175) (84), and has received seven citations by other groups since its publication only one year ago.

E47 controls the developmental integrity and cell cycle quiescence of multipotential hematopoietic progenitors

(Running title: E47 in uncommitted hematopoietic progenitors)

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5.1.1 Abstract

Little is known about the transcriptional regulators that control the proliferation of multipotent bone marrow progenitors. Understanding the mechanisms that restrict proliferation is of significant interest since the loss of cell cycle integrity can be associated with hematopoietic exhaustion, bone marrow failure, or even oncogenic transformation. Here, we show that multipotent LSKs (lineage^{-}Sca^{hi}ckit^{+}) from E47 deficient mice exhibit a striking hyperproliferation associated with a loss of cell cycle quiescence and increased susceptibility to *in vivo* challenge with a mitotoxic drug. Total LSKs contain long-term self-renewing hematopoietic stem cells (HSCs) and downstream multipotential progenitors (MPPs) that possess very limited or no self-renewal ability. Within total LSKs, we found specific developmental and functional deficits in the MPP subset. E47 knockout (KO) mice have grossly normal numbers of self-renewing HSCs but a 50-70% reduction in non-renewing MPPs and downstream lineage-restricted populations. The residual MPPs in E47 KO mice fail to fully upregulate *flk2* or initiate V(D)J recombination, hallmarks of normal lymphoid lineage progression. Consistent with the loss of normal cell cycle restraints, we show that E47 deficient LSKs have a 50% decrease in *p21*, a cell cycle inhibitor and known regulator of LSK proliferation. Moreover, enforced expression studies identify *p21* as an E47 target gene in primary bone marrow LSKs. Thus, E47 appears to regulate the developmental and functional integrity of early hematopoietic subsets in part through effects on *p21*-mediated cell cycle quiescence.

5.1.2 Introduction

The mechanisms that regulate hematopoietic self-renewal and multi-lineage differentiation potential are of great importance from both the basic biological and clinical perspectives. Primitive hematopoietic cells that repopulate all blood cell lineages reside in the bone marrow (BM) LSK population that lacks lineage markers while expressing high levels of Sca-1 and c-kit. Total LSKs are a heterogeneous population that contain HSCs with long-term (LT) hematopoietic reconstitution activity (14, 15, 85) as well as downstream MPPs that have little or no self-renewal capabilities (86, 87). HSCs continually replenish the immune system in steady-state circumstances and regenerate long-term hematopoietic functioning after stress exposure or myeloablative therapy while MPPs can rapidly give rise to multiple downstream lineages (88). Not only is it of significant interest to understand the mechanisms that confer long-term self-renewal capability to LT-HSCs, but also those that restrict the expansion and mitotic capacity of downstream MPPs.

Mounting evidence indicates that cell cycle quiescence is of vital importance for the functional integrity of both HSCs and MPPs. First, the loss of the normal restraints on LSK cell cycling is associated with stem cell exhaustion and loss of self-renewal potential. For example, genetic ablation of the cell cycle inhibitor p21 (36), the PTEN regulator of PI3 kinase gene (89, 90) or the FOXO family of transcriptional regulators (91), leads to increased cell cycle entry with loss of LT- HSC function, and bone marrow failure. Second, the ectopic acquisition of self-renewal capabilities may serve as a platform for malignant transformation. Triple deletion of the *p16*, *p19*, and *p53* genes involved in cell cycle regulation and survival was recently shown to confer long-term self-renewal capabilities to MPPs (92). While extracellular environmental cues signaling through the Notch and Wnt pathways are important for HSC activity, less is known

about the cell-intrinsic factors that govern the development, maintenance, and function of multipotent subsets (93).

The transcription factor E47 is a member of the E protein family that is encoded by the E2A gene. E47 is essential for multiple aspects of B and T lineage development including V(D)J recombination (74), enforcement of developmental checkpoints (74, 94, 95), differentiation (73, 74, 96-98), cell cycle regulation (99, 100) and survival (55, 101, 102). Furthermore, repression or absence of E47 or E2A activity has been implicated in cancer development (103). Half of E2A KO mice rapidly display T cell tumors at 3 to 10 months of age as do a proportion of mice deficient in the E47 splice product (12, 75). Translocations in which E2A is fused to PBX1 are detecTable 1n 23% of all pediatric pre B cell acute lymphoblastic leukemia (ALL) patients (11, 104, 105), and inhibition of E2A activity by the overexpression of antagonists is mechanistically linked to Hodgkin lymphoma (80). That E2A is linked to cancers of multiple lineages raises the possibility that disruption of E2A in uncommitted hematopoietic progenitors acts as a first lesion that renders cells susceptible to secondary transforming events in a lineage-dependent manner. Indeed, indirect evidence hints at a role for E proteins in the regulation of HSC or MPP integrity. Functional ablation of the E protein inhibitors Id1 or SCL/Tal-1 leads to severe defects in hematopoietic progenitor activity and function (71, 106, 107). However, direct evidence for a pivotal role of E47 within the HSC and MPP subsets has been lacking.

In this study, we demonstrate a critical role for E47 in the establishment of a robust MPP population. We found that E47 deficient LSKs exhibit hyperproliferation, a loss of cell cycle quiescence, and increased sensitivity to a cell cycle specific drug. Within total LSKs, we found specific defects in the MPP subset. While HSCs are numerically intact, downstream MPPs are significantly reduced in E47 KO mice as compared to wild type mice. Moreover, the lymphoid

differentiation potential of E47 KO MPPs is severely compromised. To establish the molecular mechanisms underlying MPP failure, we used gain of function and loss of function approaches to identify E47 target genes. Our results identify two important stem cell regulators, *p21* and *Ikaros,* as potential E47 targets within the primitive LSK population. Together, our data suggest that E47 is required for the developmental and functional integrity of MPPs through effects on cell cycle quiescence. Since E proteins are not restricted to the bone marrow, knowledge about E47 in multipotent hematopoietic progenitors may provide broader insight into the mechanisms that control multi-lineage differentiation potential in non-hematopoietic tissues.

5.1.3 Materials and Methods

5.1.3.1 Mice

E47 KO mice and H2-SVEX V(D)J recombination reporter mice (57, 74) were bred in accordance with IACUC policies at the University of Pittsburgh.

5.1.3.2 Flow Cytometry

Hematopoietic progenitors were isolated and stained for surface markers as we have reported (56, 57). Antibodies to murine surface markers were obtained from eBioscience. Primary anti-mouse Abs included AA4.1 APC (clone AA4.1), B220 APC or biotin (clone RA3-6B2), CD3 biotin (clone 2C11), CD11b biotin (clone M1/70), CD19 biotin or Cy5PE or FITC (clone MB19-1), CD27 PE (clone LG.7F9), CD34 FITC (clone RAM34), CD43 PE (clone S7), CD48 PE (clone HM48-1), CD117 PE or Cy5PE (clone 2B8), CD135 PE (clone A2F10), CD150 APC or FITC (clone 9D1), Gr-1 biotin (clone 8C5), IgM (clone 331) biotin or FITC, IL-7R PE (clone SB/14), Ly6C biotin or FITC (clone HK1.4), NK1.1 biotin (clone PK136), TER-119 biotin (clone TER-119), TCR-γδ biotin (clone UC7-13D5), and Sca-1 FITC or APC or Cy5PE (clone D7). Secondary reagents were streptavidin-Cy7-PE or streptavidin-Pacific Blue

(Molecular Probes). E2A (clone G127-32, PharMingen) intracellular staining was performed as described (108). In brief, cells were fixed with Cytofix (BD Bioscience), permeabilized with PBS-0.2% Tween 20 for 10 min at 37°C, and stained for E2A at room temperature for 30 mins. Flow cytometry was performed on a three-laser, nine-detector LSR II (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

5.1.3.3 BrdU labeling, Cell Cycle Analysis, and Fluorouracil Treatment

BrdU incorporation assays were performed as we have previously described (56, 57). Briefly, mice were injected *i.p*. with 200 μg BrdU in PBS, or PBS alone as a control, at 12-h intervals. Twenty four hours after the first injection, bone marrow was isolated, and cells were stained for surface markers and anti-BrdU FITC with BrdU flow kit (BD Bioscience) according to the manufacturer's instructions. Ki-67 intracellular staining was performed as previously described (39). To determine the G_2/M cell cycle status, cells stained with Ki-67 were subsequently washed and incubated with DAPI (5 μg/ml) for a minimum of 30 minutes at room temperature before flow cytometric analysis. For *in vivo* analysis of the restriction on cell cycle entry, mice were injected weekly with 150 mg/kg of the cell-cycle specific drug 5-fluorouracil (5-FU) or PBS *i.p*. as described (36). Animals were weighed weekly, and animals displaying a change in body weight of greater than 30%, loss of coat quality, or lethargy were promptly sacrificed accordance with university IACUC policies. For short-term experiments, mice were sacrificed 10-12 hours after 5-FU or PBS administration, and bone marrow cells harvested for surface staining.

5.1.3.4 EMSA

HSCN1c110 LSK cells are a Notch1-transduced cell line that displays multipotency and self-renewal potential *in vitro* and *in vivo* (109). Electrophoretic mobility shift assays with

HSCN1c110 nuclear extracts was performed using the μE5 probe as described (110). In brief, cells were resuspended in 10 mM HEPES (pH 7.9), 10 mM KCl, 1.0 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 1 mM PMSF, protease inhibitor mixture (PIM), and Nonidet P-40 (0.1%) and centrifuged at 8000 rpm for 5 mins. The nuclear containing pellet was solubilized in 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 2 mM PMSF, PIM, and glycerol (10%) on ice for 20 mins. The lysate was centrifuged and the nuclear containing pellet was collected. The nuclear extracts were preincubated with rabbit anti-mouse polyclonal E47 or E2A antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with radiolabeled DNA probe with 0.5 μg poly(dI-dC) as a nonspecific competitor. The binding complexes were resolved by electrophoresis in 5% polyacrylamide gel for 3 hrs at room temperature.

5.1.3.5 Retroviral transduction

Lineage-negative BM cells from E47 heterozygous mice were infected with E47-ER-huCD25 or the control bHLH-ER-huCD25 lacking the transactivation domain (103). Retroviral supernatant were obtained from the Phoenix packageing cell line using the Fugene 6 transfection kit (Roche). BM cells were depleted of lineage positive cells (NK1.1, CD11b, CD19, B220, TER-119 and Gr-1) using streptavidin microbeads (Miltenyi Biotec) according to the manufacturer's recommendation. Lineage-negative cells were pre-stimulated overnight in IMDM (Cellgro) with 20% FCS containing stem cell factor (100 ng/ml), *flk2/flt3* ligand (100 ng/ml), IL-11 (10 ng/ml), IL-6 (100 ng/ml) (Peprotech), and 1% penicillin/streptomycin. Retroviral supernatant containing 6 μg/ml polybrene (Sigma) was added to the cells, and two rounds of spin-infection were performed as described (111). After 24 hours of culture, infected cells were incubated with 4-OHT (Sigma) for 5 hours to activate the E47-ER fusion protein. Transduced

cells with a huCD25^{+} LSK phenotype were then sorted for mRNA isolation and quantitative PCR analysis.

5.1.3.6 Statistics

Multiple comparisons were performed using ANOVA followed by Tukey-Kramer HSD post-hoc analysis. Two sample comparisons were performed using the Students t Test. Differences were regarded as significant at $p < 0.05$. Analyses were performed using the JMP version 5.1 statistical software package (SAS Institute).

5.1.4 Results

5.1.4.1 E47 is Expressed and Functionally Active in Uncommitted Hematopoietic Progenitors

In examining the differential requirements for E47 activity during the earliest stages of B versus T lineage development, we found a surprising depletion of the earliest B and T lineage precursor subsets in the absence of E47. Specifically, E47 deficient mice had a virtual ablation of BM common lymphoid progenitors (CLPs), efficient progenitors to the B lymphocyte lineage, and a two-fold reduction in the frequency of thymic early T lineage progenitors (ETPs), progenitors to the T lymphocyte lineage. **Figure 3** depicts the phenotypic resolution of these subsets that are then quantified in **Table 1**. Across E47 wild type (WT), heterozygous (HET) and knockout (KO mice), BM CLPs were reduced 10-fold, consistent with our previous findings (74). Since young E47 deficient mice frequently develop thymic lymphomas of DN origin (55), we quantified ETPs in two day old animals to avoid leukemia-associated perturbations. Thymic ETPs were reduced four-fold from 485 $\pm$ 429 (n=10) to 137 $\pm$ 210 (n=6) in E47 HET versus KO mice (**Table 1**). The paucity of both CLPs and ETPs is unexpected since none of the known E47 targets are predicted to recapitulate this defect. Moreover, upstream multipotent LSKs were

reduced two-fold in frequency (**Figure 3**) and three-fold in absolute number (**Table 1**). These data suggest that E47 activity is required earlier in hematopoietic development than was appreciated based on a small sample size (74).

A careful examination throughout the earliest stages of hematopoietic development reveals that total E2A protein (E47 + E12) is detecTable 1n 72% of BM LSKs and 79% of CLPs as assessed by intracellular staining and flow cytometry (**Figure 4A**). E2A expression further increases during the pre-pro B and pro-B stages of B lineage development in terms of both the frequency of total $E2A^+$ cells and mean fluorescence intensity (**Figure 4A**), thereby extending previous observations using knockin GFP reporter mice (112, 113). Original studies indicated that the total $B220^+CD43^+$ pro B cell subset contains two distinct populations expressing different levels of E2A (114). We initially obtained this result and found that 45% of cells were positive for E2A staining (**Figure 4C)**. However, within the $B220^+CD43^+$ population which is fairly heterogeneous, B lineage potential is known to lie in the minor subset that lacks DX5, Ly6C, IgM, and CD4 expression (115). When we re-examined E2A protein levels in the population enriched for pro B potential, we found that 92% of cells are positive for E2A and that expression is uniformly high (**Figure 4C**). E2A expression is also detecTable 1n human hematopoietic progenitors and is comparably upregulated during B lineage progression, suggesting the generality of our findings across both mouse and man (**Figure 4A & D**).

Total LSKs are a heterogeneous population that contain HSCs as well as downstream MPPs. The transition from LT-HSC to MPP is associated with the acquisition of the *flk2/flt3* cytokine receptor (14). Interestingly, murine E2A expression increased from 53% $\pm$ 6.1 in the *flk2*$^-$LSK LT-HSC population to 71% $\pm$ 6.1% in the *flk2*$^+$LSK MPP subset (n=3 independent experiments; representative data shown in **Figure 4B**). These data indicate that E2A is expressed

in primary HSCs from unmanipulated mice, raising questions about the functional role of this transcription factor at this pivotal stage of development.

We exploited a model stem cell line to examine E47 binding activity in uncommitted hematopoietic progenitors (109). Notch-1 transduced HSCN1c110 is self-renewing clonal line that retains pluripotency, and gives rise to lymphoid and myeloid lineages *in vivo* (109). HSCN1c110 nuclear extracts bound to the E-box containing μE5 target probe were clearly supershifted by antibodies to E47 and E2A (**Figure 4E**). No supershifting was seen using the isotype control, demonstrating the specificity of E47 and E2A activity. Thus, E2A protein is expressed and functional in uncommitted hematopoietic progenitors but its role in this compartment remains unknown.

5.1.4.2 E47 Promotes the Development of MPPs

Total LSKs contain both long-term self-renewing HSCs and MPPs with very limited or no self-renewal ability. We analyzed the presence of each developmental compartment in E47 WT, HET and KO mice. Within total LSKs, the minority HSC subset can be resolved on the basis of SLAM marker expression (15), CD27 (16), or *flk2* (14), phenotypic schemes that enrich HSCs to varying degrees (71). We obtained identical results using all three phenotypic models. E47 WT, HET and KO mice had comparable numbers of phenotypic HSC defined as $CD150^{+}CD48^{-}$LSKs, $CD27^{-}$LSKs, or $flk2^{-}$LSKs (**Figure 5A & B** and **Table 1**). By contrast, MPPs defined as $CD150^{-}CD48^{-}$LSK, $CD27^{+}$LSK or $flk2^{+}$LSK were uniformly reduced by 50% in E47 KO versus WT mice across all three phenotypic schemes. The early developmental defect was even more pronounced in downstream lineage restricted progenitors (LRP; $CD150^{-}CD48^{+}$LSKs), cells that can give rise to B or myeloid lineages but have little T cell potential (15) and CLPs ($AA4.1^{+}Sca^{lo}IL7R^{+}lin^{-}$), cells that efficiently give rise to B cells. LRPs and CLPs were

reduced 70% and 90%, respectively. Thus, disruption of E47 did not alter the absolute number of HSCs ($p > 0.05$) but did significantly reduce MPPs and downstream LRPs and CLPs ($p < 0.05$). That identical results were obtained using all three developmental schemes emphasizes the robustness of the data, and precludes the possibility of an apparent loss of MPPs due to perturbation in any single marker used to characterize this population. Similar results were observed using the CD34 marker to distinguish LT-HSCs and MPPs, again emphasizing the generality of our findings (data not shown).

Not only are MPPs reduced in number in E47 KO mice but this population appears to be functionally comprised. The *flk2* brightest subset of MPPs contains the early lymphoid progenitor (ELP) population that first initiates *rag* expression, a key step in B lineage specification (116). We found that MPPs from E47 KO mice fail to fully upregulate the *flk2* cytokine receptor. The 25% of *flk2*^{bright} LSKs associated with lymphoid potential (23) is markedly reduced in E47 KO LSKs (**Figure 6A**). Specifically, this subset is reduced from 25.1% $\pm$ 2.3 (n=5) to 9.4% $\pm$ 3.9% (n=6); $p < 0.05$. This is an important observation because cells with the potential to undergo V(D)J recombination are exclusively contained within the *flk2*^{bright} LSK population in WT mice. While 1.2% of WT LSKs express V(D)J recombinase activity as visualized using a fluorescent recombination reporter, this *flk2*^{bright} recombination^{+} subset is completely absent in E47 KO LSKs (**Figure 6B**). In an analysis across multiple independent mice, the frequency of recombination^{+} LSKs was reduced from 1.3% $\pm$ 0.42 (n=4) to 0.05% $\pm$ 0.05 (n=5) in E47 WT versus KO mice, respectively; $p < 0.05$. Thus, E47 activity is required for the development and/or maintenance of a robust *flk2*^{bright} MPP compartment that is competent to perform V(D)J recombination.

5.1.4.3 E47 Regulates LSK Quiescence

A key component of hematopoietic integrity is cell cycle quiescence. To examine the role of E47 in multipotent progenitors, we first examined the *in vivo* requirement of E47 for the proliferation and survival of total LSKs. After two days of administration of the thymidine analogue BrdU, 40% $\pm$ 6.4 of WT/HET LSKs are $BrdU^+$ versus 66% $\pm$ 12 of KO LSKs (**Figure 7A & D**; average $\pm$ SD of three independent experiments). Elevated levels of BrdU incorporation in KO versus WT LSKs may reflect enhanced survival of labeled cells within a particular compartment or increased rates of proliferation. We found uniformly low levels of apoptosis of WT and KO LSKs directly *ex vivo* (<5% apoptosis; **Figure 7B**) as well as after overnight *in vitro* culture of rigorously purified LSKs that had been depleted of phagocytes that might otherwise clear dying progenitors (data not shown). Identical results were observed using both the mitotracker and annexin-V methods for detecting apoptotic cells (data not shown). Thus, increased BrdU incorporation is unlikely to reflect enhanced survival of E47 KO LSKs. Rather, increased BrdU incorporation likely reflects entry into the cell cycle. Direct analysis of the cell cycle status of E47 WT versus KO LSKs reveals interesting differences. While the proportion of cells in the active phases of the cell cycle ($S + G_2/M$) is comparable between WT and KO mice, the latter mice display an increased proportion of LSKs that have exited G_0. The frequency of LSKs in $S + G_2/M$ is 19.4% $\pm$ 3.4 (n=4) versus 17.4% $\pm$ 3.2 (n=5) in E47 WT versus KO mice, respectively (**Figure 7C & D**). The proliferation antigen Ki-67 is expressed in all stages of the cell cycle except for G_0, rendering the absence of this protein a sensitive marker of quiescence. The frequency of $Ki\text{-}67^{NEG}$ cells was reduced from 32.3% $\pm$ 3.3 (n=6) to 23% $\pm$ 3.1 (n=6) in WT versus KO LSKs indicating a loss of quiescence and, by consequence, increased entry into the cell cycle (**Figure 7C & D**). This cell cycle perturbation appeared to be restricted to the $Sca\text{-}1^+c\text{-}$

kit$^+$ subset of lineage-negative cells as the frequency of Ki-67^{NEG} Sca-1$^-$c-kit$^+$ progenitors was similar between WT versus KO mice, 6.2% $\pm$ 1.2 (N=6) versus 4.9% $\pm$ 0.9 (N=6), $p > 0.05$, respectively. Together, these data indicate that E47 acts to restrain LSK cell cycle entry.

We examined the biological consequence of the hyperproliferation in E47 KO LSKs by challenging the ability of these progenitors to recover in response to mitotoxic challenge. Repeated exposure to the cell cycle-specific drug 5-fluorouracil (5-FU) depletes proliferating hematopoietic progenitors (117, 118), thereby challenging the restriction on cell cycle entry of stem cells in intact animals (36). Consistent with altered patterns of cell cycling, E47 KO mice are preferentially sensitive to *in vivo* challenge with 5-FU. While all E47 KO mice died within 15 days of 5-FU administration, 90% of WT and HET mice survived beyond this point (**Figure 7E**). Moreover, our data indicate a specific loss of cell cycle integrity in specific phenotypic subsets contained within total LSKs. For this analysis, we quantified each the *flk2*$^-$ and *flk2*$^+$ LSK subsets after short-term (10-12 hours) *in vivo* challenge with 5-FU. While short-term exposure to 5-FU has little effect on the number of *flk2*$^-$ and *flk2*$^+$ LSK subsets in WT mice, these subsets are reduced two-fold and four-fold, respectively, in E47 KO mice (**Figure 7F**). These data provide clear evidence that E47 is required for the proliferative integrity of MPPs *in vivo*. Together, these data indicate that E47 KO LSKs have increased proliferation together with a loss of quiescence, an interpretation consistent with the role of E47 in restraining B and T cell precursor proliferation (99, 100).

E47 is known to restrict the proliferation of primary B and T cell progenitors (99, 100) as well as some non-hematopoietic cell lines (119). E47 has also been shown to bind to the *p21* promoter, and activate gene expression (103, 119, 120). The CDK inhibitor *p21* is of particular interest because its genetic ablation leads to a loss of quiescence (36), and consequent bone

marrow failure. Quantitative PCR analysis of sorted LSKs reveals a 50% decrease in *p21* expression in KO versus WT LSKs (**Figure 8A**), suggesting that *p21* expression may be regulated either directly or indirectly by E47. To determine the capacity of E47 to directly activate *p21* expression in primary LSKs, we performed gain of function experiments in which we infected LSKs with a tamoxifen-inducible form of E47 (E47-ER) that allows the selective induction of E47 following tamoxifen exposure. E47-ER activation by 4-OHT induced *p21* transcript abundance suggesting E47 regulates *p21* in primary LSKs (**Figure 8B**). Enforced expression of E47 also induced *Ikaros*, a key regulator of early hematopoietic differentiation (**Figure 8B**). By contrast, no changes were detecTable 1n the other cell cycle regulators *gfi1*, *cdk6* or *c-myb*, indicating the specificity of the *p21* and *Ikaros* expression alterations. Indeed, the hyperproliferation of E47 deficient LSKs is strikingly reminiscent of *p21* knockout LSKs which exhibit loss of quiescence associated with severe hematopoietic reconstitution deficits (36).

5.1.5 Discussion

To date, knowledge about the transcription factors that regulate the integrity of the individual HSC and MPP compartment within the multipotent LSK population has been limited. Here, we define a critical role for the transcriptional factor E47 in the developmental and functional integrity of BM LSKs, with specific requirement in the MPP subset. Not only is the number of MPPs significantly reduced in E47 KO mice as shown by multiple independent phenotypic schemes but the lymphoid differentiation potential of E47 KO MPPs is severely compromised. In addition, E47 KO MPPs have reduced expression of the essential cytokine receptor *flk2/flt3* and an absence of V(D)J recombinase activity, defects that are associated with a profound reduction in the earliest B and T lineage progenitors in these deficient mice. Moreover, we show that total LSKs from E47 KO animals exhibit hyperproliferation and a loss of G_0

quiescence. Reciprocal gain of function and loss of function studies identify the cell cycle inhibitor *p21*, a known regulator of hematopoietic integrity, as an E47 target gene.

Our data highlight an important role for E47 in the balance between proliferation and differentiation of hematopoietic progenitors toward the lymphoid lineages. We show that E47 KO MPPs exhibit hyperproliferation and loss of quiescence at the expense of lymphoid differentiation. Inappropriate entry into the cell cycle has been shown to inhibit lineage-specific differentiation events (121). The cell cycle regulator CDK6 that is specifically expressed in proliferating cells appears to block differentiation to the myeloid lineage (121). Ectopic expression of CDK6 enhances proliferation but inhibits differentiation of primary murine myeloid progenitors. As another example, the orphan nuclear receptor Nurr1 promotes dopamine cell differentiation through cell cycle arrest (122). Established literature also shows that expression of the cell cycle regulator *p21* is upregulated during the differentiation of myeloid cells (123) and non-hematopoietic oligodendrocytes (124), suggesting that *p21* might regulate cell differentiation through its cell cycle regulatory function. Furthermore, both loss of function and gain of function experiments performed here suggest that the key cell cycle regulator *p21* is an E47 target in primary LSK progenitors. Thus, E47 appears to promote the differentiation of MPPs towards lymphoid lineage while controlling cell cycle quiescence.

Our data identify the key regulator of early hematopoietic differentiation *Ikaros* as a potential E47 target. Disruption of Ikaros activity in E47 deficient LSKs may contribute to the severe lymphoid differentiation defects in E47 KO MPPs. Like E47, Ikaros is essential for robust B and T lymphocyte development (125). The B cell arrest in E47 KO mice and Ikaros KO mice occurs at similar stages, with severe defects in the CLP compartment and reduced *flk2* expression in MPPs. Also, in accordance with our finding of E47 KO MPP deficits, previous studies showed

that Ikaros null mice have severe defects in HSC function as well as MPP differentiation deficits (126, 127). Here, we show E47 deficient mice exhibit developmental and differentiation perturbations in both of these compartments. Therefore, our finding of an interaction between E47 and Ikaros warrants further investigation.

E47 has been suggested to control the cell cycle progression of hematopoietic as well as non- hematopoietic cells. Mice lacking one or both alleles of E47 or the E2A parent gene exhibit hyperproliferation in primary B (100) and T lineage progenitors (99) as well as CLPs (74). Consistent with these findings, we demonstrate that E47 acts to restrain proliferation by controlling the cell cycle quiescence of LSKs (**Figure 7**). Thus, in primary lymphocytes, E47 uniformly restrains cell cycle proliferation. Observations in cell lines are more heterogeneous, suggesting that E47 activates or inhibits proliferation in a cell type specific manner (119, 128). Several key cell cycle regulators have been identified as E47 targets including *p21* and *p16*. E47 has been found to physically interact with the *p21* promoter and induce *p21* expression the HeLa cell line (119). Our data provide convincing evidence that *p21* is a potential E47 target in primary LSKs and multipotent hematopoietic progenitors. Since *p21* is of vital importance in the differentiation and self-renewal of hematopoietic as well as non-hematopoietic tissues, we propose that E47 may control early hematopoietic development and differentiation through interaction with its immediate downstream target *p21*.

Together, our results define a role for the transcription factor E47 in the developmental integrity of bone marrow MPPs. We show that E47 is required for the formation of a robust MPP subset that is capable of lymphoid lineage progression. We also show that that E47 regulates the proliferative integrity of multipotent progenitor subsets through effects on *p21*. Recent studies have found that MPPs with oncogenic mutations display hyperproliferation and increased self-

renewal ability (92), indicating that loss of quiescence in MPPs might be associated with tumorigenesis. As mentioned above, MPPs from mice mutant for three tumor suppressor genes (*p53*, *p16* and *p19*) showed hyperproliferation accompanied by the abnormal acquisition of long-term renewal capabilities, suggesting the potential for transformation (92). Furthermore, MLL-GAS7 oncoprotein transformed MPPs displayed significantly heightened proliferation as well as induced leukemias of multiple lineages in lethally irradiated mice, indicating that loss of constraints on MPP proliferation might offer the opportunity for malignancy (129). Consistent with the hyperproliferation and loss of quiescence of E47 KO MPPs, T cell leukemia is frequently seen in E47 and E2A KO mice (12). In humans, disruption of E2A activity is associated with the cancers of B and T lineage (80, 81, 130). The link between cell cycle restraint defects in E47 deficient MPPs and transformation of the lymphoid lineages remains to be investigated.

5.1.6 Acknowledgements

We sincerely thank Bonnnie Blomberg, John Choi, Dewayne Falkner, Daniela Frasca, Barbara Kee, Kees Murre, Xiao-Hong Sun and Will Walker for reagents and technical advice. We greatly appreciate critical input from Binfeng Lu, Kay Medina, and Richard Steinman.

5.1.7 Tables and Figures

Table 1. Early hematopoietic defects in mice lacking one or two copies of E47.

		wild type[§]	heterozygote[§]	knockout[§]
Bone marrow				
total bone marrow adult		31 ± 9 (n=18)^{A}	29 ± 13 (n=30)^{A}	20 ± 8 (n=28)^{B}
total LSKs		33,129 ± 13,802 (n=15)^{A}	24,226 ± 14,648 (n=11)^{AB}	11,729 ± 7,231
LT-HSC				
$CD150^{+}CD48^{-}$ LSK		868 ± 220 (n=6)^{ns}	735 ± 326 (n=4)^{ns}	721 ± 326 (n=8)^{ns}
$CD27^{-}$ LSK		460 ± 198 (n=8)^{ns}	340 ± 269 (n=3)^{ns}	283 ± 159 (n=7)^{ns}
$flk2^{-}$ LSK		11,156 ± 5,120 (n=7)^{ns}	9,757 ± 8,060 (n=3)^{ns}	10,029 ± 3,918 (n=8)^{ns}
MPPs				
$CD150^{-}CD48^{-}$ LSK		1,931 ± 1,010 (n=6)^{A}	1,892 ± 1,133 (n=4)^{AB}	723 ± 160 (n=7)^{B}
$CD27^{+}$ LSK		34,714 ± 14,360 (n=8)^{A}	16,138 ± 4,852 (n=3)^{AB}	12,528 ± 12,105
$flk2^{+}$ LSK		36,111 ± 13,625 (n=7)^{A}	25,396 ± 23,953 (n=3)^{AB}	14,561 ± 8,376 (n=8)^{B}
$flk2^{bright}$ LSK		14,463 ± 5,346 (n=5)^{A}	na	2,468 ± 1,461(n=6)^{A}
lineage restricted				
$CD150^{-}CD48^{+}$ LSK		56,338 ± 16,146 (n=3)^{A}	na	13,917 ± 5,605 (n=4)^{B}
lymphoid specific				
CLPs		8,216 ± 4,741 (n=4)^{A}	7,970 ± 4,574 (n=16)^{A}	625 ± 354 (n=14)^{B}
Thymus				
total thymocytes x 10^{5}	<12 hrs old	na	18.0 ± 6.7 (n=8)^{A}	4.8 ± 2.0 (n=5)^{B}
	24-48 hrs old	20.4 ± 6.8 (n=4)^{A}	19.5 ± 7.9 (n=8)^{A}	3.9 ± 1.1 (n=4)^{B}
ETPs	<12 hrs old	na	342 ± 127 (n=8)^{A}	126 ± 31 (n=5)^{B}
	48 hrs old	na	485 ± 429 (n=10)^{A}	137 ± 210 (n=6)^{B}

Thymus or bone marrow tissue isolated from the indicated mice were examined for the presence of hematopoietic progenitors subsets contained within the LSK subset ($lineage^{-}$, sca^{hi}, kit^{hi}).

Thymic tissue was analyze from newborn mice to avoid perturbations associated with thymic leukemias apparent in young adult mice. n = number of individuals. [§]

$p<0.05$, ANOVA followed by Tukey HSD for multiple comparisons or Student's t test for pairwise analysis. Significant differences between groups are indicated by the A or B superscript; ns, not significant; na, data not available.

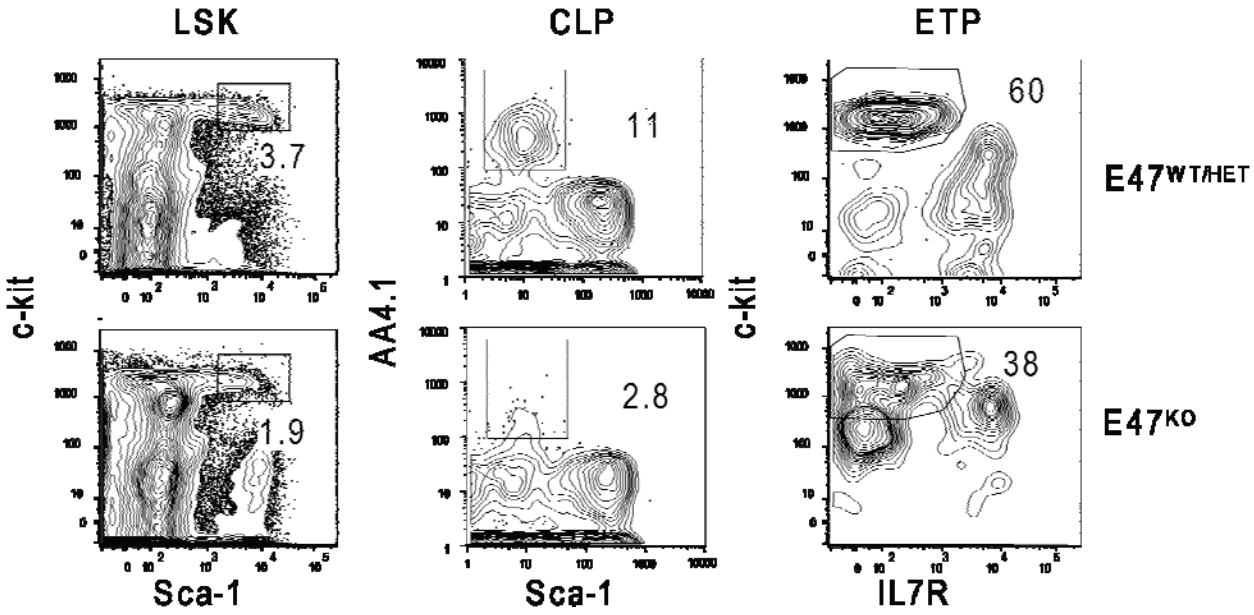

Figure 3: Disruption of early hematopoietic progenitors in E47 deficient mice

BM from young adult E47 WT/HET or KO mice was stained to resolve total LSKs ($Lin^{-}Sca\text{-}1^{hi}c\text{-}kit^{+}$) or CLPs ($AA4.1^{+}Sca\text{-}1^{lo}IL7R^{+}lin^{-}$) by flow cytometry. Thymocytes from forty eight hour old pre-leukemic E47 WT/HET or KO mice were resolved for ETPs ($c\text{-}kit^{+}IL7R^{-}CD44^{+}CD25^{-}Lin^{-}$). The data are representative of 6-30 independent animals.

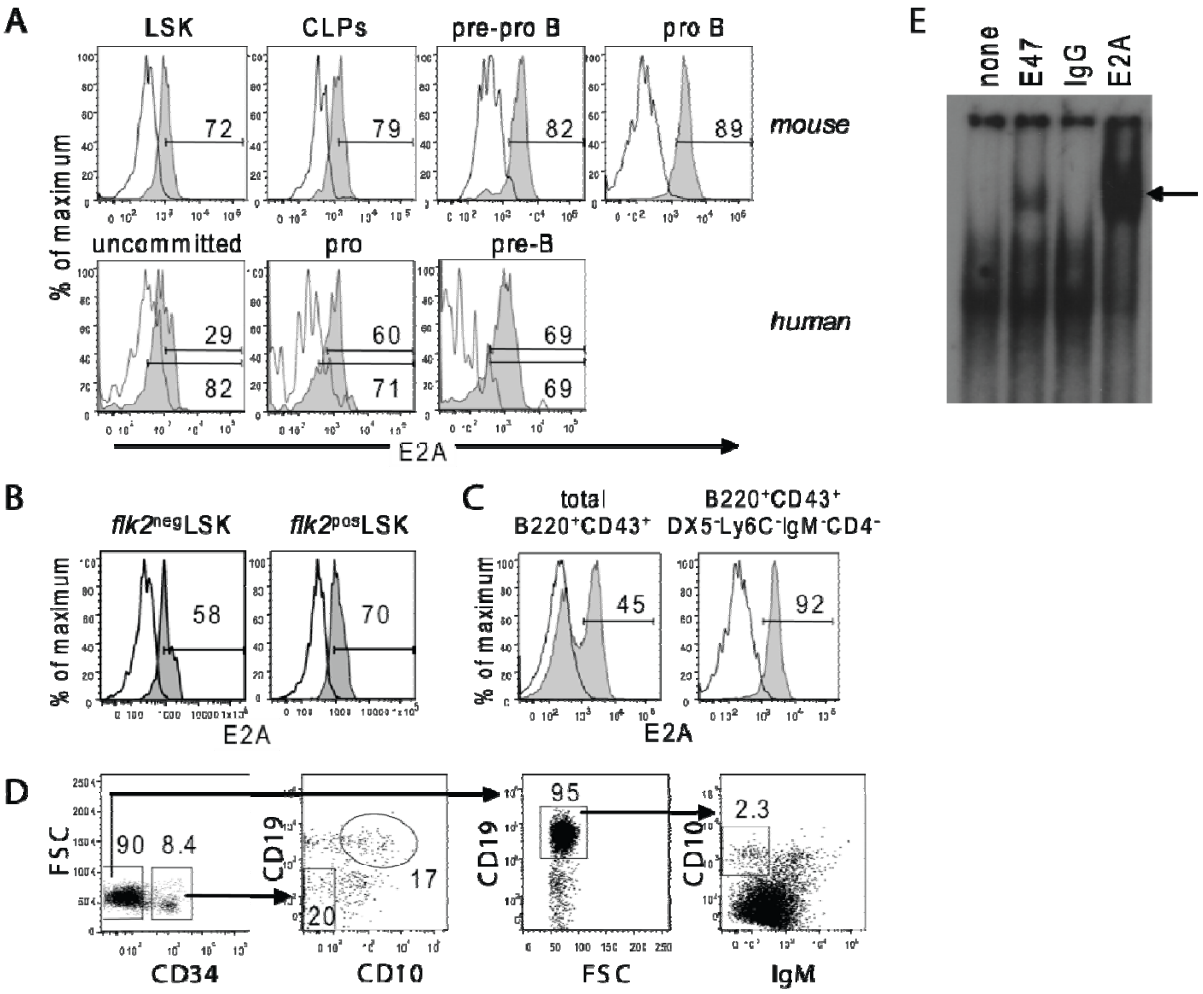

Figure 4: The transcription factor E47 is expressed and functionally active in uncommitted hematopoietic progenitors

A) Murine and human BM cells stained to resolve the indicated subsets were fixed and permeabilized to detect intracellular E2A (shaded histograms) or the isotype control (open histograms). Murine LSKs and CLPs were resolved as in Figure 1 while murine pre-pro B and pro B were defined as $B220^{+}CD43^{+}DX5^{-}Ly6C^{-}IgM^{-}$ cells that lacked or expressed CD19, respectively. Human bone marrow cells were resolved as uncommitted BM progenitors ($CD34^{+}CD10^{-}CD19^{-}$), pro-B cells ($CD34^{+}CD10^{+}CD19^{+}$), and pre-B cells ($CD34^{-}CD19^{+}IgM^{-}$).

Due to variation in background fluorescence across human B cell precursor subsets, the gating is shown relative to the background staining in each individual subset (upper gate) as well as by applying a uniform gate across all populations (lower gate). B) Total murine LSKs were further resolved based on *flk2* expression. C) Murine pro-B cells resolved as total $B220^{+}CD43^{+}$ were further refined as $B220^{+}CD43^{+}DX5^{-}Ly6C^{-}IgM^{-}$ BM cells. D) Gating strategy to resolve the human B cell subsets depicted in A. E) EMSA analysis of E47 activity in an LSK cell line. Nuclear extracts prepared from HSCN1c110 cells were pre-incubated in the presence of antibodies to E47, E2A (E47 + E12) or control IgG and then incubated with the radiolabeled E5 DNA probe. The arrow indicates the supershift. The data are representative of 3-5 independent mice or primary human samples (A-D) or 2 independent experiments (E).

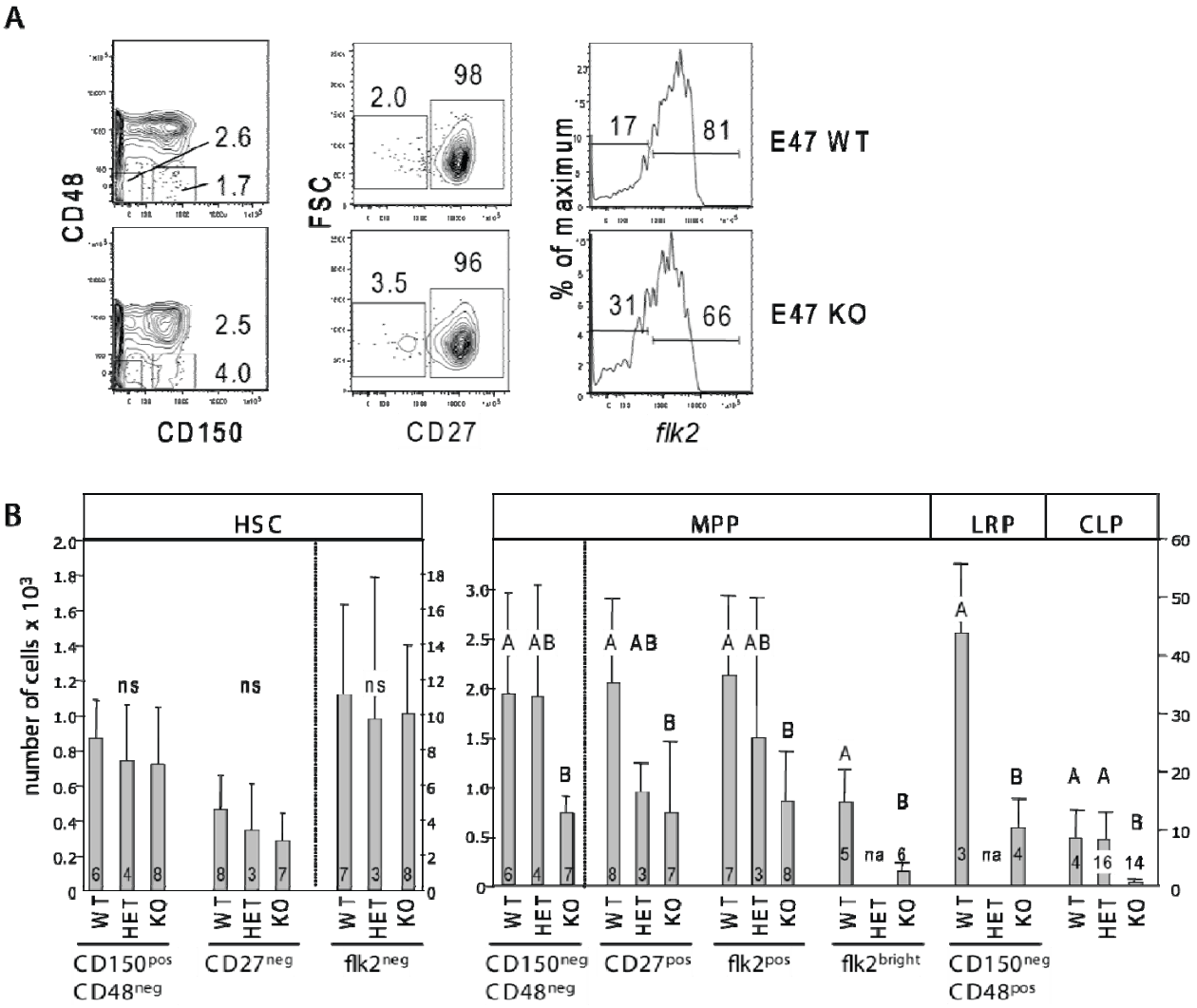

Figure 5: E47 is required for the developmental integrity of MPPs

A) BM from young adult E47 WT/HET or KO mice was stained to resolve HSCs and MPPs using three independent phenotypic schemes. HSCs were resolved as *flk2/flt3*$^{-}$ LSKs, CD150^{+}CD48^{-}LSKs or CD27^{-}LSKs. B) BM LSKs from E47 WT, HET or KO mice were stained to resolve the indicated subsets as described in Table 1. The number within or over each bar indicates the number of mice used to calculate mean $\pm$ SD. The letters A and B indicate

statistical significance as determined in an ANOVA followed by Tukey-Kramer HSD post-hoc analysis, $p < 0.05$. ns, not significant.

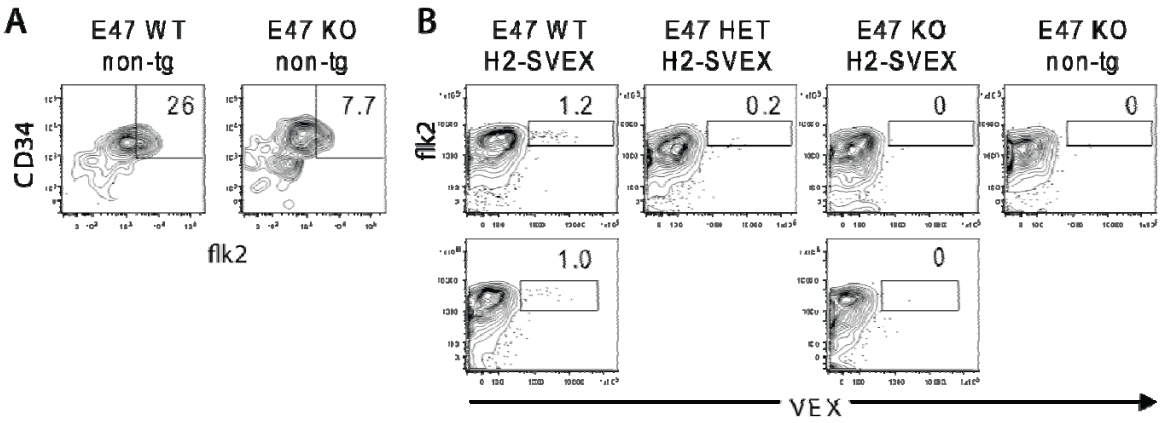

Figure 6: Lymphoid differentiation potential is compromised in E47 deficient MPPs

A) BM LSKs from E47 WT or KO mice were analyzed for the presence of the brightest *flk2*$^{+}$ cells enriched for lymphoid potential. B) E47 KO mice were crossed to the H2-SVEX V(D)J recombination reporter strain in which the VEX variant of green fluorescent protein indicates V(D)J recombinase activity in live cells. non-tg, non-transgenic. The data are representative of five (A) or four (B) independent experiments.

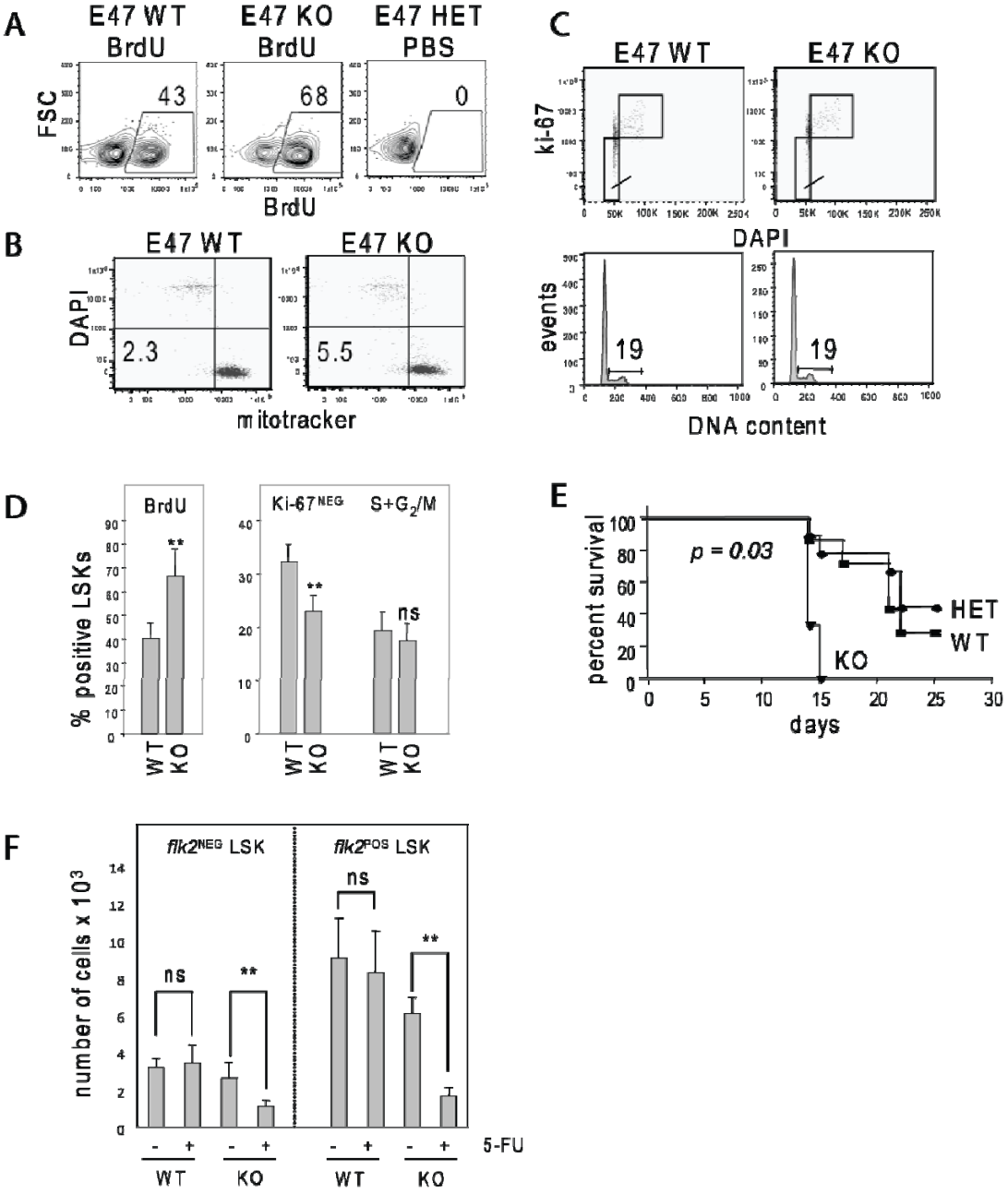

Figure 7: Disruption of LSK quiescence in the absence of E47

A) BM from E47 WT or KO mice treated with BrdU for 48 hours was stained to identify LSKs followed by intracellular staining with anti-BrdU antibodies. Background fluorescence was determined by injecting E47 HET mice with PBS followed by the identical staining procedures.

The data are representative of 3 independent experiments. B & C) Total BM LSKs from E47 WT or KO mice were fixed and stained with antibodies to Ki-67, mitotracker, or DAPI. The percent of cells in the gates is indicated. D) Cumulative data from A-C (3-4 independent mice per group) were analyzed by the Wilcoxon Rank Sum test. $**p < 0.05$; ns, not significant. E) The cell cycle specific drug 5-FU was administered weekly to E47 WT (n=5), HET (n=5) or KO (n=3) mice, and survival outcome examined. The data are depicted as Kaplan-Meier Survival curves. The data are representative of 2 independent experiments. F) BM from E47 WT and KO mice treated with 5-FU or PBS for 10 to 12 hours were stained to identify *flk2/flt3*$^-$ LSKs and *flk2/flt3*$^+$ LSKs. The data are representative of 3-4 pairs of age-matched mice per group.

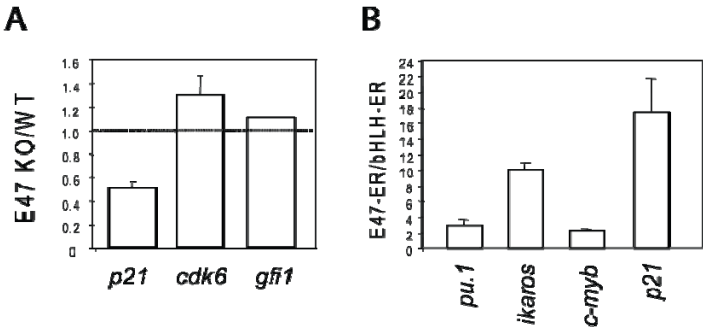

Figure 8: The transcription factor E47 regulates the expression of cell cycle regulator *p21* in LSKs

A) LSKs sorted from E47 WT or KO mice were examined for the expression of *p21*, *cdk6* or *gfi1* by real time RT-PCR. The data are normalized to β-actin. Levels of gene expression are presented as E47 KO/WT ratio. The data represent the mean of four independent analyses from three different sorts (*p21*), three independent analyses from two different sorts (*cdk6*), or two independent analyses from two sorts (*gfi1*); standard deviation is not depicted for *gfi1* since only two data points are available. B) Lin^- BM from E47 HET mice transduced with E47-ER-huCD25 or the control vector bHLH-ER-huCD25 were incubated with 4-OHT to activate E47. Cells were harvested, $huCD25^+$ LSKs were sorted by FACS, and mRNA was isolated for QPCR. The β-actin expression ratio in E47-ER/bHLH-ER was set as 1, and the expression of the indicated genes was then normalized based on actin. The data are representative of 2-4 independent sorts (A) and 3 independent experiments (B).

5.2 SPECIFIC AIM 2 AND SECOND MANUSCRIPT

The following manuscript has been submitted for publication. This study defined a critical cell autonomous role of E47 in regulating the long-term functional potential of HSCs. We found that cell intrinsic E47 is dispensable for the short-term myeloid differentiation, but is required for the long-term differentiation and self-renewal of HSCs.

Cell intrinsic E47 is dispensable for short-term myeloid development, but is required for long-term differentiation potential and self-renewal activity of hematopoietic stem cells

(running title: E47 in HSC integrity)

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5.2.1 Abstract

The immune system is constantly replenished by a rare population of hematopoietic stem cells (HSC) residing in the bone marrow of adult organisms. E-proteins, the widely expressed basic helix-loop-helix (bHLH) transcription factors, appear to contribute to HSC activity, but their specific cell intrinsic functions remain undefined. In contrast to a recent report, we show that E47 is dispensable for the short-term myeloid differentiation of HSCs. In our quantitative assays, E47 deficient progenitors show competent myeloid production *in vitro* and *in vivo* as well as under burden of a pathogen mimic. We also show that long-term myeloid and lymphoid differentiation is compromised due to the progressive loss of the self-renewal potential of HSCs *in vivo*. Compromised self-renewal of E47 null HSCs is associated with over-proliferation and premature exhaustion under replication stress. These observations suggest that cell-intrinsic E47 is dispensable for the short-term myeloid repopulation activity of HSCs and, by contrast, that E47 plays an essential cell-autonomous role in the self-renewal of HSCs by preventing hyperproliferation-associated exhaustion following replication stress.

5.2.2 Introduction

Despite rapid progress in deciphering the cellular and environmental cues involved in HSC self-renewal and repopulation, the specific transcriptional regulators that control the functional integrity of HSCs are still being defined (34, 49). Recent studies implicate E proteins, a family of bHLH transcription factors, in controlling the maintenance and lineage repopulation activities of HSCs [3,4]. The E protein inhibitor Id1, has been shown to modulate the self-renewal and differentiation of long-term HSCs (71). E2A, an essential bHLH transcription factor in immune system development, has also been suggested to contribute to HSC maintenance and early lineage commitment [4-6]. However, the precise roles and mechanisms of E proteins in regulating HSC dynamics including the size of functional HSC pool, the long-term HSC persistence, and the short-term HSC myeloid differentiation, remain unclear. In addition, recent studies conflict on whether E47 promotes myeloid development (131) or prevents myeloid development (132) of uncommitted hematopoietic progenitors.

Little is known about the transcription regulatory pathways that control the size of the long-term HSC pool. While multiple groups uniformly suggested that E47 is required for the development of early hematopoietic progenitors, including the non-self renewing multi-potent progenitors (MPPs) and the downstream lymphoid-myeloid primed MPPs (LMPPs) (83, 131, 133), results from the self-renewing HSC pool are discordant. Two groups found normal numbers of HSCs in E47 knockout (KO) mice, using three independent phenotypic schemes including the flk2$^-$ LSK (lineage$^-$ Sca-1$^+$ c-kit$^+$), CD27$^-$ LSK, and CD150$^+$CD48$^-$ LSK definitions (83, 133). Another group, however, showed a reduction of HSCs in mice lacking E47 using a slightly different phenotypic definition CD150$^+$flk2$^-$ LSK (131). Since none of the above phenotypic schemes defines an entirely pure population of long-term HSCs, the results from all

these studies might reflect the discrepancy between phenotypic HSCs and functional HSCs (89, 134, 135). Indeed, only 1 in 3 $CD150^{+}CD48^{-}$ LSKs has functional long-term HSC (LT-HSC) properties in young mice, and this ratio is even lower in aged mice (15, 89). Another concern is that E47 might directly regulate the expression of specific markers, rendering some of these surface proteins imprecise phenotypic indicators of long-term HSC subsets. Therefore, the precise role of E47 in regulating the size of functional HSC pool remains to be definitively established, and may be best assessed by quantitative *in vivo* limit dilution adoptive transfer assays rather than by simple resolution of phenotypic HSC subsets.

Numerous advances define a requirement for E proteins in hematopoiesis (84). E proteins have been established as essential transcription factors in the lymphoid lineage differentiation (73-75). Conversely, E47 is not required for megakaryocyte/erythroid potential as the clonal frequency of megakaryocytes is virtually identical between wild-type (WT) and E47-deficient progenitors (133). However, the role of E proteins in myeloid lineage development is controversial. A previous report showed that myeloid progenitors are reduced in E2A deficient mice, suggesting that E2A promotes myelopoiesis (131). In contrast, another recent study found that a variant of E47 prevents myeloid lineage differentiation of LMPPs by *in vitro* culture assays (132). A third study showing that mice lacking the Id inhibitor of E47 activity have normal myeloid differentiation (71), suggests that E proteins may be dispensable at least for short-term myeloid activity. These conflicting observations might reflect the different roles of E proteins within hematopoietic progenitors (cell autonomous) versus in the hematopoietic progenitor cell niche (cell non-autonomous). Indeed, both cell autonomous and cell non-autonomous roles for the Id inhibitors of E47 activity are being defined (54, 71). A careful separation of the cell intrinsic versus extrinsic effects of E47, with specific attention to

quantitative differences, is essential for a precise understanding of the specific functions of E proteins in myelopoiesis.

In this study, we performed quantitative *in vivo* and *in vitro* assays to examine the cell intrinsic role of E47 in regulating the functional potential of HSCs including their short-term activation and myeloid differentiation, and the size of the functional HSC pool. We found that E47 deficient bone marrow progenitors showed functional niche engraftment. Unexpected relative to recent findings, E47 null HSCs became effectively activated and demonstrated competent short-term myeloid differentiation potential in response to transplantation stress in wild-type hosts *in vivo* or lipopolysaccharide (LPS) stimulation *in vitro*. However, the long-term repopulation and self-renewal activities of HSCs were severely compromised. Self-renewal defects of E47 null HSCs are cell-intrinsic, and are associated with hyperproliferation and premature exhaustion under transplantation stress. Quantification of this defect revealed a 3-fold reduction in the frequency of functional HSCs in E47 deficient mice by limiting dilution adoptive transfer assays *in vivo*. Together, these observations quantified a cell-intrinsic role of E47 in regulating the long-term self-renewal, but not the short-term activation and myeloid differentiation of hematopoietic stem cells.

5.2.3 Materials and Methods

5.2.3.1 Mice

E47 KO mice generated on an FVB/N background (73) were backcrossed to C57BL/6 mice for eleven generations. CD45.1 C57BL/6 mice were purchased from The Jackson Laboratory. Mice were bred in accordance with Institutional Animal Care and Use Committee policies at the University of Pittsburgh School of Medicine.

5.2.3.2 Flow Cytometry

Hematopoietic progenitors were stained as we have reported. Primary and secondary antibodies (Abs) to murine surface markers were from eBioscience. Primary anti-mouse Abs included Sca-1 FITC or APC or Cy5PE ; c-Kit PE or Cy5PE ; CD3 biotin or Cy5PE; CD4 Cy5PE or PE; CD8 APC or biotin or Cy5PE; CD11b biotin; CD19 biotin or Cy5PE; B220 APC or biotin; NK1.1 biotin; TER-119 biotin; CD135 PE ; CD150 APC; CD48PE; CD135 PE; Gr-1 biotin; CD14 FITC; CD45.1 PE; and CD45.2 FITC. Secondary reagents were streptavidin-Cy7-PE or streptavidin-Pacific Blue. Flow cytometry was performed on a four-laser, twelve-detector LSR II (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

For apoptosis analysis, surface stained cells were incubated with Annexin V (BD Biosciences) and DAPI at room temperature for 15 mins according to the manufacturer's instruction. To detect the level of reactive oxygen species (ROS), the cells were loaded with 10 μM Carboxy-H2DCFDA (Invitrogen) at 37°C for 15 mins before analysis.

5.2.3.3 BrdU Incorporation and Cell Cycle Analysis

BrdU incorporation and cell cycle analysis has been described by us. Briefly, mice were injected *i.p.* with 200 μg BrdU in PBS, or PBS alone as a control, at 12-h intervals. 24 h after the first injection, bone marrow was isolated and cells were stained for surface markers using the anti-BrdU FITC flow kit (BD Biosciences) according to the manufacturer's instructions. Ki-67 intracellular staining and cell cycle analyses were performed as we described (83).

5.2.3.4 Cell Culture

Flk2$^-$ LSKs were sorted as described. Briefly, lineage-marker (CD3, CD19, CD11b, Gr-1, Ter119, NK1.1, B220) negative bone marrow cells were depleted by magnetic automated cell separation (MACS), and stained with surface antibodies to flk2, Sca1, and c-kit. The indicated

populations were doubled sorted by flow cytometry cell sorting (FACS) on a three laser, 11 detector FACS Aria (BD Biosciences).

For the LTC-IC (Long-Term Culture-Initiating Cell) assay, flk2$^-$ LSKs cells were double sorted directly onto S17 stromal cells in 96-well plates (136) at the concentration indicated in the figure legend. After 35 days, cultures overlaid with methocult M3434 (StemCell Technologies) and colony forming potential was assessed after 7 days according to the manufacturer's protocol. For serial replating assays, primary cultures were trypsinized after 10 days and hematopoietic progenitors replated by sorting on fresh S17 stromal cells in 96 well plates for another 25 days before colony forming potential was assessed using methocult.

For *in vitro* colony-forming assay, flk2$^-$ LSKs cells were double sorted directly onto methocult M3434 (StemCell Technologies) at a limit dilution dose (1, 2 and 5 cells per well) in 96 well plates. Colony forming potential was assessed after 7 days by morphology scored according to the manufacturer's protocol.

For serum-free medium culture assays, double sorted flk2$^-$ LSKs were cultured with X-VIVO 15 medium (Biowhittaker) supplemented with 10% BSA (StemCell Technologies) and the following recombinant murine cytokines (Peprotech): 20 ng/ml stem cell factor, 50 ng/ml flk2/flt3 ligand, 10 ng/ml IL-3 and 50 ng/ml thrombopoietin, in the presence or absence of 10 μg/ml LPS (Sigma).

5.2.3.5 Adoptive Transfer Assays

For limiting-dilution competitive repopulation assays, serial dilutions of bone marrow cells from wild-type or E47 knockout littermates on the CD45.2 background were mixed with 2×10^5 CD45.1 wild-type competitor cells and injected into lethally irradiated (10 Gy) CD45.1 recipient mice via the tail vein. Repopulation in the peripheral blood of the recipient mice was

measured at 16 weeks post transplant and competitive reconstitution unit was calculated according to Poisson statistics by L-Calc software (StemCell Technologies).

For serial transplantation, 2×10^6 wild-type or E47 deficient CD45.2 bone marrow cells were injected into lethally irradiated CD45.1 recipient mice. Sixteen weeks post transplantation, 2×10^6 bone marrow cells from primary recipients were serially transferred into lethally irradiated CD45.1 secondary recipients. Multi-lineage reconstitution was examined monthly in the peripheral blood and at 16 weeks post transplant in the spleen of both the primary recipients and the secondary recipients.

For niche engraftment analysis, 2×10^6 wild-type or E47 knockout CD45.2 bone marrow cells were injected into lethally irradiated CD45.1 recipient mice. The recipient mice were sacrificed at 2 weeks post-transplant, and the number of bone marrow $CD45.2^+$ $flk2^-$ LSKs was counted.

5.2.3.6 Statistics

The statistical significance of differences between group means ($p<0.05$) was established using Student's t test. The frequencies of progenitors in limiting dilution assays were calculated according to Poisson statistics by L-Cal software (StemCell Technologies).

5.2.4 Results

5.2.4.1 E47 KO HSCs Have Efficient *In Vitro* Myeloid Differentiation under Pathogen-free Conditions and after LPS stimulation

The precise cell intrinsic role of E47 in regulating the myeloid differentiation potential of HSCs remains unclear, with one study suggesting that E proteins promote myeloid development (131) and other studies suggesting otherwise (71, 132). Not only do HSCs produce myeloid lineage cells in response to colony stimulating factors (CSF) but recent work identifies a CSF-

independent pathway of myeloid differentiation driven by bacterial products (137). Since LPS induces E47 expression in mature lymphocytes (110), we wondered if the potential for E47 to control myelopoiesis would be enhanced in response to this pathogen mimic.

We confirmed the expression of the LPS co-receptor CD14 on E47 WT versus KO flk2$^-$ and flk2$^+$LSKs by flow cytometry. Comparable levels of CD14, in terms of both percentage and mean fluorescence intensity, were detecTable 1n WT and E47 KO flk2$^-$ LSKs, suggesting that E47 is dispensable for surface expression of this LPS co-receptor (**Figure 9A**, *left panel*, and data not shown). In contrast, decreased intensity of CD14 staining is detected in E47 KO flk2$^+$ LSKs as compared to WT flk2$^+$ LSKs (*right panel*), possibly due to the selective loss of the flk2^{brightest} LMPP subset within total flk2$^+$ LSKs in the null mice relative to wild-type [5,6].

Focusing on the HSC enriched flk2$^-$ LSK subset, we examined the requirement for E47 in myeloid differentiation under defined conditions (**Figure 9B & C**). We found that 27.4% $\pm$ 4.13 versus 26.4% $\pm$ 4.49 of CD11b$^+$ cells emerged from E47 KO versus heterozygous (HET) flk2$^-$ LSKs after culture in serum–free medium in the absence of LPS for 3 days, suggesting that E47 KO HSCs have efficient myeloid differentiation under pathogen-free conditions (n= 9 wells, $p<0.05$). This finding is unexpected given that myeloid lineage progenitors as well as total colony forming unit (CFU)-G are reduced 2-fold in E2A germline knockout mice (131). High dose LPS (10 μg/ml) [18] increased the percentage of CD11b$^+$ cells from both E47 KO and E47 HET progenitors to a similar degree. Specifically, 46.3% $\pm$ 6.72 versus 39.6% $\pm$ 4.84 of CD11b cells emerged from E47 KO versus HET flk2$^-$ LSKs in the presence of LPS. While the percentage was similar, the absolute number of CD11b cells emerging from E47 KO flk2$^-$ LSKs was increased relative to WT; the role of E47 in proliferative activity is further characterized in

detail below. Thus, the presence of LPS further increased the numbers of $CD11b^+$ cells, and the magnitude of this effect was proportional across WT and KO progenitors.

To carefully evaluate the myeloid developmental potential of progenitors at the single cell level in the absence of confounding proliferative effects, we performed *in vitro* limit dilution CFU assays (**Figure 9D**). $Flk2^-$ LSKs were seeded in methocult at 1, 2 and 5 cells per well and cultured for 7 days. Since hyperproliferation increases colony size, but not colony number, the results obtained from this assay permit sensitive discrimination between hyperproliferation of E47 KO progenitors and the per-cell differentiation potential. We found that WT and E47 KO $flk2^-$ LSKs have comparable colony-forming activity both in the presence and absence of LPS. Specifically, 39.4% versus 35.4% of WT versus E47 KO $flk2^-$ LSKs gave rise to myeloid colonies in the absence of LPS. LPS stimulation increased the frequency of myeloid colony-forming cells to 50.7% versus 48.5% for WT versus E47 KO $flk2^-$ LSKs, respectively (n=48 wells per group, $p>0.05$). Together, these observations demonstrate that E47 KO HSCs can be efficiently activated for rapid lineage differentiation by LPS, and that E47 does not appear to restrain or promote myeloid potential in this compartment.

However, one previous report found reduced myeloid progenitors in E2A deficient mice (23). Given the recent finding of a role for E proteins both within hematopoietic progenitors and in the bone marrow microenvironment (54), we reasoned that the divergence in these observations might be due to the cell autonomous role of E47 within hematopoietic progenitors versus its cell non-autonomous role in the microenvironment. Resolving this issue requires examination of E47 null HSCs within a wild-type *in vivo* environment.

5.2.4.2 E47 null HSCs Have Competitive Short-term Myeloid Repopulation, but Defective Long-term Repopulation Activities

Within the HSC enriched flk2$^-$ LSK subset, LT-HSCs can be more specifically identified using the CD150 and CD48 SLAM markers (15). We found that the frequencies of these immunophenotypically characterized LT-HSCs (CD150$^+$CD48$^-$ LSKs) are relatively similar: 0.0050% $\pm$ 0.0038 cells versus 0.0049% $\pm$ 0.0034 cells in the bone marrow of WT versus E47 KO mice (n= 6 mice, data not shown). However, whether these HSCs displayed competent short-term and long-term myeloid repopulation activity remains unclear. This is a particularly important point since even the SLAM LSK phenotypic definition does not resolve a pure HSC subset [11].

To definitively assess the cell intrinsic effect of E47 on the myeloid repopulation *in vivo*, we performed competitive adoptive transfer assays in which the developmental potential of E47-deficient progenitors is compared relative to WT progenitors, and measured the short-term and long-term lineage repopulation in WT recipients. For these analyses, we focused on the short-lived Gr-1$^+$ subset since whose presence is a sensitive indicator of ongoing hematopoiesis (136). We found comparable short-term reconstitution of Gr-1$^+$ myeloid cells by E47 null or WT donor bone marrow through 12 weeks post transplantation (**Figure 10A**). Specifically, 40% $\pm$ 15 versus 45% $\pm$ 12 of Gr-1$^+$ cells were CD45.2$^+$ in the peripheral blood of the mice reconstituted by WT versus KO donor cells at 8 weeks post transplant, and 56% $\pm$ 12 versus 41% $\pm$ 18 of Gr-1$^+$ cells were CD45.2$^+$ for WT versus KO donor cells at 12 weeks post transplant, respectively (n=5 mice, $p>0.05$). These data suggest that cell intrinsic E47 is dispensable for short-term myeloid differentiation of E47 null HSCs in the context of a wild-type developmental environment, supporting the *in vitro* findings in **Figure 9**.

However, significantly reduced myeloid reconstitution by E47 null donor cells was observed at long-term points (**Figure 10A**). Hematopoiesis at 16 weeks is thought to reflect the contribution of LT-HSCs as short-lived subsets cannot durably sustain reconstitution at this point (136). At 16 weeks post transplant, 72% $\pm$ 10 versus only 39% $\pm$ 18 of Gr-1^+ cells were CD45.2^+ in the peripheral blood of the mice reconstituted by WT versus KO donor cells, respectively (*left panel*, n=5 recipients per group, $p<0.05$). Likewise, the percentage of CD45.2^+ Gr-1^+ cells in the spleens of the recipient mice decreased from 75% $\pm$ 13 for WT donor cells to 42% $\pm$ 16 for E47 KO donor cells (*right panel*, n=5 recipients per group, $p<0.05$). Not surprisingly given known defects in lymphoid potential, E47 deficient donor bone marrow cells fail to reconstitute detectable T cells or B cells in the presence of an equal number of competitor cells (**Figure 10B**, n=8 recipients, $p<0.05$). Together, these observations suggest that E47 KO HSCs can efficiently mediate rapid short-term myeloid lineage repopulation under transplantation stress, but that long-term repopulation activities fail to persist.

5.2.4.3 E47 null HSCs Display Poor Self-renewal Efficiency *In Vivo*

We performed serial transplantation under non-competitive conditions to examine the cell-autonomous role of E47 in maintaining the self-renewal potential of LT-HSCs. Consistent with our *in vitro* findings, E47 KO bone marrow showed competent myeloid reconstitution in primary WT recipients. Comparable numbers of donor derived Gr-1^+ cells were detected in both the peripheral blood and spleen in mice transplanted with WT or E47 KO donor bone marrow (**Figure 11A**). However, significant repopulation defects were observed in the secondary recipients of E47 null bone marrow. Reconstitution of Gr-1^+ cells derived from CD45.2 E47 deficient donor bone marrow was decreased as early as 8 weeks, and at 16 weeks post transplant, E47 null donor bone marrow showed about a two-fold reduction in both the peripheral blood and

the spleen. Specifically, the number of WT versus KO donor derived CD45.2^{+}Gr-1^{+} cells was 1,114 $\pm$ 196 versus 619 $\pm$ 293 per μl in the peripheral blood, and 10,858 $\pm$ 2,087 versus 6,108 $\pm$ 1,922 per spleen (n=6 to 8 mice, $p<0.05$). The observation that myeloid repopulation is grossly normal in the primary recipients but significantly reduced in the secondary recipients suggests progressive depletion of HSC repopulation activity caused by diminished self-renewal. Had the E47 defect been restricted to short-lived HSCs or MPPs, then the magnitude of reconstitution would be similar across primary and secondary recipients, which is not the case.

Similar progressive loss of repopulation activity was observed for T lymphoid reconstitution (**Figure 11B**). T cell reconstitution by E47 deficient bone marrow was reduced by 5 fold in the peripheral blood and 3 fold in the spleen of primary recipients, and a much more severe defect of E47 KO donor bone marrow was observed in secondary recipients (a 9-fold decrease in peripheral blood and 10-fold decrease in the spleen; n=6 to 8 mice, $p<0.05$). That E47 deficient bone marrow showed progressive loss in both myeloid and T lymphoid reconstitution in secondary recipients indicated severe defects in the long-term *in vivo* self-renewal of HSCs. This finding contrasts with the apparent integrity of myeloid production in the short term.

5.2.4.4 Mechanisms Underlying HSC Exhaustion

We performed a series of *in vivo* assays to examine the mechanisms underlying the functional defects of E47 KO HSCs. First we examined whether E47 KO HSCs can mediate functional niche engraftment, the critical step subsequent to bone marrow homing. In this assay, we measured the number of donor derived flk2^{-} LSKs engrafted two weeks after adoptive transfer of CD45.2 WT or E47 KO bone marrow cells into CD45.1 hosts. Comparable numbers of CD45.2^{+} flk2^{-} LSKs (3616 $\pm$ 623 versus 4604 $\pm$ 639) were detected in the bone marrow of

mice reconstituted by WT or E47 KO bone marrow cells (**Figure 12A,** *left panel,* n=3 mice, p>0.05). Therefore, E47 KO HSCs do not have detectable defects in niche engraftment. In contrast to short time points, at 16 weeks post transplant, the number of $CD45.2^{+}$ $flk2^{-}$ decreased from 4184 $\pm$ 2174 in mice reconstituted by WT donor cells to 2174 $\pm$ 818 in those reconstituted by E47 KO donor cells (**Figure 12A**, *right panel,* n=8 mice, p<0.05). The reduction of E47 KO HSCs at later time points suggests premature exhaustion under transplantation stress.

HSC exhaustion can be associated with poor survival or abnormal energy metabolism [21-23]. However, we found similar frequencies of apoptosis, 20.3% $\pm$ 2.5 versus 17.9% $\pm$ 5.8 for the E47 KO or WT derived $flk2^{-}$ LSKs isolated either directly *ex vivo* from donor animals (data not shown) or at 12 weeks post transplant, a time point at the onset of discordance between WT and KO persistence (**Figure 713B**, *top row,* n=3 mice, p>0.05). Also, the mean fluorescence intensity of ROS in E47 KO or WT derived $flk2^{-}$ LSKs was comparable at 12 weeks post transplant, 285 $\pm$ 13 versus 234 $\pm$ 43, suggesting normal ROS metabolism of E47 KO HSCs even following adoptive transfer stress (*bottom row,* n=3 mice, p>0.05). Therefore, neither apoptosis nor abnormal energy metabolism overtly contribute to E47 KO HSC exhaustion.

We have previously shown that E47- deficient total LSKs exhibit a 50% reduction in p21 transcript (83). Consistent with our previous findings, transcripts of the cell cycle inhibitor *p21*were reduced 3 fold in E47-deficient $flk2^{-}$ LSKs as compared to WT $flk2^{-}$ LSKs (**Figure 14A,** n=4 independent sorts, p<0.05). Indeed, the HSCs in *p21* KO mice also displayed severe long-term self-renewal defects that are strikingly similar to the defects of E47 null HSCs [24]. Thus *p21* might play an important role in linking the cell cycle kinetics abnormalities and functional defects of E47 null HSCs. We then assessed the cell cycle status of E47 null versus WT HSC enriched $flk2^{-}$ LSKs at steady-state homeostasis. While $flk2^{-}$ LSKs were numerically comparable

in WT versus E47 null mice (**Figure 14B**), E47-deficient flk2$^-$ LSKs showed hyperproliferation as indicated by a loss of G_0 quiescent cells and increased percentage of the actively cycling cells (**Figure 14C**). Specifically, the proportion of Ki67 negative quiescent cells was 70.3% $\pm$ 3.0 versus 56.0% $\pm$ 3.2 in WT and E47 KO flk2$^-$ LSKs, respectively (n=3 mice, $p<0.05$). Conversely, the proportion of cells in the S+G_2+M phases increased from 10.1% $\pm$ 1.1 to 15.1% $\pm$ 4.3 in these WT versus KO flk2$^-$ LSKs (n=3 mice, $p<0.05$). Thus, E47 restricts the proliferation of HSCs at steady-state homeostasis, a result consistent with our previous findings.

The magnitude of proliferative defects was even more severe following adoptive transfer stress. Three weeks after adoptive transfer of E47 KO and WT donor bone marrow cells into wild type recipients, E47 null flk2$^-$ LSKs exhibited strikingly greater proliferation relative to WT (**Figure 14D**). Cells residing in G_0 were decreased from 31.7% $\pm$ 5.8 for WT donor derived flk2$^-$ LSKs to 18.2% $\pm$ 7.2 for E47 KO donor derived flk2$^-$ LSKs (*left panel*, n = 3 mice, $p<0.05$). Reciprocally, the proportion of cells in S+G_2+M phases increased from 18.3% $\pm$ 6.8 for donor derived WT flk2$^-$ LSKs to 27% $\pm$ 2.0 for donor derived E47 KO flk2$^-$ LSKs in these mice (**Figure 14D***, middle panel*). Furthermore, the BrdU incorporation increased from 47% $\pm$ 17 for WT flk2$^-$ LSKs to 71.6% $\pm$ 6.5 for donor derived E47 KO flk2$^-$ LSKs (*right panel*, n=3 mice, $p < 0.05$). These observations suggest that cell-intrinsic E47 regulates the proper cell cycle activation of HSCs under transplantation stresses. Thus, not only do E47 null progenitors exhibit hyperproliferation in the context of an E47 KO background (**Figure 14C**) but also in the context of a wild type background (**Figure 14D)**, suggesting cell-autonomous requirements.

5.2.4.5 Quantitative Analysis of LT-HSCs Defects in E47 Deficient Mice

A quantitative study of the defects in E47 deficient long-term HSCs in the context of wild type developmental environment is essential for a clear understanding of cell-autonomous

requirement for E47. We first examined the role of E47 in regulating the long-term colony-initiating potential of HSCs *in vitro*. Double sorted flk2$^-$ LSKs were cultured at limiting-dilution doses for 35 days, a time frame that is selected for cells with long-term hematopoietic reconstitution potential, after which functional differentiation was assessed using methocult (136). We found a 2-fold reduction in colony forming cells in the absence of E47. As shown in **Figure 15A** (*left panel*), the frequency of colony forming cells decreased from 1 in 58 of WT flk2$^-$ LSKs to 1 in 126 of E47 KO flk2$^-$ LSKs ($p<0.05$). Serial long-term culture assays were then performed to measure the frequency of progenitors capable of serial repopulation activity. The frequency of colony forming cells that survived serial re-plating was reduced 3 fold from 1 in 618 versus 1 in 2,355 of WT versus E47 KO flk2$^-$ LSK, respectively (*right panel*, $p<0.05$). Thus, E47 deficient HSCs showed severe cell-autonomous defects in colony forming activities and the magnitude of these defects are progressive during serial passage.

To directly quantify the cell intrinsic role of E47 in regulating the size of functional long-term HSCs *in vivo*, we performed limiting dilution adoptive transfer assays in which 3-fold serial dilutions of test donor bone marrow are competed against a constant number of competitor bone marrow cells. Blood reconstitution analyses at 16 weeks post transplantation, a time point reflecting the long-term repopulation activity of HSCs, revealed a 3-fold reduction in the frequency of long-term repopulating cells derived from the bone marrow of E47 deficient mice as compared to wild type mice (**Figure 15B**). Specifically, the frequency of functional long-term HSCs from WT and KO mice was 1/10,947 and 1/27,203, respectively (**Table 2**, n=8 recipient mice/group, $p<0.05$). That the donor cells were functioning in the context of WT environments demonstrates specific cell intrinsic defects of E47 null donor HSCs.

5.2.5 Discussion

In this study, we quantified the cell-intrinsic requirement for the transcription factor E47 in the functional integrity of HSC differentiation and persistence. E47 null HSCs showed competent short-term myeloid differentiation, but compromised long-term multi-lineage repopulation activity in both *in vivo* quantitative and qualitative adoptive transfer assays and in defined cultures *in vitro*. We also found severe defects in the long-term self-renewal capabilities of E47 KO HSCs associated with cell-autonomous hyperproliferation under replication stress. No obvious effects on niche engraftment, apoptosis or metabolic disruption were detectable. That myeloid development remains appreciably intact despite egregious disruption in each lymphoid potential, self-renewal, and proliferative activity helps to pinpoint the specific biological activities of HSCs for which cell-intrinsic E47 is required.

Previous studies have established that E47, cell-intrinsic or otherwise, is dispensable for restriction to the megakaryocyte/erythroid (MEP) lineages (133) and is required for lymphoid lineage potential (98). Absent from this developmental cascade is a clear understanding of myeloid lineage potential. While the germline loss of E47 inhibits myelopoiesis (131), suggesting that E47 is required for myeloid potential, mice deficient in the E protein inhibitor Id1 exhibit normal short-term myeloid activity (71). Moreover, forced expression of a variant form of E47 restricts myeloid development of LSKs (132), rendering the role of E47 unclear. Here, we carefully show that E47-deficient progenitors have competent short-term myeloid potential *in vivo* and also under two *in vitro* conditions, pathogen free circumstances or under the stress of a pathogen mimic. These results indicate that E47 is dispensable for myeloid restriction of flk2$^-$ LSKs, and that previously observed defects in myeloid output (131) can be attributed to a basic loss of HSC integrity over time rather than a specific defect in myeloid potential *per se*. Rather,

our studies are consistent with findings in Id1 knockout mice that short-term myelopoiesis is unaffected by perturbed E protein activities while long-term myelopoiesis is compromised due to HSC failure (71).

We identified a striking hyperproliferation of donor-derived E47 deficient HSCs under transplantation stress. Cell cycle regulation has been established as an important mechanism governing the long-term self-renewal potential of HSCs (34). For instance, in mice lacking the key cell cycle inhibitors p21 or p16, hyperproliferation caused an increase in the number of phenotypic HSCs under steady-state circumstances, but the repopulation activities of HSCs were gradually exhausted during serial transplantation (36, 134). In the E47 deficient mice, we and others found normal numbers of phenotypic HSCs under steady-state homeostasis (83, 133), but a 3-fold reduction in the frequency of functional HSCs by competitive adoptive transfer assays (**Figure 13B, 14B,** and **Table 2**). As E47 deficient progenitors show efficient engraftment two weeks after adoptive transfer (**Figure 12A,** *left panel*), these findings cannot be explained by a failure to productively lodge in the bone marrow. Rather, this divergence is likely due to exhaustion of E47 KO HSCs under replication stress in the transplantation condition. Indeed, the number of $flk2^-$ LSKs derived from E47 KO donor cells was reduced by 2–fold at 16 weeks after transplantation as compared to WT donor derived $flk2^-$ LSKs (**Figure 12A,** *right panel)*, indicating HSC exhaustion under transplantation stress in the context of a wild type environment. Similar exhaustion of HSCs was observed after administration of mitotoxic drug 5-FU (83). Together, these observations suggest that E47 may prevent the premature exhaustion of HSCs under replication stress, possibly by acting on cell cycle regulation.

An important consideration is that the divergence between the number of phenotypic HSCs in steady state and functional HSCs by adoptive transfer experiments might also be influenced by a loss of true LT-HSCs in the immunophenotypically characterized progenitor populations in E47 KO mice. Similar reduced engraftment efficiency per phenotypic HSC has been observed in aged mice. The frequency of long-term repopulating cells within the highly purified $CD150^{+}CD48^{-}$ LSKs decreased from about 0.5 in the young mice to less than 0.16 in the old mice [10]. This reduced engraftment activity per phenotypic HSC in old mice is more severe using other phenotypic schemes such as $Lin^{-}Thy\text{-}1^{low}Sca1^{+}c\text{-}Kit^{+}$ (135). Indeed, established literature showed that E47 expression is significantly decreased in other hematopoietic subsets, such as B cell progenitors, in old mice (110, 138). Thus, the relationship between loss of E47 and stem cell ageing warrants further investigation.

Together, our results quantify the cell-intrinsic requirement for the transcription factor E47 in the functional integrity of bone marrow LT-HSCs. We show that E47 maintains the size of a functionally robust HSC pool and prevents the premature exhaustion of long-term self-renewal of HSCs under replication stress. We also show that E47 is dispensable for short-term rapid lineage differentiation under transplantation stress or after LPS challenge. Not only does premature exhaustion of HSCs significantly reduce the efficiency of long-term hematopoiesis in response to hematopoietic injury, microbial challenge, ageing and other replication stresses, but also hyperproliferation and constant activation of HSCs under these persistent stresses might result in accumulation of mutations and provide the molecular and cellular basis for tumorigenesis. Indeed, disruption of E2A or E47 activity is associated with the cancers of both lymphoid and myeloid lineages in humans and mice (10-12, 80). Thus, further investigations on

the role of E47 in HSC replication and transformation may provide new insights into tissue damage repair, ageing and cancer development.

5.2.6 Acknowledgements

This work is supported by NIH R01 AI079047 (Borghesi). We thank Dewayne Falkner for excellent cell sorting, and Christine Milcarek and Kathee Martincic for helpful advice. We gratefully acknowledge critical comments from Micke Sigvardsson, Lisa Denzin and Kim Payne.

5.2.7 Authorship Contribution

QY performed all figures except 6A which was performed by LB. QY and LB designed experiments and wrote the manuscript. BE contributed key ideas to the development of this work. All authors read and approved the final paper.

5.2.8 Tables and Figures

Table 2: Limiting dilution competitive reconstitution unit

Donor Genotype	Cell dose	Negative recipients (%) 8 mice/group	CRU (95% CI)
E47 WT	200,000	0	1/10974 (1/14923, 1/7807)
	66,667	0	
	22,222	12.5	
	7778	50	
E47 KO	200,000	0	1/27203 (1/36769, 1/20125)
	66,667	12.5	
	22,222	37.5	
	7778	75	

The indicated doses of E47 WT or KO donor bone marrow cells were mixed with a constant dose (200,000 cells) of CD45.1 competitor WT bone marrow cells, and transplanted into lethally irradiated CD45.1 recipient WT recipient mice. Blood reconstitution was examined at 16 weeks after adoptive transfer and, the threshold for positive engraftment was set as 1%. 95% confidence interval (CI) is indicated.

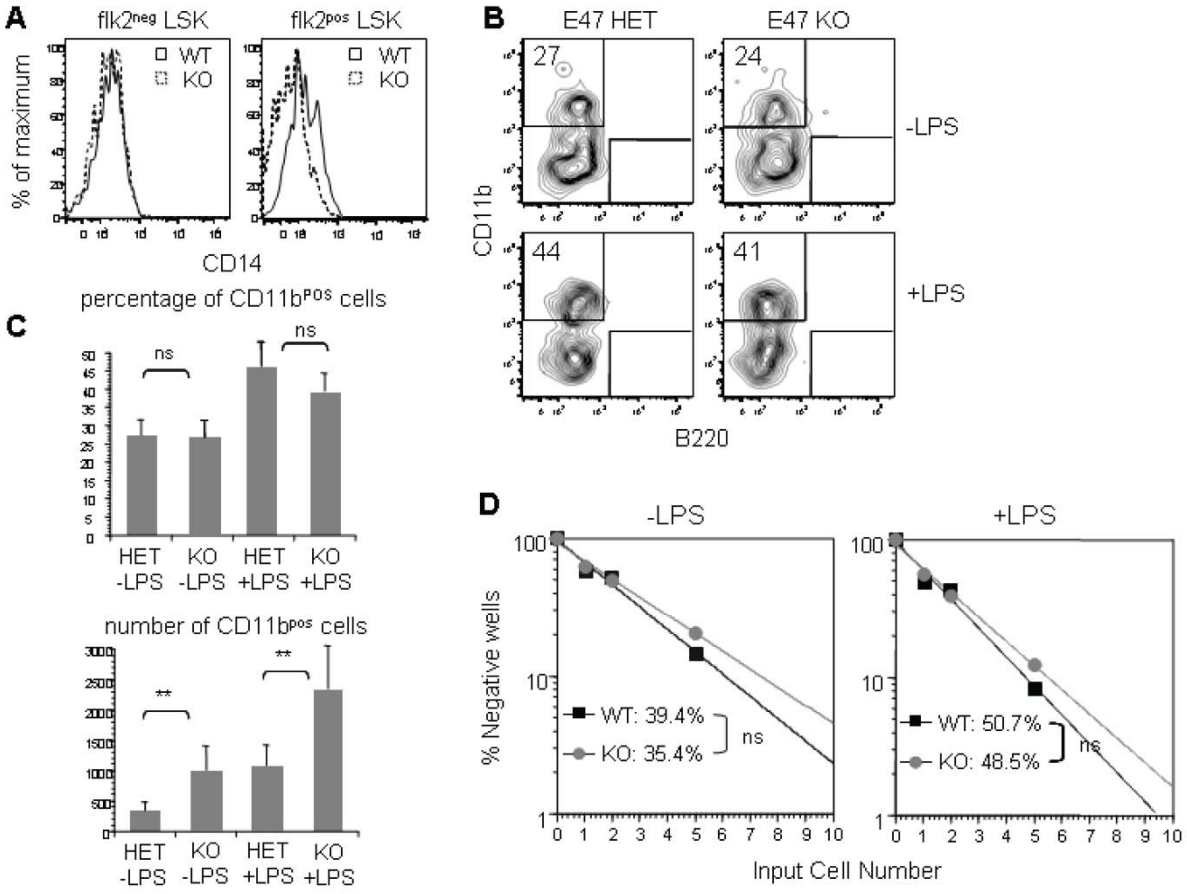

Figure 9. E47 deficient HSCs show efficient myeloid differentiation under pathogen free conditions and after stimulation with a pathogen mimic

(A) The expression of CD14 on flk2$^-$ LSKs and flk2$^+$ LSKs from WT and KO littermates was examined by flow cytometry analysis. The data are representative of two independent experiments. (B & C) Flk2$^-$ LSKs from E47 HET or KO mice were cultured at 300 cells per well in 96-well plates for 72 hours in the presence or absence of 10 μg/ml LPS. Cells were harvested, counted and stained with lineage specific antibodies. The number and frequency of CD11b$^+$ myeloid cells were measured. n=9 wells. (**$p<0.05$, ns = not significant). The data are representative of two independent experiments. (D) Flk2$^-$ LSKs from E47 HET or KO mice were

cultured in methocult at 1, 2 and 5 cells per well in 96-well plates for 7 days in the presence or absence of 10 μg/ml LPS. Wells with CFU-G, M colonies were scored positive. The frequency of colony forming cells was calculated according to Poisson statistics. n=48 wells per group. The data are representative of two independent experiments.

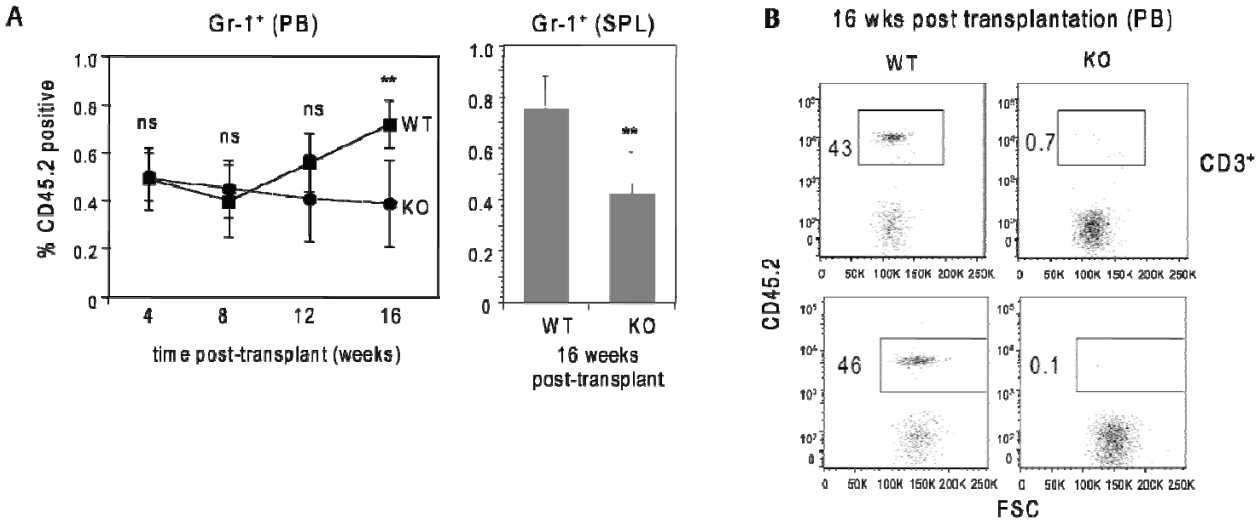

Figure 10. E47 deficient bone marrow cells display compromised long-term competitive repopulation activity *in vivo*

(A) CD45.2 WT and E47 KO bone marrow cells (2 x 10^5) mixed with an equal number of CD45.1 competitor cells were adoptively transferred into lethally irradiated CD45.1 recipient mice. The proportion of test donor derived ($CD45.2^+$) Gr-1^+cells was monitored every 4 weeks in the peripheral blood of the recipient mice. The recipients were sacrificed at 16 weeks post transplant and myeloid lineage repopulation in the spleen was measured. n=5 mice ($p<0.05$). The data are representative of two independent experiments. (B) Competitive lymphoid repopulation activity of WT and E47 KO bone marrow cells was measured at 1:1 test donor : competitor ratio. The percentage of test donor derived ($CD45.2^+$) lymphoid cells in the peripheral blood of the recipient mice at 16 weeks post transplant is shown. n=8 mice. (PB: peripheral blood; SPL: spleen).

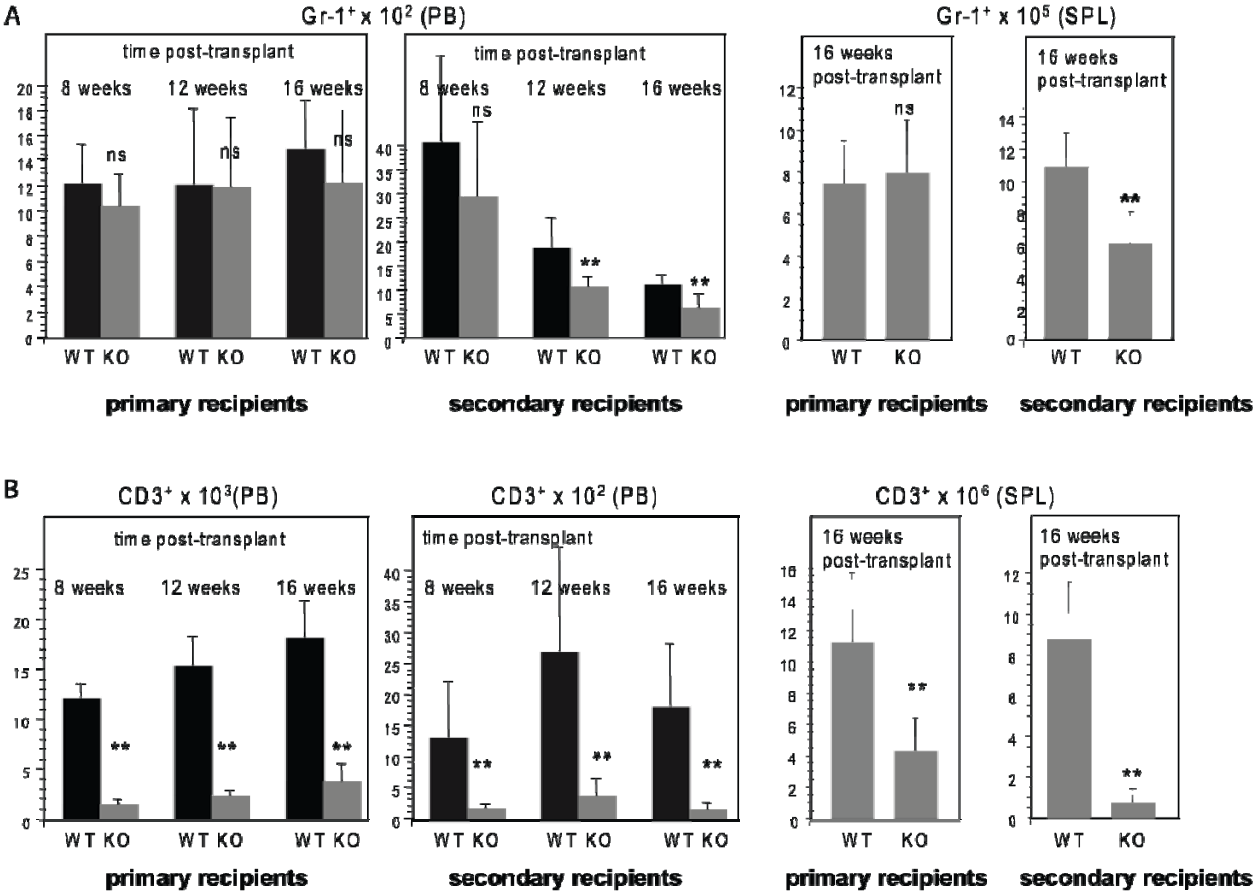

Figure 11. E47 deficient HSCs display poor self-renewal efficiency *in vivo*

Serial transplantation was performed to examine the long-term self-renewal efficiency of HSCs from WT and E47 KO mice. The bone marrow cells from WT and E47 KO littermates were first adoptively transferred into primary recipients for 16 weeks, and then serially transferred into secondary recipients. Blood reconstitution was measured monthly. Recipients were sacrificed at 16 weeks post transplant and lineage reconstitution in the spleen was examined. Myeloid or lymphoid reconstitution efficiency was indicated by the number of donor derived $CD45.2^+$ $Gr\text{-}1^+$ cells (A) or $CD3^+$ cells (B), respectively. n=6 to 8 mice per group (**$p<0.05$).

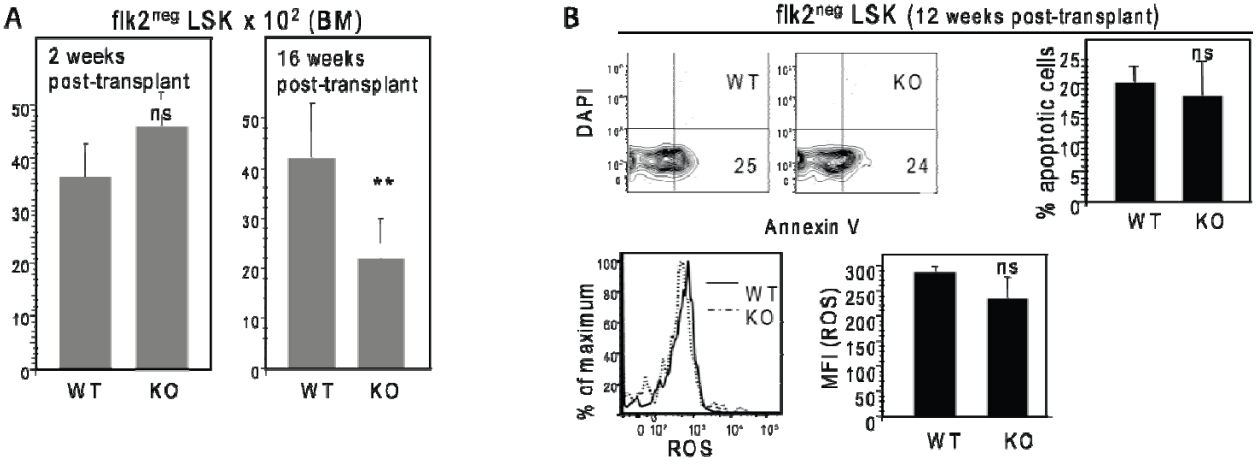

Figure 12. E47 null HSCs show normal homing, niche engraftment, apoptosis rate and ROS metabolism

(A) CD45.2 WT or E47 KO bone marrow cells (2 x 10^6) were injected into lethally irradiated CD45.1 recipient mice. The number of donor derived flk2$^-$ LSKs was examined at 2 weeks and at 16 weeks post transplant. n=3 mice for 2 weeks post transplant, n=8 mice for 16 weeks post transplant. (B) Lethally irradiated CD45.1 recipient mice were adoptively transferred with E47 KO or WT CD45.2 donor bone marrow cells, and sacrificed at 12 weeks post transplant. The bone marrow cells from these recipients were stained with antibodies to resolve donor derived CD45.2 flk2$^-$ LSKs, and then labeled with Annexin V and DAPI for apoptosis analysis, or with H_2DCFDA to measure the level of reactive oxygen species. n=3 mice. (**$p<0.05$, ns = not significant)

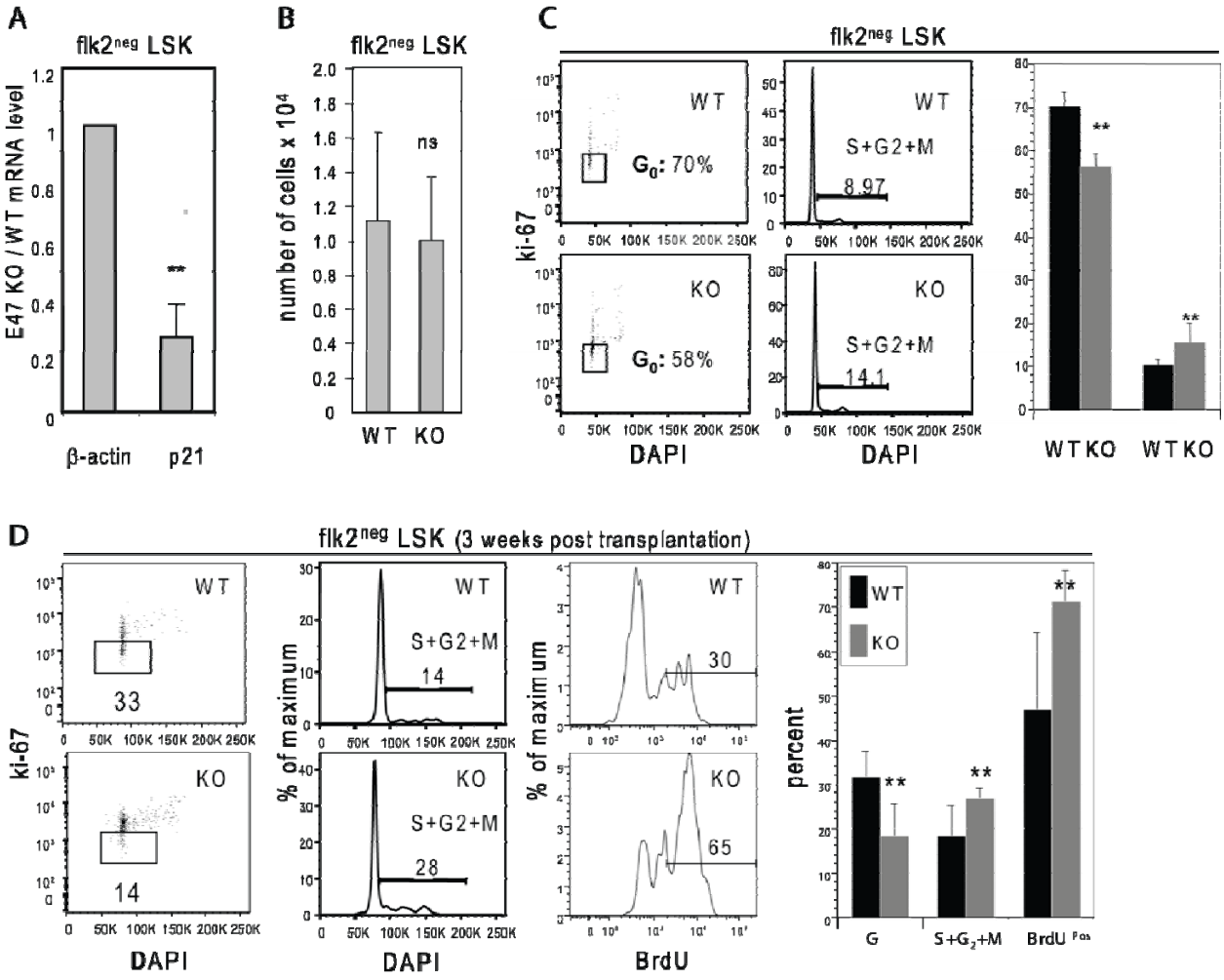

Figure 13. E47 null HSCs displayed hyperproliferation under steady state and following transplantation stress

(A) Flk2$^-$ LSKs from WT and E47 KO littermates were sorted by flow cytometry, and the expression of *p21* and *β-actin* was examined by quantitative reverse-transcription PCR. The data are presented as KO/WT ratios for each transcript. n=4 independent sorts (**$p < 0.05$). (B) Bone marrow cells from WT and E47 KO littermates were stained with cell surface antibodies to resolve HSC enriched flk2$^-$ LSKs, and the number of flk2$^-$ LSKs was counted. n=11 mice. (C) Surface stained flk2$^-$ LSKs from E47 WT or KO mice were fixed and then stained with

antibodies to the Ki67 proliferation antigen and DAPI for cell cycle analysis. n=3-4 mice (**p < 0.05). (D) Lethally irradiated mice reconstituted with E47 KO or WT CD45.2 donor bone marrow cells were sacrificed at 3 weeks post transplant. 100 µg BrdU was injected into recipient mice at 12-hour interval for 24 hours before sacrifice. The donor derived CD45.2 flk2$^-$ LSKs were fixed and stained with antibodies to BrdU, Ki67, or DAPI for proliferation and cell cycle analysis. n=4 mice. (**p<0.05, ns = not significant)

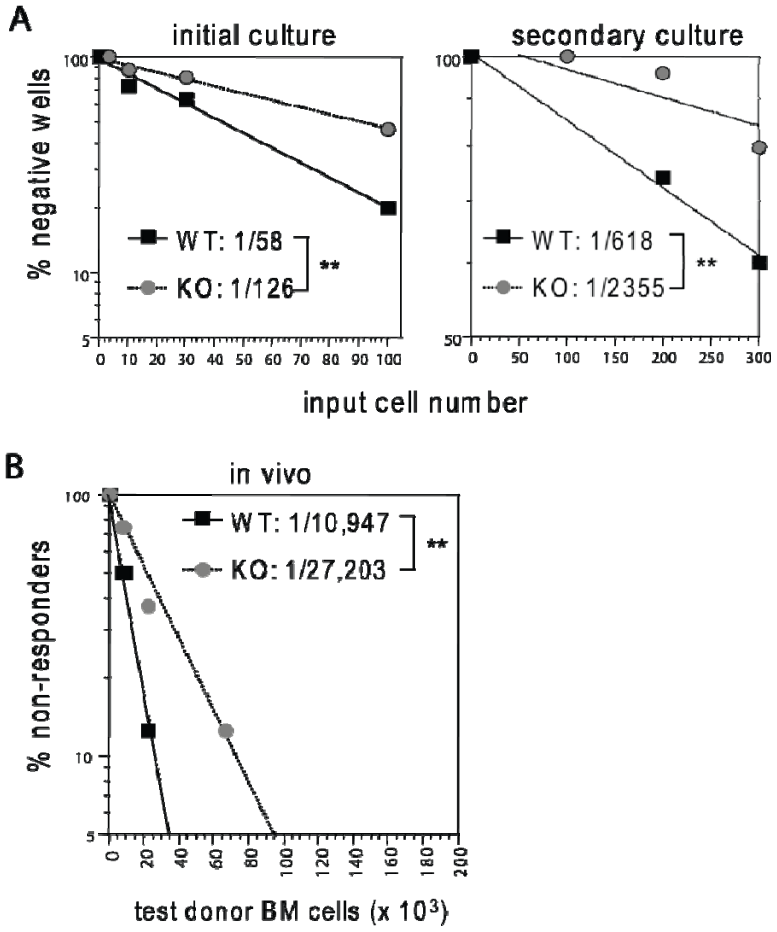

Figure 14. Quantitative analysis of LT-HSC defects in E47 deficient mice

(A) LTC-IC assay was performed with double sorted flk2$^-$ LSKs from WT and E47 KO littermates to determine the frequency of long-term colony forming cells. Plotted is the percentage of wells that did not give rise to colonies after a single plating (left panel) or serial-replating (right panel) at the indicated input cell numbers. The frequency of long-term colony forming cells was calculated according to Poisson statistics. (B) Limit dilution doses (0.67×10^5, 0.22×10^5, 0.073×10^5) of CD45.2 WT and E47 KO bone marrow cells mixed with a constant number (2×10^5) of CD45.1 competitor cells were adoptively transferred into lethally irradiated CD45.1$^+$ recipient mice. The graph depicts the percentage of CD45.1 recipient mice that has less

than 1% of donor CD45.2^{+} Gr-1^{+} cells at 16 weeks following adoptive transfer of the indicated donor cell numbers. The frequency of functional HSCs was calculated using Poisson statistics. Eight recipient mice were used at each cell dose per genotype. (**$p<0.05$).

6.0 SUMMARY AND CONCLUSION

In this study, we investigated the role of transcription factor E47 in the earliest, multipotent stages of hematopoiesis. We found that E47 is required for the proper development of MPPs, and for the long-term self-renewal and muti-lineage repopulation activity of HSCs. Our observations also suggested that E47 prevents the cell cycle hyper-activation and premature exhaustion of these early progenitors under hematopoietic stress. Finally, we identified the cell cycle inhibitor and HSC regulator p21 as a key target of E47 in the mutilpotent HSC and MPP subsets. Not only may diminished self-renewal of the primitive bone marrow progenitors result in inefficient hematopoietic regeneration in responses to ageing and hematopoietic injury, but also unchecked cell cycle activation might provide the cellular and molecular basis for malignant transformation (92). By linking the transcription factor E47 with the development and functional potential of the primitive bone marrow stem/progenitor cells, our study may provide new insights into the transcriptional gene regulatory networks underlying early hematopoiesis and how they go awry in hematopoietic malignancies.

In this project, we have not only defined the specific roles of E47 in regulating the integrity of early hematopoietic progenitors *in vivo,* but also have investigated the cellular and molecular mechanisms using a number of molecular biology approaches. Using loss of function and gain of function assays, we found that E47 regulates the expression of the key cell cycle inhibitor p21. Indeed, the HSC defect of E47 deficient mice is strikingly similar to that of *p21* KO mice (36, 83). The HSCs in both p21 deficient and E47 deficient mice displayed severely

compromised long-term self-renewal potential as indicated by progressive loss of multi-lineage repopulation activity. Thus, we hypothesize that E47 might regulate the long-term self-renewal activity of HSCs through effect on p21. Our ongoing experiments are aimed at establishing a causal relationship between E47 and p21. Specifically, we will compare the HSCs from the $E47^{+/-}$ $p21^{+/-}$ compound heterozygous mice with those from the $E47^{+/-}$ or $p21^{+/-}$ heterozygous mice to determine whether E47 and p21 function in the same pathway. We expect that the cell cycle and self-renewal defects of the $E47^{+/-}$ $p21^{+/-}$ HSCs will be close to $E47^{+/-}$ HSCs, and will be more severe than the $p21^{+/-}$ HSCs. The results obtained from this experiment will suggest whether p21 acts as a downstream target of the E47 pathways in regulating HSC integrity. Furthermore, we have also found severe defects in the lymphoid differentiation of HSCs and MPPs in E47 deficient mice, which cannot be explained by p21 deficiency. Therefore, E47 most likely acts through multiple pathways to regulate the self-renewal, development and differentiation of early hematopoietic progenitors. The specific experiments to identify other major molecular targets of E47 and our expectation will be detailed later in the "Future Direction" section.

Proper hematopoietic development requires not only coordinated actions of cell intrinsic regulators, but also an intact bone marrow microenvironment (49-52). It is thus important to carefully discriminate the cell intrinsic versus cell non-autonomous role of E47 in early hematopoiesis. By adoptively transferring the E47 deficient HSCs into wild-type recipients, we identified a remarkable cell intrinsic role for E47 in regulating the long-term functional potential of HSCs. However, whether E47 also contributes to the integrity of the bone marrow niche remains to be determined. Indeed, E47 has been found to regulate the proliferation and differentiation of osteoblasts, the main type of cells forming the bone marrow osteoblastic niche

for HSCs (79). Also, both cell autonomous and cell nonautonomous role for the E47 inhibitor, ID1, in early hematopoiesis have been defined (71). Further studies, such as reverse adoptive transfer, will be helpful in determining whether E47 plays a direct role in regulating the cell non-autonomous HSC niches. Specifically, in the reverse adoptive transfer experiments, the wild type donor HSCs will be transplanted into the E47 deficient recipient mice. The development of the WT HSCs in the bone marrow microenvironment of the E47 deficient mice will thus reveal whether E47 plays a cell nonautonmous role in regulating the HSC activity.

Together, our study highlighted a critical cell intrinsic role for E47 in regulating the development and functional integrity of multipotent hematopoietic stem cells and progenitors. Our observations also suggest that E47 prevents hyperproliferation and premature exhaustion of primitive bone marrow progenitors, possibly through effects on p21. Given the important role for E47 in hematopoietic development and malignant transformation, further studies to decipher the underlying molecular mechanisms might improve our understanding and/or treatment of hematopoietic damage repair, ageing and cancer development.

7.0 IMPICATIONS FOR FUTURE RESEARCH

The observations from this thesis project revealed a critical role for E47 in regulating the long-tem self-renewal and multi-lineage differentiation of HSCs and MPPs. The findings from this project have also opened up several important questions for future research. Two unanswered major questions are the specific underlying mechanisms of E47 activity, and the other major roles for E47 in early hematopoiesis. These gaps might be filled by future studies that focus on: 1) the specific downstream molecules and pathways through which E47 regulates the integrity of HSCs and MPPs? 2) whether and how E47 contributes to the transition from MPPs to downstream lymphoid progenitors?

First of all, my observations identified p21 as a potential target of E47 in multipotent HSCs and MPPs, yet other key downstream targets of E47 remain to be deciphered. A high-throughput gene expression assay, such as a genome-wide transcriptome micro-array analysis, will be very helpful in identifying the specific targets of E47 in the primitive multipotent stem/progenitor cells. The genes that we should prioritize are those involved in hematopoietic differentiation, cell cycle regulation and stem cell function, because my results in this thesis have suggested a critical role for E47 in regulating the lineage differentiation, cell cycle quiescence, and long-term self-renewal of HSCs and MPPs. For example, it will be very worth examining the expression of the key transcription factors in early lymphoid differentiation such as Ikaros and PU.1 (125, 139), the critical cell cycle regulator such as p16 and p53 (92, 134), and the HSC niche regulators such as Notch receptors and WNT signaling molecules (52, 53). Notably, E47

has been linked to notch expression (65), and a direct relationship between these molecules in HSCs has not been examined.

Another major unanswered question is whether and how E47 regulates the transition from MPPs to downstream lymphoid progenitors. Despite a 2-3 fold reduction of MPPs in E47 deficient mice, these mice demonstrated a even more severe defects in the downstream lymphoid progenitors (10 fold reduction in CLPs and 5 fold reduction in ETPs), suggesting a disruption in the transition from MPPs to these downstream lymphoid progenitors. Yet how E47 contributes to the early lymphoid differentiation of MPPs remains unclear. My results in this thesis showed that forced expression E47 induced the expression of Ikaros, another transcription factor that is required for the development of CLPs and downstream B progenitors. Thus, we hypothesize that E47 might regulates the transition from MPPs to CLPs through effects on Ikaros. To test this hypothesis, we can examine whether enforced expression of Ikaros in MPPs by retrovirus transduction can partially rescue the development of CLPs *in vivo* and *in vitro*. This study might provide new clues in deciphering the transcription regulatory network in early lymphopoiesis. Another important observation is that E47 deficient MPPs lack CCR9 expression (133), the key molecule that is critical for the migration of MPPs to the thymus where the progenitors begin to seed the T cell compartments (25). Thus, E47 might regulate the early T cell development partially through effects on the settling of extrathymic progenitors into the thymus, the initial step of thymic T cell development. Direct intrathymic injection of E47 deficient MPPs might be helpful in determining whether E47 contributes to this earliest step of T cell development. We will expect that the defects in T cell progenitors could be partially alleviated by direct intrathymic injection of MPPs, suggesting a role for E47 in the settling of extrathymic

progenitors into thymus. These ideas will form a basic for a mechanistic understanding of how E47 influences the early lymphoid differentiation of HSCs and MPPs.

Finally, since the majority of the experiments in this study are performed using mouse models, future studies to extend these findings in human beings might be extremely helpful. For example, we can use *in vitro* assays to compare the functional activity of the $CD34^{+}$ hematopoietic stem/progenitor cells between healthy volunteers and the patients with germline mutations of the TCF3 gene, such as TCF3 haplo-insufficiency or TCF3 involved chromosomal translocations. We would expect that HSCs with these TCF3 mutations display abnormal cell cycle kinetics and defects in long-term multi-lineage differentiation potential, which renders them susceptible to malignant transformation by secondary damage during development. Together, it is of great public health importance to study the molecular mechanisms that regulate the self-renewal and differentiation of stem cells in normal hematopoiesis and in blood malignancies, and our observations on the role of E47 in early hematopoiesis might yield new insights into the development of stem cells and how they become aberrant in human diseases.

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