GENETIC DISSECTION OF SELF-RENEWAL PATHWAYS DURING HEMATOPOIETIC DIFFERENTIATION

by

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ABSTRACT

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Hematopoietic stem cells (HSCs) differentiate into mature blood cells; while maintaining themselves for the lifetime of the organism because of their ability to self-renew. *Bmi1* is required for the maintenance of HSCs and lymphocytes. While examining the deletion of Bmi1 targets in *Bmi1$^{-/-}$* mice, we found that *p16^{Ink4a}* deletion did not alleviate defects in HSCs and lymphocytes, whereas *p19^{Arf}* deletion partially restored lymphocytes but not HSCs. Deletion of both *p16^{Ink4a}* and *p19^{Arf}* partially restored HSC functions. *Trp53* deletion also partially rescued the lymphocytes and HSCs. However, none of these deletions completely restored maintenance of HSCs and lymphocytes. We conclude that the targets of Bmi1 differentially maintain subsets of hematopoietic cells. In HSCs these results suggest that Bmi1 might not require the direct action of p19^{Arf} for

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regulation of Trp53, and could possibly directly regulate Trp53 turnover or mark Trp53 transcriptional targets for epigenetic silencing.

We went on to examine the impact of deleting $p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}/Trp53$ on hematopoietic cells. There were no defects in the output of mature cells, suggesting that these deletions do not compromise differentiation. We show that bone marrow cells from mice deficient in three genes downstream of Bmi1 - $p16^{Ink4a}$, $p19^{Arf}$, and $Trp53$ (triple mutant mice)- have an approximately 10 fold increase in cells able to reconstitute hematopoiesis. This increase is associated with the acquisition of long-term reconstitution capacity by cells whose phenotype is c-kit^{+}Sca-1^{+}Flt3^{+}CD150^{-}CD48^{-}Lineage^{-}, which defines multipotent progenitors (MPPs) in wild-type mice. The pattern of triple mutant MPP response to growth factors resembles that of wild-type MPPs but not wild-type HSCs. MPPs lack the ability to self-renew and are destined to stop proliferating after a finite number of cell divisions. The molecular mechanisms that limit the proliferation capacity of MPPs and more mature progenitors are not understood. These results demonstrate that Ink4a/Arf and Trp53 play a central role in limiting the expansion potential of MPPs. This has implications for oncogenesis due to the fact that these pathways are commonly repressed in cancer, suggesting a mechanism by which early progenitor cells could gain the ability to self-renew and become malignant with further oncogenic mutations.

CHAPTER ONE

Introduction

The advent of multicellularity coupled with a requirement for cell proliferation over the life span of an organism in order to maintain tissue homeostasis, has probably driven the evolution of a stem cell compartment that is separate for the bulk of terminally differentiated cells within tissues. With an increase in cell number and tissue complexity, these organisms had to develop specialized cell types and restrict life-long proliferation to a subset of cells within tissues. A stem cell can be pluripotent, capable of generating all three germ layers, as seen in the case of embryonic stem cells and germ cells. Multipotent stem cells on the other hand are lineage specific and operate as the driving force of tissue specific regeneration in adults. Stem cells achieve their longevity and persist due to their capacity to self-renew. Self-renewal is a mitotic division where one or both daughter cells retains the undifferentiated stem cell identity as well as the replication and differentiation potential of the parent cell (Clarke and Fuller, 2006; Kondo et al., 2003; Shizuru et al., 2005; Weissman and Shizuru, 2008).

In the hematopoietic system, hematopoietic stem cells (HSCs) are the source of continual regeneration, and are the only cells capable of generating the full spectrum of differentiated cells found in hematopoietic tissues; they persist by self-renewal divisions for the lifetime of the animal, thereby maintaining hematopoiesis (Figure 1.1). The

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existence of a self-renewing multipotent progenitor in the bone marrow was proven in a series of experiments by Till and McCulloch (Till and McCulloch, 1961; Till and McCulloch 1963; Weissman and Shizuru, 2008) and this subsequently led to attempts to isolate them. The development of antibodies against cell surface markers found on blood cells and technologies allowing for efficient cell separation led to the isolation of HSCs based on immunophenotypic markers and permitted the testing of their function in transplantation and *in vitro* assays (Weissman and Shizuru, 2008). This enabled the detailed characterization of the hematopoietic hierarchy (Figure 1.1) (Weissman and Shizuru, 2008).

One of the central goals in stem cell biology is the understanding of the mechanisms regulating self-renewal. Chapter 2 of this thesis is a published review of the pathways that play a role in regulating HSC self-renewal (Akala and Clarke 2006). In the hematopoietic system, the ability to prospectively isolate relatively pure populations of HSCs enables gene expression analysis and the transplantation of genetically modified HSCs allows for the study of pathways that are required for their maintenance. Our lab has studied the mechanisms regulating self-renewal by analysis of the differential gene expression profile of adult murine HSCs (Park et al., 2002). In this study we found a difference in the expression of a number of transcripts, including *Bmi1*, *Notch1, BMP4,* and *Mcl1* between HSCs and their more differentiated progeny. Subsequently *Mcl1* and *Bmi1* have been conclusively determined to be required for HSC survival and self-renewal (Opferman et al., 2005; Park et al., 2003).

The differential expression of *Bmi1* between HSCs and their mature progeny led to the examination of the phenotype of $Bmi1^{-/-}$ mice. These mice had a progressive

decrease in the number of hematopoietic cells as they matured, as well as neurological deficits expressed by an ataxic gait and posterior skeletal transformation (Park et al., 2003;van der Lugt et al., 1994). $Bmi1^{-/-}$ mice are normal at birth and then develop a hypocellular bone marrow as they mature and eventually die about 2 months after birth (Park et al., 2003; van der Lugt et al., 1994). As the progressiveness of the hematopoietic defect suggested a potential defect in the maintenance of hematopoiesis efforts were focused on characterizing HSC function in $Bmi1^{-/-}$ mice. This analyses led to our discovery that $Bmi1$ is selectively required for the maintenance of adult, but not fetal HSCs; thereby explaining the progressive nature of the phenotype where Bmi1 is not required for the ontogeny of HSCs, but for the maintenance of HSCs in adult mice after the transition from fetal to adult hematopoiesis.

In the bone marrow of $Bmi1^{-/-}$ mice, $p16^{Ink4a}$ and $p19^{Arf}$, products of the alternatively spliced $Ink4a/Arf$ locus, were upregulated, as well as the Trp53 induced gene, $Wig1$ (Park et al., 2003). The overexpression of $p16^{Ink4a}$ in HSCs in $vitro$ led to their senescence, while the overexpression of $p19^{Arf}$ resulted in Trp53 dependent apoptosis (Park et al., 2003). These results suggest that the activation of these pathways in HSCs is detrimental to their maintenance. Bmi1 represses the $Ink4a/Arf$ locus (Itahana et al., 2003; Jacobs et al., 1999), and the deletion of the $Ink4a/Arf$ locus in a $Bmi1$ deficient background can rescue the ability of embryonic fibroblast to proliferate (Jacobs et al., 1999). In chapter 3 of this thesis, published in the supplementary section of Akala et al., 2008, we have attempted to carefully analyze the role of $p16^{Ink4a}$, $p19^{Arf}$, the $Ink4a/Arf$ locus and $Trp53$ in HSCs. We also examined the role of these pathways on lymphocyte maintenance since pathways regulating HSC self-renewal such as Bmi1, also

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play a role in the maintenance of long lived lymphocyte populations (Park et al., 2003).We discovered that unlike neural stem cells, the deletion of either $p16^{Ink4a}$ or $p19^{Arf}$ alone was not capable of rescuing the HSC defects of *Bmi1* null mice. However, $p19^{Arf}$ deletion in *Bmi1* null mice, but not that of $p16^{Ink4a}$, partially rescued the lymphocytic defects of *Bmi1* null mice. A partial rescue of HSC function required the deletion of the entire *Ink4a/Arf* locus. The most striking discovery was that deletion of *Trp53* also resulted in a partial rescue of HSC function in *Bmi1* null mice. This was surprising because we expected the results of the deletion of *Trp53* in *Bmi1* null mice on HSC function to be identical to that of $p19^{Arf}$ deletion in *Bmi1* null mice, due to previous results that showed that the effects of $p19^{Arf}$ on HSCs require the activation of Trp53 (Park et al., 2003). Our recent results suggest that Bmi1 targets differentially maintain hematopoietic cells and that Bmi1 targets Trp53 for HSC maintenance in a $p19^{Arf}$-dependent and independent manner.

In chapter 4 of this thesis, published in Akala et al., 2008, we sought to characterize the role that the deletion of $p19^{Arf}$, *Trp53*, and the entire *Ink4a/Arf* locus played in the maintenance of hematopoiesis. We discovered that in young mice, there was no appreciably impact of these deletions. Others have confirmed our results that these deletions have more of an effect in the context of stem cell aging (Dumble et al., 2007; Janzen et al., 2006; Stepanova and Sorrentino, 2005). Due to the fact that a prior study in the lab showed that there are multiple genetic determinants that regulate the frequency of HSCs and their restricted progeny (Morrison et al., 2002), we decided to study the impact of the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, and *Trp53* (triple mutant) on hematopoiesis. We discovered an approximately 10 fold increase in the frequency of

triple mutant bone marrow cells capable of long-term hematopoietic reconstitution of lethally irradiated recipient mice. This increase is associated with the acquisition of long-term reconstitution capacity by early multipotent progenitors (MPPs) immediately downstream of HSCs. However, in more differentiated myeloid progenitors, the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, and $Trp53$ did not affect their longevity. With these studies we have uncovered a mechanism that limits the proliferative capacity and self-renewal of early progenitors in the hematopoietic system. The inability to remove self-renewal constraints on more mature progenitors indicates that with progressive differentiation the controls restricting self-renewal increase (Figure 1.2) (Clarke and Fuller, 2006). These results have implications for oncogenic transformation because this expansion of long-lived cells potentially reveals a mechanism of how these genes or their pathways that are frequently mutated in cancer contribute to oncogenesis (Berggren et al., 2003; Burke et al., 2005; Esteller et al., 2000; Weber et al., 2002; Zolota et al., 2007). These aberrantly expanded progenitor populations can become a fertile ground for more mutations that then lead to full malignant transformation.
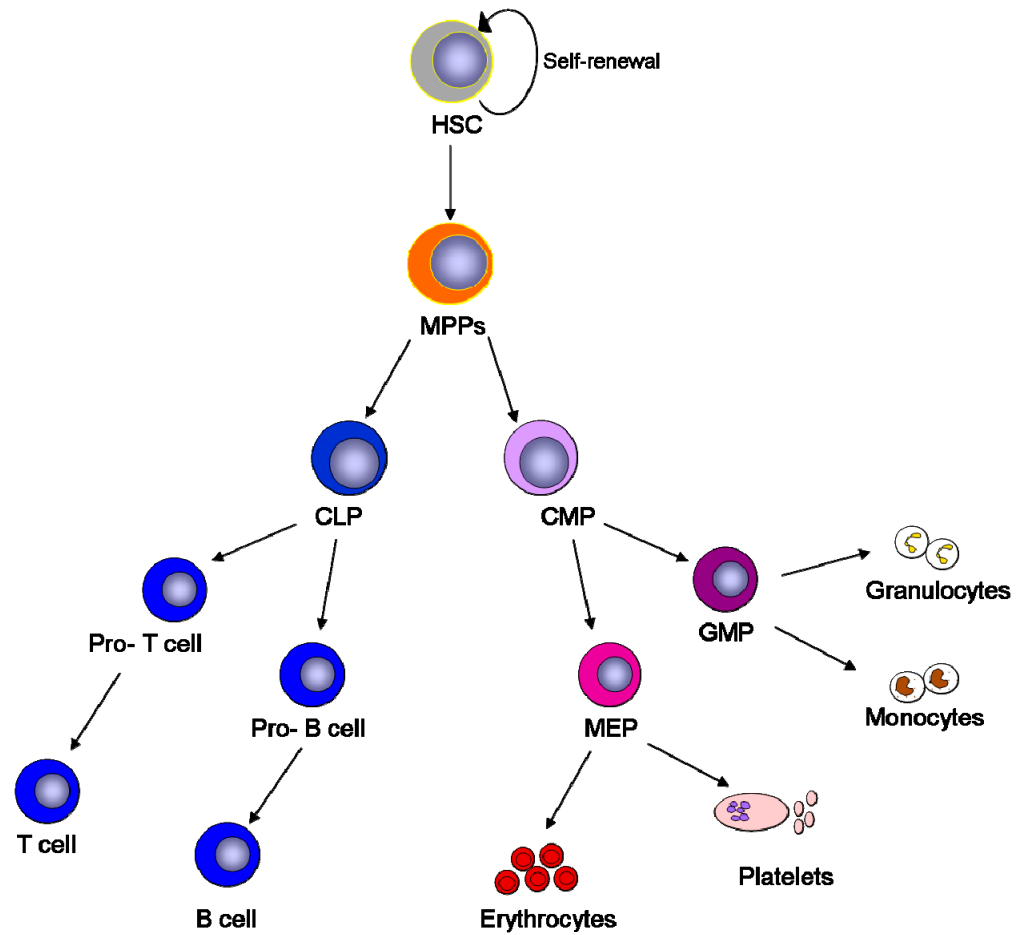
Figure 1.1 Normal hematopoiesis. The hematopoietic stem cell sits at the apex of the hematopoietic system, self-renewing in order to continually regenerate the full spectrum of mature blood cells.

HSCs: Hematopoietic Stem Cells MPPs: Multipotent Progenitors
CMP: Common Myeloid Progenitors CLP: Common Lymphoid Progenitors
GMP: Granulocyte Monocyte Progenitors MEP: Megakaryocyte-Erythroid Progenitors
Pro-B cell: Progenitor B cells Pro-T cell: Progenitor T cells
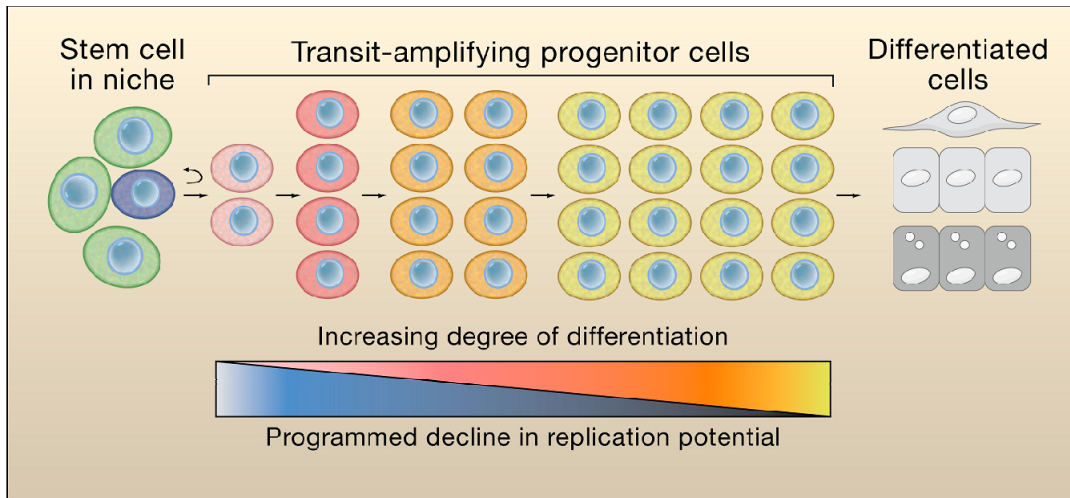
Courtesy of Cell Press, Clarke et al., 2006. Cell Volume #124, pages 1111-1115

Figure 1.2 Loss of progenitor replicative potential with progressing differentiation from the stem cell. The differentiation from the stem cell to early progenitors, to more mature progenitors, and eventually mature terminally differentiated cells, is accompanied by increasing restrictions that limit regenerative capacity. In the hematopoietic system we observe a progressive restriction in regenerative capacity as differentiation proceeds from hematopoietic stem cells to short lived multipotent Progenitors, to even more short lived myeloid progenitors.

CHAPTER TWO

Hematopoietic Stem Cell Self-renewal

ABSTRACT

Recent studies have begun to elucidate the mechanisms controlling hematopoietic stem cell (HSC) self-renewal. Self-renewal requires the integration of survival signals and proliferation controls with the maintenance of an undifferentiated state. This demands a complex crosstalk between extrinsic signals from the microenvironment with the cell intrinsic regulators of self-renewal. The Polycomb protein, Bmi1, is absolutely required for the maintenance of both adult HSCs and neural stem cells. Evidence from studies in murine and human embryonic stem (ES) cells, point to the dynamic role of polycomb group proteins, in concert with master transcriptional regulators, in actively maintaining an undifferentiated state; similar mechanisms may apply to multiple types of stem cells. Recently, new regulators of HSC maintenance have been identified such as MCL-1, Tel/Etv6, Gfi-1, PTEN, and STAT5. In order to better understand HSC self-renewal, we need to comprehend how these pathways are coordinated.

INTRODUCTION

Hematopoiesis is a tightly regulated process in which a rare pool of hematopoietic stem cells (HSCs) gives rise to the lymphohematopoietic system consisting of red blood cells, platelets, granulocytes, macrophages, B- and T-lymphocytes. In order to maintain hematopoietic homeostasis throughout the lifetime of the animal, this pool of HSCs must be maintained. This is achieved by the process of self-renewal, a specialized cell division where one or both of the daughter cells remain undifferentiated and retain the replication potential of the parent. One of the central goals in stem cell biology is to understand the mechanisms that regulate self-renewal. While stem cells from different organs may vary in their developmental potential; all stem cells must self-renew and regulate the relative balance between stem cell maintenance and regeneration of the mature cells of an organ.

While the phenotypic and functional properties of HSCs have been extensively characterized, we have just begun to understand how self-renewal is regulated. Recent research has begun to shed light on the different pathways involved in this process. In order for a stem cell to renew it must survive and at the same time produce both undifferentiated and differentiated progeny. Therefore the self-renewal program must include genes that regulate these processes and have the capacity to integrate them effectively. This review will touch on some of the pathways that are known to be involved in regulating HSC regeneration (Figure 2.1).

CELL INTRINSIC HSC SELF-RENEWAL PATHWAYS

Anti-apoptotic proteins

Stem cell maintenance requires that proliferation pathways remain functional while differentiation, senescence and cell death pathways are repressed. There is a large body of evidence that suppression of apoptosis is required for HSC survival. Evidence of this is directly shown in studies using transgenic mice constitutively expressing *BCL-2* in all hematopoietic tissues. The forced expression of the oncogene *BCL-2* results in increased numbers of transgenic HSCs *in vivo* and gave them a competitive edge over wild-type HSCs in competitive reconstitution experiments (Domen et al., 2000; Domen et al., 1998), suggesting that cell death plays a role in regulating the homeostasis of HSCs. Recently another anti-apoptotic BCL-2 family member, MCL-1 has been shown to be required for HSC survival. (Opferman et al., 2005).

Transcription Factors

The transcription factor Tel/Etv6, an Ets related-transcriptional repressor is also required for HSC maintenance. Conditional inactivation of *Tel/Etv6* in HSCs rapidly leads to the depletion of *Tel/Etv6*-deficient bone marrow. However, Tel/Etv6 is not required for the maintenance of committed progenitors and when it is conditionally inactivated in most hematopoietic lineages it does not affect their differentiation or survival (Hock et al., 2004b). At the moment the mechanism by which Tel/Etv6 regulates adult HSC renewal is not known. Study of the downstream targets it represses should shed light on other players essential for HSC maintenance.

The homeobox (*Hox*) genes encode transcription factors that regulate embryonic body patterning and organogenesis. They also play a role in both stem cell maintenance and hematopoietic differentiation. Overexpression of *HoxB4* in bone marrow leads to expansion of HSCs *in vivo* and *in vitro*, therefore appearing to be a positive regulator of

HSC self-renewal (Antonchuk et al., 2002b; Krosl et al., 2003; Miyake et al., 2006; Sauvageau et al., 1995). It therefore came as a surprise when *HoxB4*-deficient mice had normal hematopoietic development but exhibited only mild proliferative HSC defects (Brun et al., 2004). In an attempt to determine if this was due to compensatory mechanisms the entire HoxB cluster was deleted, however this did not lead to major defects in hematopoiesis (Bijl et al., 2006), possibly due to compensation by HoxA4 and/or HoxC4.

Gfi-1, a Zinc-finger repressor, has been recently implicated as a new regulator of HSC self-renewal. Two groups independently determined that Gfi-1 controls self-renewal of HSCs by restraining their proliferative potential (Hock et al., 2004a; Zeng et al., 2004). They showed that *Gfi-1*-deficient HSCs display increased proliferation rates, but are functionally compromised in competitive repopulation and serial transplantation assays. Gfi-1 may exert it effects on HSC proliferation by regulation of the cell cycle inhibitor $p21^{CIP1/WAF1}$. *p21* mRNA expression levels are dramatically lower in the *Gfi-1*-deficient HSCs (Hock et al., 2004a). $p21^{CIP1/WAF1}$ itself has been implicated in the regulation of HSCs and in its absence HSCs have an impaired serial transplantation capacity(Cheng et al., 2000). Another cell cycle inhibitor, $p18^{INK4C}$ has also been shown to affect HSC self-renewal. The absence of $p18^{INK4C}$ leads to increased HSC self-renewal (Yu et al., 2006; Yuan et al., 2004). Therefore, intricate control of the cell cycle and proliferation machinery is required for self-renewal regulation.

The JAK–STAT pathway is a common downstream pathway of cytokine signaling that promotes hematopoiesis. Constitutive activation of the transcription factors of the STAT (signal transducer and activator of transcription) family, particularly STAT3

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and STAT5 are frequently detected in leukemias, lymphomas and solid tumors (Yu and Jove, 2004). In order to evaluate their role in HSCs, constitutively active STAT mutants were used to activate signaling in HSCs. Activation of STAT5 in HSCs led to dramatic expansion of multipotent progenitors and promoted HSC self-renewal *ex vivo* (Kato et al., 2005). Deletion of STAT5 resulted in profound defects in hematopoiesis and markedly reduced ability of the mutant cells to repopulate the bone marrow of lethally irradiated mice (Snow et al., 2002). In a mouse model of myeloproliferative disease (MPD), sustained STAT5 activation in HSCs induced fatal MPD, suggesting that the capacity of STAT5 to promote self-renewal of hematopoietic stem cells is crucial to MPD development (Kato et al., 2005). Another group showed that transduction of adult mouse bone marrow cells with a constitutively activated form of STAT3 increased their regenerative activity in lethally irradiated recipients, while the transduction of these cells with a dominant negative form of STAT3 suppressed their regenerative activity (Chung et al., 2006). These studies suggest that STAT proteins play a role in HSC self-renewal and potentially in other tissues owing to the wide range of solid tissue and blood malignancies that harbor constitutively activated STATs.

Signal Transducers

The PTEN tumor suppressor, a modulator of several major signaling pathways has recently been implicated as a regulator of HSC self-renewal and an initiator of leukemogenesis (Kim et al., 2006; Zhang et al., 2006b). It functions by inhibiting signaling through the AKT pathway. Although *Pten* deletion initially leads to a transient expansion in HSCs numbers, the HSCs were depleted over time. *Pten*-deficient HSCs engraft normally in recipient mice, but are unable to sustain multi-lineage hematopoietic

reconstitution. When *Pten*-deficient HSCs were transplanted into irradiated mice, they were only capable of short-term multi-lineage hematopoietic reconstitution and could not stably engraft irradiated recipients long-term (Yilmaz et al., 2006b; Zhang et al., 2006b). The exact mechanisms by which PTEN exerts its effects are not known.

Polycomb group Proteins

Recent studies have also shown the importance of Polycomb pathways and their interaction in regulation of HSC self-renewal and lineage restriction. In particular, members of PRC1 (Polycomb Repression Complex 1) such as *Bmi1*, *Mel-18*, and *Rae28* have been implicated. *Bmi1* plays an important role in regulating the proliferative activity of stem and progenitor cells. It is required for the self-renewal of both adult HSCs and neural stem cells (Molofsky et al., 2003; Park et al., 2003). Bmi1 enhances symmetrical expansion of the stem cell pool through self-renewal, induces a marked *ex vivo* expansion of MPPs, and increases the ability of HSCs to repopulate bone marrow *in vivo* (Iwama et al., 2004). Leukemic cells lacking *Bmi1* undergo proliferation arrest, differentiation, and apoptosis leading to the lack of maintenance of leukemia in a mouse transplant model (Lessard and Sauvageau, 2003). In *Bmi1*-deficient bone marrow, there is an upregulation of cell cycle inhibitors $p16^{Ink4A}$, $p19^{ARF}$, and the Trp53 induced gene *Wig1*, with a downregulation of apoptosis inhibitor *AI-6*. This suggests a mechanism where Bmi1 functions by modulating proliferation and preventing apoptosis (Park et al., 2004) (Figure 2.2). Bmi1 has also been shown to regulate the expression of *Hox* genes which are required for differentiation during hematopoiesis (Park et al., 2003; van der Lugt NM, 1996).

Loss or knockdown of another Polycomb gene, *Mel-18*, leads to increased expression of *Hoxb4* (Kajiume et al., 2004), *Mel-18*-deficient bone marrow had an increase in overall HSC numbers but when transplanted HSCS showed a decrease in their activity due to arrest in G_0 phase of the cell cycle. *Rae28*-deficient HSCs were defective in their long-term repopulating ability in serial transplantation experiments (Kim et al., 2004c; Ohta et al., 2002). Taken together, these studies show the importance of the *Polycomb* genes in HSC self-renewal and maintenance of the blood system.

Transcriptional repression by Polycomb group (PcG) proteins is essential for maintenance of HSC identity. We demonstrated that Bmi1, part of the Polycomb Repressive Complex 1 (PRC1), is necessary for self-renewal of adult HSCs and subsequently also the self-renewal of neural stem cells. Part of the mechanism by which it functions is by repression of genes that promote lineage specification, cell death, and cell cycle arrest (Molofsky et al., 2003; Park et al., 2003). More recently, PcG complexes have been shown to be essential for maintenance of the undifferentiated state in murine embryonic stem (ES) cells and human ES cells by directly repressing a large number of developmental regulators (Boyer et al., 2006; Lee et al., 2006). Young and colleagues showed that the Nanog, Oct4 and Sox2 transcription factors, essential for ES cells to maintain pluripotency, bind many of the same target genes (Boyer et al., 2005; Boyer et al., 2006; Lee et al., 2006). PcG complexes bind to and presumably repress the expression of a subset of these genes linked to differentiation (Boyer et al., 2006; Lee et al., 2006).This represents a dynamic repression of genes required for differentiation and presents a scenario where PcG proteins act as transcription repressors by cooperating with a specific set of transcription factors in stem cells. Some target genes include

members of the *Hox* family which are important for induction of differentiation.

Expression of *Hox* genes, which are involved in differentiation, are repressed in the ES

cells by Polycomb (Boyer et al., 2006; Lee et al., 2006). Thus, PcG complex repression is

also obligate for ES cell identity. Taken together, these studies suggest that differentiation

is the default state during stem cell self-renewal requires active repression of transcription

factors that prevent self-renewal.

CELL EXTRINSIC REGULATORS OF HSC RENEWAL

It has been proposed the HSCs occupy a specific microenvironment, the niche,

composed of the support cells surrounding the HSCs, signals from these support cells and

the extracellular matrix play a role sustaining them in the bone marrow (Li and Xie,

2005). Genetic studies have demonstrated that osteoblasts are a crucial cellular element

of the HSC niche in the bone marrow (Calvi et al., 2003; Zhang et al., 2003).Work by

Arai et al. uncovered mechanisms where the fate of HSCs is controlled by signals from

components of the niche (Arai et al., 2004). In the study the authors find that in the bone

marrow,Tie2 expressing HSCs reside in the niche in association with Ang-1 expressing

osteoblast and that Tie2/Ang-1 signaling promotes HSC quiescence in the niche, thereby

preserving self-renewal capability (Arai et al., 2004). This provides functional evidence

for the role of the niche in regulation of HSC self-renewal.

The Notch pathway is a highly conserved signal transduction pathway that

functions as a master regulator of cell fate in a wide range of developmental processes

and systems. In a recent study using a transgenic Notch reporter mouse, Notch signaling

was shown to be highly active in HSCs and downregulated as they differentiate *in vitro*

and only a small fraction of mature cells had Notch reporter activity *in vivo* (Duncan et al., 2005). Inhibition of Notch signaling in adult HSCs, also led to accelerated differentiation *in vitro* and depletion of HSCs *in vivo*. This suggests that Notch signaling is important for HSC maintenance of an undifferentiated state and ability to undergo self-renewing divisions (Duncan et al., 2005). Enhanced activation of *Notch1* (Stier et al., 2002) or *Notch 4* (Int3) (Ye et al., 2004) contributed to increased HSC self-renewal and consequent differentiation inhibition providing further evidence for the role of Notch signaling in HSCs. Interestingly, *Notch1*-deficient HSCs were able to reconstitute mice with inactivated *Jagged-1*, a ligand of the Notch receptor, in the BM stroma even under competitive conditions, excluding an essential role for Jagged-1-mediated Notch signaling during hematopoiesis (Mancini et al., 2005). Work by Mancini et al. indicates that Jagged1-mediated Notch1 signaling is dispensable for both self-renewal and differentiation of HSCs. A possible explanation for the contradictory results of these groups is probably due to the redundancy in the mammalian Notch pathway; where there are four different Notch receptors that can bind five different ligands.

Like the above mentioned Notch pathway, the Wnt pathway has also been implicated in the regulation of HSC self-renewal. The Wnt pathway is a signaling cascade that acts during embryonic development as well as in the adult, directing events such as cell proliferation and differentiation. It has been shown that purified Wnt3A can promote self-renewal of HSCs in hematopoietic reconstitution experiments (Willert et al., 2003). Another group observed that there is LEF-1/TCF reporter activity in HSCs transplanted into lethally irradiated mice, suggesting that they respond to Wnt signaling *in vivo* (Reya et al., 2003) The same group performed retroviral overexpression studies

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suggesting that ß-catenin plays a key role in HSC self-renewal. Overexpression of a dominant active form of ß-catenin in HSCs led to enhanced self-renewal capacity *in vitro* with preservation of stem cell markers. HSCs expressing this construct also had increased reconstitution potential *in vivo* compared with control HSCs (Reya et al., 2003). In contrast, HSCs transduced with *Axin*, which negatively regulates ß-catenin by enhancing its degradation, had reduced growth potential *in vitro* as well as a significant reduction in the ability to reconstitute hematopoeisis in irradiated mice (Reya et al., 2003). The reciprocal outcome of these experiments led the authors to suggest that ß-catenin mediated Wnt signaling is important in HSC self-renewal. However, the results of another group shows that mice in which ß-catenin has been conditionally deleted via Cre-loxP mediated excision, reveal no hematolymphoid defects (Cobas et al., 2004), thereby suggesting that the Wnt signalling effects in HSCs might be modulated via alternative pathways.

Sonic hedgehog (Shh) signaling has also been implicated in the regulation of self-renewal by the finding that human HSCs (CD34$^+$Lin$^-$CD38$^-$) exhibited increased self-renewal in response to Shh stimulation in vitro, albeit in combination with other growth factors (Bhardwaj et al., 2001). Recent studies have begun to tackle the complex orchestration of these signaling pathways. For example, interaction between the Notch and Wnt signaling pathways was shown where Notch signaling was required for Wnt-mediated maintenance of undifferentiated HSCs (Duncan et al., 2005).

CONCLUSION

In this review, we identify a number of regulators that are involved in the control of HSC self-renewal. Recent evidence suggests that the HSC niche plays an integral role by providing signals necessary for renewal including Ang-1. In addition, developmental pathways such as Notch, Wnt, and Shh appear to be used by the niche to regulate HSC properties. There are also cell intrinsic factors that modulate HSC regeneration. Since leukemia involves abnormal expansion of self-renewing cells, it is not surprising that some transcription factors that have been implicated in leukemogenesis, such as TEL/ETV6 and STAT5, have also been implicated in HSC self-renewal.

Finally, Bmi1 and other members of the *Polycomb* family of transcriptional repressors have been shown to play a role in HSC renewal. Indeed, *Polycomb* has been implicated in the maintenance of stem cells in multiple types of stem cells including neural stem cells and ES cells. *Polycomb* appears to function by repressing genes involved in the induction of apoptosis, senescence and differentiation. This suggests that non self-renewing cell divisions are the default state for stem cells, and that transcriptional repression of these non-stem cell fates is needed for successful self-renewal.

Self-renewal

Intrinsic Factors

Bmi1
Gfi-1
PTEN
STAT5
Tel/Etv6
p21
p18
MCL-1
Mel-18
Rae28
HoxB4

Extrinsic Factors

Notch
Wnt
Shh
Tie2/Ang-1

HSC
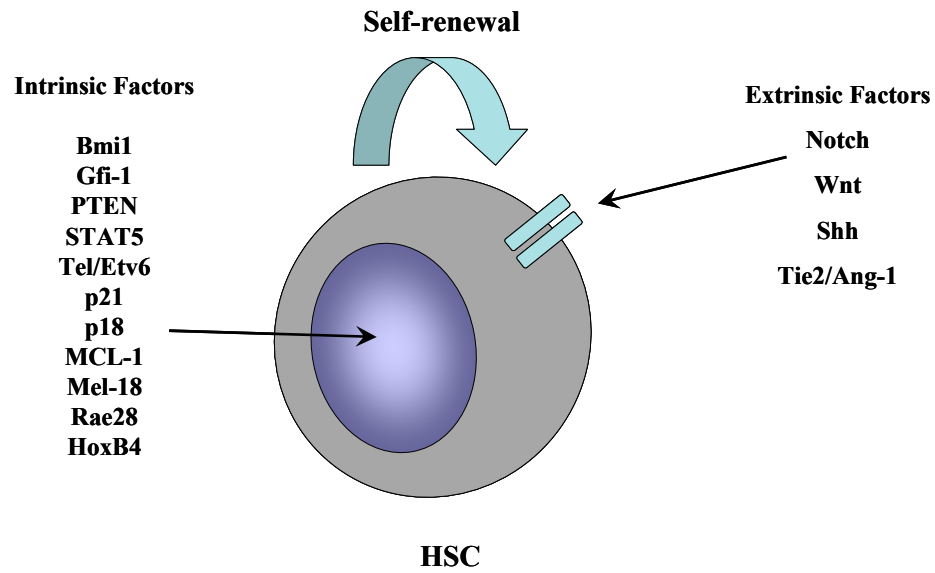
Figure 2.1 Known regulators of HSC maintenance. The above factors have been implicated in the maintenance of HSCs. This includes factors that are intrinsic to the HSC and extrinsic factors from the niche.
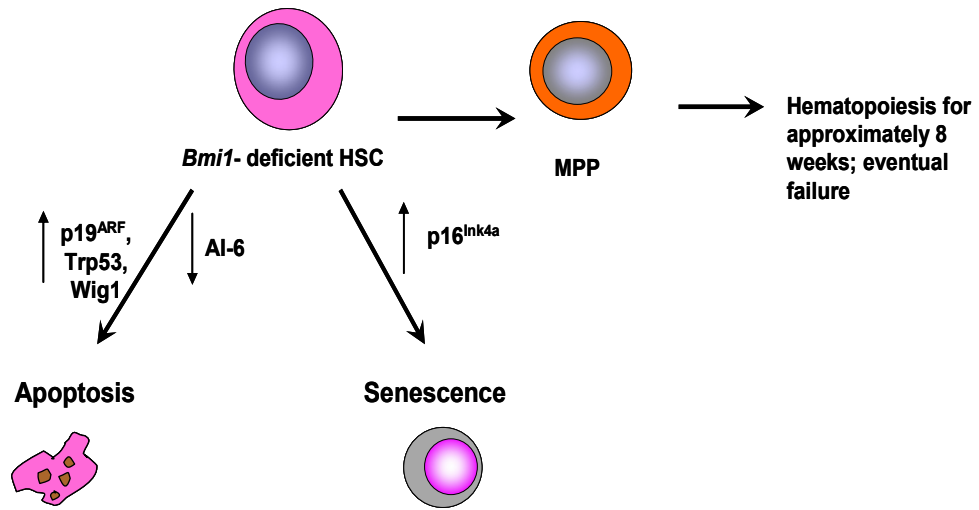
Figure 2.2 Impact of *Bmi1* loss on HSCs. In the absence of *Bmi1*, HSCs fail to self-renew and are depleted, eventually resulting in hematopoietic failure.

CHAPTER THREE

Differential rescue of hematopoietic stem cells and lymphocytes in *Bmi1* deficient mice by deletion of $p16^{Ink4a}$, $p19^{Arf}$, and *Trp53*

ABSTRACT

$Bmi1^{-/-}$ mice have defects in maintenance of adult hematopoietic stem cells (HSCs) as well as lymphocytes while overexpressing $p16^{Ink4a}$ and $p19^{Arf}$. Here we show that $p16^{Ink4a}$ deletion did not alleviate defects in HSCs and lymphocytes, whereas $p19^{Arf}$ deletion partially restored lymphocytes, but not HSCs, in $Bmi1^{-/-}$ mice. Deletion of $p16^{Ink4a}/p19^{Arf}$ partially restored HSC functions. *Trp53* deletion also partially rescued the lymphocytes and HSCs. However, none of the deletions restored maintenance of HSC and lymphocytes completely. Therefore, $p16^{Ink4a}$ and $p19^{Arf}$ and other Bmi1 targets differentially maintain different hematopoietic compartments. Furthermore, Trp53 is also involved in adult HSC maintenance in both p19^{Arf}-dependent and independent manners.

INTRODUCTION

In the blood system, Hematopoietic stem cells (HSCs) and memory lymphocytes are the only long-lived populations. In mice, HSCs constituting less than 1 in 10,000 of the bone marrow cells, have the exclusive ability to maintain hematopoiesis. The defining property of stem cells is their capacity to self-renew. Self-renewal is a unique cell division in which one or both of the resulting daughter cells remains undifferentiated and retains the ability to give rise to another stem cell with a similar capacity to both proliferate and differentiate as the parental cell. The significance of self-renewal is best illustrated by the hematopoietic system (Morrison et al., 1995). In the bone marrow, both HSCs and multipotent progenitor cells (MPPs) can give rise to the different types of mature blood cells. However, only HSCs can self-renew and maintain hematopoiesis for the life of the animal. Although MPPs share differentiation potential with HSCs, they cannot self-renew and thus, unlike HSCs, transplanted MPPs only transiently reconstitute the recipient's hematopoietic system in mice (Morrison and Weissman, 1994). Therefore, the reconstitution potential of this population of cells is restricted as upon division they give rise to daughter cells with a reduced capacity to proliferate in comparison to the parental cell.

Bmi1 is necessary for the maintenance of both postnatal HSCs and postnatal neural stem cells of the central and peripheral nervous system (Molofsky et al., 2003; Park et al., 2003) as well as the proliferative potential of leukemic stem cells (Lessard and Sauvageau, 2003). Data from the mammary gland and intestine also suggest a role for Bmi1 in the maintenance of regenerative compartments in these tissues (Pietersen et al., 2008; Sangiorgi and Capecchi, 2008). Bmi1 has also been shown to be essential for

cerebellar development and is sometimes overexpressed in human medulloblastomas (Leung et al., 2004), mantle cell lymphomas (Bea et al., 2001), colorectal cancers (Kim et al., 2004b) and breast cancers (Kim et al., 2004a). Its expression is associated with poor prognosis in B-cell non-Hodgkin lymphomas (van Kemenade et al., 2001). In *Bmi1$^{-/-}$* mice, replication of both HSCs and neural stem cells is defective. Although Bmi1 has profound effects on the proliferation and/or survival of stem cells, other cell populations are less severely affected. *Bmi1$^{-/-}$* mice have normal progenitor cell numbers and in stark contrast to HSCs and neural stem cells, the mutant blood and brain progenitor cells are able to proliferate and differentiate. Thus, at birth the cellular architecture of the brain and bone marrow is relatively normal. Since the stem cell pools are not maintained, the mice develop progressive blood and brain abnormalities which contribute to premature mortality. *Bmi1$^{-/-}$* mice also have a defect in lymphocyte development (Park et al., 2003; van der Lugt et al., 1994)}. Interestingly, like HSCs, memory lymphocytes are also a long-lived cell population (Gourley et al., 2004). Therefore, Bmi1 seems to be necessary for the maintenance of both long-lived hematopoietic cell populations.

Bmi1 is a member of the *Polycomb* family of proteins that function as epigenetic repressors of gene transcription (Orlando, 2003; Simon and Tamkun, 2002). *Polycomb* complexes repress transcription through their modification of histones (Lund and van Lohuizen, 2004). In mammals the polycomb group proteins form two major complexes, polycomb repressive complexes 1 and 2 (PRC1 and PRC2). The PRC2 complex possesses histone H3-K27 methyltransferase activity and helps establish gene expression patterns (Bernstein et al., 2006; Fischle et al., 2003), while the PRC1 complex, of which Bmi1 is a member, has E3 ubiquitin ligase activity capable of ubiquinating histone H2A

and geminin (an inhibitor of DNA replication) , maintains the stable expression of these patterns (Ohtsubo et al., 2008; Wang et al., 2004). Bmi1 targets are thought to include *Hox* genes (van der Lugt et al., 1996) as well as the *Ink4a/Arf* locus (Jacobs et al., 1999), which generates *p16^{Ink4a}* and *p19^{Arf}* due to usage of alternative transcriptional start sites resulting in different first exons (Sherr, 2001). p16^{Ink4a} inhibits cyclin-dependent kinase 4 (cdk4) and cdk6 by binding to cyclin D, resulting in hypo-phosphorylation of retinoblastoma gene product (Sharpless et al., 2001), which in turn binds and inhibits the E2F transcription factor, thus preventing transcription of E2F target genes important for the G1/S transition (Collins and Sedivy, 2003). p19^{Arf} sequesters MDM2 and inhibits Trp53 degradation, thus inducing cell cycle arrest and apoptosis mediated by Trp53 (Honda and Yasuda, 1999; Weber et al., 1999; Zhang et al., 1998). Mutations in *p16^{Ink4a}* and *p19^{Arf}* are frequently found in many types of human cancers, implicating them as key regulators of immortalization/senescence checkpoints. Expression of *p16^{Ink4a}* and *p19^{Arf}* is often not detected in normal tissues and these genes function as inhibitors of abnormal proliferation.

Mice lacking *Bmi1* showed the induction of both *p16^{Ink4a}* and *p19^{Arf}* in various hematopoietic and neuronal tissues indicating that the *Ink4a/Arf* locus is one of the major targets of Bmi1 in these tissues (Molofsky et al., 2003; Park et al., 2003). In neural stem cells, *p16^{Ink4a}* deficiency partially restored the ability of *Bmi1* deficient stem cells to self-renew based on neurosphere formation assays (Molofsky et al., 2003). Fibroblasts from *Bmi1^{-/-}* mice have reduced proliferation in tissue culture and this defect is partly rescued by deletion of both *p16^{Ink4a}* and *p19^{Arf}* (Jacobs et al., 1999). Overexpression of *p16^{Ink4a}* and *p19^{Arf}* in HSCs caused cells to stop proliferating and to undergo Trp53-dependent

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apoptosis (Park et al., 2003; van der Lugt et al., 1994). Deletion of $p19^{Arf}$ was able to partially rescue neural stem cells and the deletion of *Trp53* was able to partially rescue the lymphocyte compartment in *Bmi1*$^{-/-}$ mice (Bruggeman, 2005; Molofsky et al., 2005). We therefore investigated whether deletions of *Ink4a* locus genes and *Trp53* would rescue the hematopoietic defects of *Bmi1*$^{-/-}$ mice. We find that unlike in neural stem cells, neither the lymphocyte nor the hematopoietic stem cell compartments are restored by $p16^{Ink4a}$ deletion, whereas lymphocyte, but not HSC, numbers are partially restored by $p19^{Arf}$ deletion. Deletion of both $p16^{Ink4a}$ and $p19^{Arf}$ partially rescued both lymphocytes and HSCs. Deletion of *Trp53* also partially restored the functions of lymphocytes and HSCs of *Bmi1*$^{-/-}$ mice. Our studies demonstrate that $p16^{Ink4a}$ and $p19^{Arf}$ have differential roles in hematopoiesis and repression of these genes and *Trp53* or its targets is part of the mechanism by which *Bmi1* functions to sustain HSCs. We also demonstrate that *Trp53* appears to be genetically downstream of Bmi1 in a $p19^{Arf}$ dependent and independent manner.

RESULTS

$p16^{Ink4a}$ deletion does not rescue the hematopoietic defects of *Bmi1*$^{-/-}$ mice.
        Bmi1 represses the *Ink4a/Arf* locus genes, $p16^{Ink4a}$ and $p19^{Arf}$ (Jacobs et al., 1999). To understand the role of these *Bmi1* target genes in hematopoietic stem cell function, we generated various transgenic mice deficient for $p16^{Ink4a}$, $p19^{Arf}$ or *Trp53* in a *Bmi1*$^{-/-}$ background to determine if they could rescue the hematopoietic defects observed in *Bmi1*$^{-/-}$ mice. Consistent with previous reports, *Bmi1*$^{-/-}$ mice have an approximately 90% reduction in total lymphocyte counts in the peripheral blood, with normal neutrophil and

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monocyte counts (Figure 3.1a) (van der Lugt et al., 1994). Their hematocrit and platelet

counts were also within the normal range (Figure 3.1b, 3.1c). Peripheral blood analysis of

$Bmi1^{-/-}p16^{Ink4a-/-}$ mice shows that they have a 90% reduction in peripheral blood

lymphocytes similar to that of $Bmi1^{-/-}$ mice (Figure 3.1a). Examination of B lymphocytes

in the bone marrow and spleen of $Bmi1^{-/-}p16^{Ink4a-/-}$ mice using the pan B lymphocyte

marker B220 shows they have a 5-fold reduction in the B220$^+$ population compared to

wild-type (Figure 3.2a, 3.2b). This is comparable to the B220$^+$ population found in the

bone marrow and spleen of $Bmi1^{-/-}$ mice. The percentage of double positive CD4$^+$CD8$^+$

thymocytes in $Bmi1$ mutant mice was 25% of wild-type, and further deletion of $p16^{Ink4a}$

did not increase the double positive CD4$^+$CD8$^+$ population (Figure 3.3a). Single positive

CD4$^+$ and CD8$^+$ thymocytes are also not rescued by the deletion of $p16^{Ink4a}$ in $Bmi1^{-/-}$

mice (Figure 3.3b, 3.3c)

We next analyzed HSCs of $Bmi1^{-/-}p16^{Ink4a-/-}$ mice by flow cytometry. As reported

previously (Park et al., 2003), there was an average 2-fold reduction of HSCs in the $Bmi1^{-/-}$ mice ranging from normal to zero frequencies (Figure 3.4). This is consistent with

previous results that the primary defect in the hematopoietic system of $Bmi1$ mutant mice

is in the stem cell and lymphocyte compartments. The stem cell compartment of $Bmi1^{-/-}$
$p16^{Ink4a-/-}$ mice also demonstrated an average 2-fold reduction in HSCs, indicating that

deletion of $Bmi1^{-/-}p16^{Ink4a-/-}$ was not able to restore the $Bmi1$ HSC phenotype.

The number of stem cells measured by flow cytometry analysis of both mutants

was probably an overestimate of the true frequency of HSCs. HSCs represent less than 1

out of 10,000 bone marrow cells. With such rare cell populations, false positive events

can lead to overestimation of the population. To determine the functional potential of

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HSCs, competitive reconstituting experiments were carried out by injecting 5×10^5 bone marrow cells obtained from 4- to 6-week old mice into lethally irradiated recipients, and monitored for 20 weeks. Flow cytometry analysis of peripheral blood at 20 weeks post-transplantation indicated that bone marrow cells from wild-type mice efficiently reconstituted both myeloid and lymphoid cells as shown by donor derived (CD45.2$^+$) Gr-1$^+$, Mac-1$^+$, CD3$^+$ and B220$^+$ cells, while $Bmi1^{-/-}$ bone marrow cells showed no capacity to reconstitute all lineages in the transplanted animals (Figure 3.5), confirming the previous result that $Bmi1^{-/-}$ mice were unable to maintain HSCs. Furthermore, there was no evidence of long-term engraftment when 5×10^5 bone marrow cells obtained from 4- to 6-week old $Bmi1^{-/-}p16^{Ink4a-/-}$ were transplanted (Figure 3.5). In secondary transplant experiments, where 1 million bone marrow cells from primary recipient mice were injected into secondary lethally irradiated congenic recipient mice, there were donor derived cells in mice reconstituted with wild-type bone marrow but no donor derived cells in mice reconstituted with $Bmi1^{-/-}$ or $Bmi1^{-/-}p16^{Ink4a-/-}$ bone marrow cells, indicating that $Bmi1^{-/-}p16^{Ink4a-/-}$ mice were not able to maintain HSCs (Figure 3.6). Therefore, $p16^{Ink4a}$ was unable to rescue either the lymphocytes or HSCs of $Bmi1^{-/-}$ mice. These results are different from central nervous system (CNS) stem cells, where deletion of $p16^{Ink4a}$ partially rescued brain defects of $Bmi1^{-/-}$ mice (Molofsky et al., 2005).

$p19^{Arf}$ deletion partially rescues lymphocytes but not HSCs of $Bmi1^{-/-}$ mice.

In peripheral blood, $Bmi1^{-/-}p19^{Arf-/-}$ mice had approximately twice the number of lymphocytes as the $Bmi1^{-/-}$ mice, while their neutrophil and monocyte counts were normal (Figure 3.1a). Their hematocrit and platelet counts were also with in normal range

(Figure 3.1b, 3.1c). The percentage of B220$^+$ B cells in the bone marrow was similar to wild-type, while the percentage in the spleen of the double mutants was ~80% of wild-type mice (Figure 3.2a, 3.2b). The percentage of double positive CD4$^+$CD8$^+$ thymocytes was essentially identical to wild-type mice (Figure 3.3a), as well as that of single positive CD4$^+$ thymocytes (Figure 3.3b). Although single positive CD8$^+$ thymocytes were rescued, the percentage was ~91% of wild-type (Figure 3.3c). HSC analysis by flow cytometry showed deletion of $p19^{Arf}$ did not restore the HSC frequency of $Bmi1^{-/-}$ mice, and this was further confirmed by competitive reconstitution experiments that showed no engraftment of cells from $Bmi1^{-/-}p19^{Arf-/-}$ bone marrow in primary recipient mice (Figure 3.4, 3.5). Secondary transplant experiments also showed no reconstitution, indicating that there were no detectable HSCs in $Bmi1^{-/-}p19^{Arf-/-}$ bone marrow (Figure 3.6). Therefore, $p19^{Arf}$ can partially rescue the lymphocyte compartment in hematopoietic tissues but not HSCs. Interestingly, deletion of $p19^{Arf}$ rescues CNS stem cell defects in $Bmi1^{-/-}$ mice (Bruggeman, 2005; Molofsky et al., 2005), suggesting that different Bmi1 target genes critical for self-renewal are tissue specific.

Deletion of the *Ink4a/Arf* locus rescues lymphocytes but incompletely rescues HSCs of *Bmi1$^{-/-}$* mice.

Mice mutant for *Bmi1* and both $p16^{Ink4a}$ and $p19^{Arf}$ showed higher peripheral blood lymphocyte counts compared to when $p19^{Arf}$ alone was deleted (Figure 3.1a). However, it was still only 60% of the wild-type. As seen in the case of the other mutants, $Bmi1^{-/-}p16^{Ink4a-/-}p19^{Arf-/-}$ mice had normal neutrophil and monocyte counts (Figure 3.1a), as well as hematocrit and platelet counts with in normal range (Figure 3.1b, 3.1c). In bone marrow and spleen, deletion of *Ink4a/Arf* restored 80% of the B220$^+$ population

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compared to that of the wild-type mice (Figure 3.2a). In the thymus the percentage of double positive CD4$^+$CD8$^+$ thymocytes was normal (Figure 3.3a), as were single positive CD4$^+$ thymocytes (Figure 3.3b). Although single positive CD8$^+$ thymocytes were rescued, the percentage was ~94% of wild-type (Figure 3.3c), quite similar to the result seen with *Bmi1$^{-/-}$p19$^{Arf-/-}$* mice. The similarity in the pattern of lymphocyte rescue between *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* mice and *Bmi1$^{-/-}$p19$^{Arf-/-}$* mice as opposed to of *Bmi1$^{-/-}$p16$^{Ink4a-/-}$* mice, suggests that p19^{Arf} is the crucial effector of Bmi1 required for the maintenance of the lymphocyte compartment (Figure 3.7).

The HSC frequency measured by flow cytometry indicated that *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* mice have much lower HSC frequency than wild-type (Figure 3.4). Competitive repopulation was used to measure functional HSCs in *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* mice. To do this, 5 x10^5 bone marrow cells from each group of mice were infused into lethally irradiated recipients. As expected, wild-type cells were able to reconstitute the bone marrow of all 12 transplanted control mice. In contrast, bone marrow cells from *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* mice were able to reconstitute the bone marrow of 9 out of 19 recipient mice (Figure 3.5, Table 1). Bone marrow cells in mice engrafted with *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* bone marrow cells were able to reconstitute the bone marrow of 5 out of 7 recipient mice in secondary transplantation experiments indicating that indeed *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* mice contain some HSCs in their bone marrow capable of self-renewal (Figure 3.6, Table 3.1). Again, not all mice were engrafted in the secondary transplants of the mutant cells indicating that self-renewal is still impaired in these mutant mice. These data suggest that other Bmi1 target gene(s) in addition to the *Ink4a/Arf* locus play a role in self-renewal of HSCs.

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***Trp53* deletion partially rescues hematopoietic defects of *Bmi1*$^{-/-}$ mice.**

p19^{Arf} has been shown to be a positive regulator of Trp53 via its binding to the

Trp53 inhibitor Mdm2. Mdm2 degrades Trp53, through its E3 ubiquitin ligase activity,

targeting Trp53 for proteasome-mediated degradation (Moll and Petrenko, 2003).

Therefore, it was expected that *Bmi1*$^{-/-}$*Trp53*$^{-/-}$ mice would display similar phenotypes

compared to *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$ mice. As noted for the other mutant mice their neutrophil,

monocyte, and platelet counts, as well as hematocrit were normal (Figure 3.1a-c). *Bmi1*$^{-/-}$

Trp53$^{-/-}$ mice peripheral blood lymphocyte counts were essentially identical to that of

Bmi1$^{-/-}$*p19*$^{Arf-/-}$ mice (Figure 3.1a); however the B220^{+} populations in bone marrow and

spleen (Figure 3.2a, 3.2b) were lower than *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$ mice. In addition, the

percentage of double positive CD4^{+}CD8^{+} thymocytes, single positive CD4^{+} thymocytes,

and single positive CD8^{+} thymocytes were also lower than that of *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$ mice

(Figure 3.3). The fact that the rescue of lymphocytic defects in *Bmi1*$^{-/-}$ mice by *Trp53*

deletion is not comparable to that affected by the deletion of *p19*^{Arf} suggests, that there

are targets other than Trp53 suppressed by Bmi1 through *p19*^{Arf} that play a role in

maintenance of the lymphocyte compartment.

Flow cytometry analysis showed that *Bmi1*$^{-/-}$*Trp53*$^{-/-}$ mice had reduced HSC

frequency compared to wild-type, but slightly higher than *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$ mice (Figure

3.4). The results of HSC activity of *Bmi1*$^{-/-}$*Trp53*$^{-/-}$ mice by competitive reconstitution

were rather unexpected. Unlike *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$ bone marrow cells, which showed no

ability for long-term blood reconstitution, the blood of 15 of 19 lethally irradiated mice

were reconstituted by bone marrow cells from *Bmi1*$^{-/-}$*Trp53*$^{-/-}$ mice (Figure 3.5 and Table

3.1). In secondary transplant experiments, bone marrow cells from 17 of 22 mice were

able to repopulate the secondary recipient mice (Figure 3.6 and Table 3.1), indicating that

Trp53 deletion is capable of partially restoring the HSC maintenance defect in *Bmi1*$^{-/-}$ mice. However, because we do not see rescue in every case, *Trp53* deletion in *Bmi1*$^{-/-}$ mice provides an incomplete rescue of the HSC maintenance defect of these mice. These data also suggest that Bmi1 potentially interacts with Trp53 or Trp53 targets in a p19^{Arf}-independent manner.

DISCUSSION

Self-renewal is the hallmark property that defines stem cells. When a stem cell divides, it has many cell fate decisions. It can self-renew making another stem cell with a similar capacity to proliferate and differentiate as the parental cell. It can differentiate into a progenitor cell, with similar ability to differentiate as the parental cell, but a significantly reduced proliferative ability. Finally, it can undergo senescence or programmed cell death. Bmi1 is a polycomb gene whose function is to repress expression of target genes making it an ideal regulator of stem cell fate decisions. Indeed, Bmi1 modulates expression of genes such as members of the *Hox* family and *Ink4a/Arf* known to modulate development and survival.

Our studies demonstrate that Bmi1 targets critical for maintaining long-lived cell populations have some cell-type specificity. Mutation of either *p16*^{Ink4a} or *p19*^{Arf} can partially rescue neural stem cells in *Bmi1*$^{-/-}$ mice (Bruggeman, 2005; Molofsky et al., 2005). We and others find that *p19*^{Arf} mutation can rescue lymphocytes in *Bmi1*$^{-/-}$ mice (Bruggeman, 2005), but neither *p16*^{Ink4a} nor p19^{Arf} mutations alone can rescue HSCs in *Bmi1*$^{-/-}$ mice in competitive reconstitution experiments. When both *p16*^{Ink4a} and *p19*^{Arf}

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are mutant, then HSCs are partially rescued in $Bmi1^{-/-}$ mice. Thus, rescue of lymphocytes, neural stem cells and HSCs differs in the $Bmi1$ mutants.

Deletion of $Ink4a/Arf$ locus genes and $Trp53$ in $Bmi1^{-/-}$ mice has differential effects on hematopoiesis. However, none of the deletions completely restored the $Bmi1$ deficient phenotypes, indicating that there are other cell type and tissue specific downstream targets that exist for Bmi1. It has been shown that some of the Hox family genes are repressed by Bmi1 (Alkema et al., 1995; van der Lugt et al., 1996). Semi-quantitative RT-PCR indicated that expression of $Hoxb4$, which is important for HSC development (Antonchuk et al., 2002a) and $Hoxb3$ were not altered in $Bmi1^{-/-}$ mice while $Hoxa9$, which functions in cell fate decisions of hematopoietic progenitors (Lawrence et al., 1997), was slightly up-regulated in thymus and bone marrow of $Bmi1^{-/-}$ mice (Park et al., 2003) indicating that Bmi1 does not suppress the expression of Hox members important for HSC maintenance (Park et al., 2003). It would be of importance to identify other targets of Bmi1 to understand the role of Bmi1 in HSC function. Increased expression of the Trp53-target gene $Wig-1$ in $Bmi1^{-/-}$ bone marrow is consistent with induction and activation of the $p19^{Arf}$ pathway in $Bmi1^{-/-}$ hematopoietic cells (Park et al., 2003). Wig-1 is a double-stranded RNA binding protein and inhibits tumor growth in $vitro$, suggesting that it may function in stress-induced Trp53 responses (Mendez-Vidal et al., 2002). The observation that $Trp53$ deficient mice have a slight increase in the numbers of stem cells is consistent with the notion that Trp53 is a downstream effecter of Bmi1 (TeKippe et al., 2003). How Bmi1 directly regulates Trp53 remains to be established.

That mutation of *Trp53* could partially rescue HSCs in *Bmi1*$^{-/-}$ mice was surprising since *p19*^{Arf} mutations could not, since activation of Trp53 is a major function of p19^{Arf}. This suggests that Bmi1 might be able to bypass p19^{Arf} for regulation of Trp53 (Figure 3.7). Trp53 is phosphorylated by (ataxia telangiectasia mutated) ATM or (ATM and Rad3-related) ATR on serine 15 upon cellular stress such as ionizing radiation, oxidative damage, and escapes from Mdm2-mediated ubiquitination and subsequent degradation (Shieh et al., 1997). Recently the regulation of oxidative stress via ATM, p38 MAPK, and FoxOs has been implicated in the maintenance of HSCs (Ito et al., 2004; Ito et al., 2006; Tothova et al., 2007). The RING-finger domain of Mdm2 is responsible for binding to Trp53, and Bmi1 also has a RING-finger domain, therefore it is possible that Bmi1 could bind Mdm2 to promote the activity of Mdm2 or Trp53 to prevent post-translational modification of Trp53 necessary for stabilization, thereby preventing cell cycle arrest and apoptosis. Alternatively, Bmi1 may regulate Trp53 transcription via an epigenetic mechanism (Figure 3.7).

In summary, we find that Bmi1 must regulate multiple downstream targets in the long-lived cell populations that depend on it for their maintenance. Of the different cell types investigated to date, it is remarkable that several genes are necessary for rescue of HSCs, but not lymphocytes or neural stem cells in mice mutant for *Bmi1*. This may be a result of the need to safeguard against the leukemic transformation of cells with such a high potential for proliferation. Investigation of role of *p19*^{Arf}, p16^{Ink4a}, *Trp53* and other Bmi1 target genes in normal stem cells self-renewal should lead to new insights into how the regulation of this process is disrupted in malignant stem cells.

MATERIALS AND METHODS
Mice

Bmi1$^{-/-}$ mice used in this study has been described (Park et al., 2003). *Trp53*$^{-/-}$ mice

(B6.129S2-*Trp53*^{tm1Tyj}), originally developed by Jacks et al. (Jacks et al., 1994) was

purchased from the Jackson Laboratory, *p16*$^{Ink4a-/-}$ mice (FVB/N.129-*Cdkn2a*^{tm2Rdp})

(Sharpless et al., 2001) and *p16*$^{Ink4a-/-}$*p19*$^{Arf-/-}$ mice (B6.129-*Cdkn2a*^{tm1Rdp}) (Serrano et al.,

1996) were obtained from Mouse Models of Human Cancers Consortium (NCI-

Frederick). *p19*$^{Arf-/-}$ mice (Kamijo et al., 1999) were obtained from Dr. Charles Sherr (St.

Jude Children's Research Hospital, TN). All mice were bred with BA mice (C57Bl/Ka-

1.1/Thy1.1) and backcrossed for 5 generations, and verified for Thy1.1, H2^{b}, and CD45.2

homozygosity by peripheral blood analysis using flow cytometry. Mice heterozygous for

multiple genes were generated by crossing heterozygous mice for each gene, and these

heterozygous mice were used to generate mice with homozygous mutations. In some

cases, homozygous mutant mice with heterozygous *Bmi1* allele were used to increase the

chance of obtaining double knockout mice. All mice used for this study were maintained

at the University of Michigan Animal Facility or Stanford Animal Facility in accordance

with the guidelines of both Institutional Animal Care Use Committees.

Flow cytometry

Bone marrow cells were flushed from the tibias and femurs of mice and stained

with a cocktail of rat monoclonal antibodies against lineage markers (CD3, CD4, CD5,

CD8, Gr-1, Mac-1, B220, and Ter119), Sca-1, c-kit, CD135, and Thy1.1 as described

(Christensen and Weissman, 2001). Cells were analyzed by flow cytometry using a

Vantage fluorescence-activated cell sorter (Becton Dickinson) or FACSAria cell sorter

(Becton Dickinson). The $Lin^-CD135^-Thy-1.1^{lo}Sca-1^+c-kit^+$ population was used to calculate the HSC frequency (Christensen and Weissman, 2001). For lineage analysis, both bone marrow cells and splenocytes were stained with antibodies against Gr-1, Mac-1, CD3, B220, and TER119, and thymocytes with antibodies against CD3, CD4, and CD8. For peripheral blood analysis, red blood cells were lysed with hypotonic buffer, and nucleated cells were stained with antibodies against CD45.2, Gr-1, Mac-1, CD3, and B220. All antibodies were directly conjugated with fluorescence colors and purchased from e-Bioscience or BD Pharmingen.

Long-term competitive reconstitution.

C57Bl/Ka-CD45.1/Thy1.2 congenic mice were lethally irradiated (1140 rads) using a Pantak Therapax DXT 300 Model X-ray unit (PANTAK, East Haven, CT) at a dose rate of approximately 3 Gy/min. The radiation was delivered in two doses at 4 hrs apart. The next day, mice were competitively reconstituted by retro-orbital venous sinus injection of whole bone marrow cells from donor mice mixed with radio-protective dose of 200,000 bone marrow cells from un-irradiated C57Bl/Ka-CD45.1/Thy1.2 mice. Peripheral blood was drawn monthly up to 20 weeks to monitor reconstitution by donor type (CD45.2) myeloid and lymphoid cells as described above. Mice that had more than 1% donor-derived ($Ly5.1^+$) cells in both lymphoid ($CD3^+$ and $B220^+$) and myeloid ($Gr-1^+$ and $Mac-1^+$) subpopulations were considered to be repopulated by donor cells. The secondary bone marrow transplant was performed using 1 million whole bone marrow cells.

Statistics. Statistical analysis was performed using the unpaired Student's t-test.

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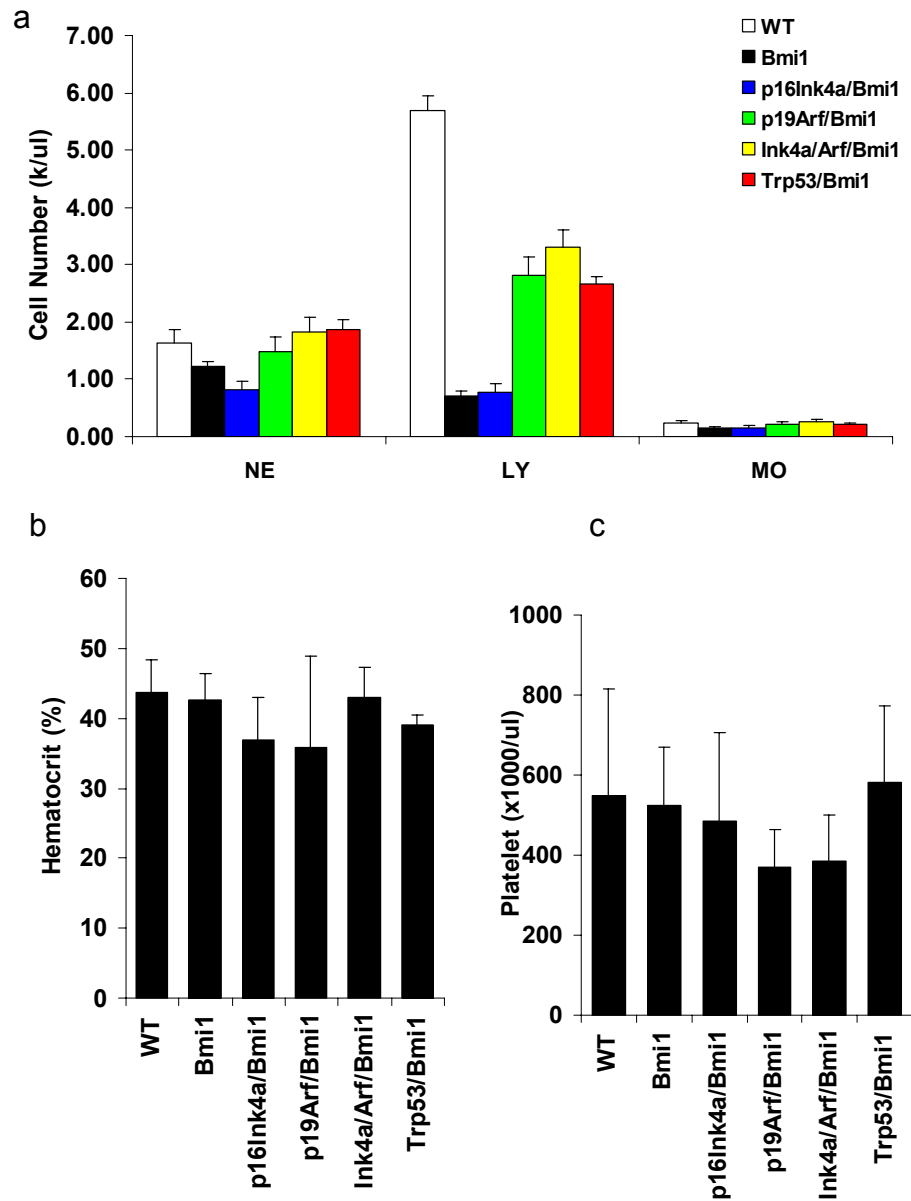
Figure 3.1 Peripheral blood analysis. a, Peripheral blood was collected from mice and analyzed for complete blood count and differential. Averages and standard deviations for neutrophil (NE), lymphocyte (LY) and monocyte (MO) counts from 34 wild-type, 20 $Bmi1^{-/-}$, 16 $Bmi1^{-/-}p16^{Ink4a-/-}$, 10 $Bmi1^{-/-}p19^{Arf-/-}$, 12 $Bmi1^{-/-}p16^{Ink4a-/-}p19Arf^{-/-}$, and 6 $Bmi1^{-/-}Trp53^{-/-}$ mice are shown. b, Hematocrit. c, Platelet count.
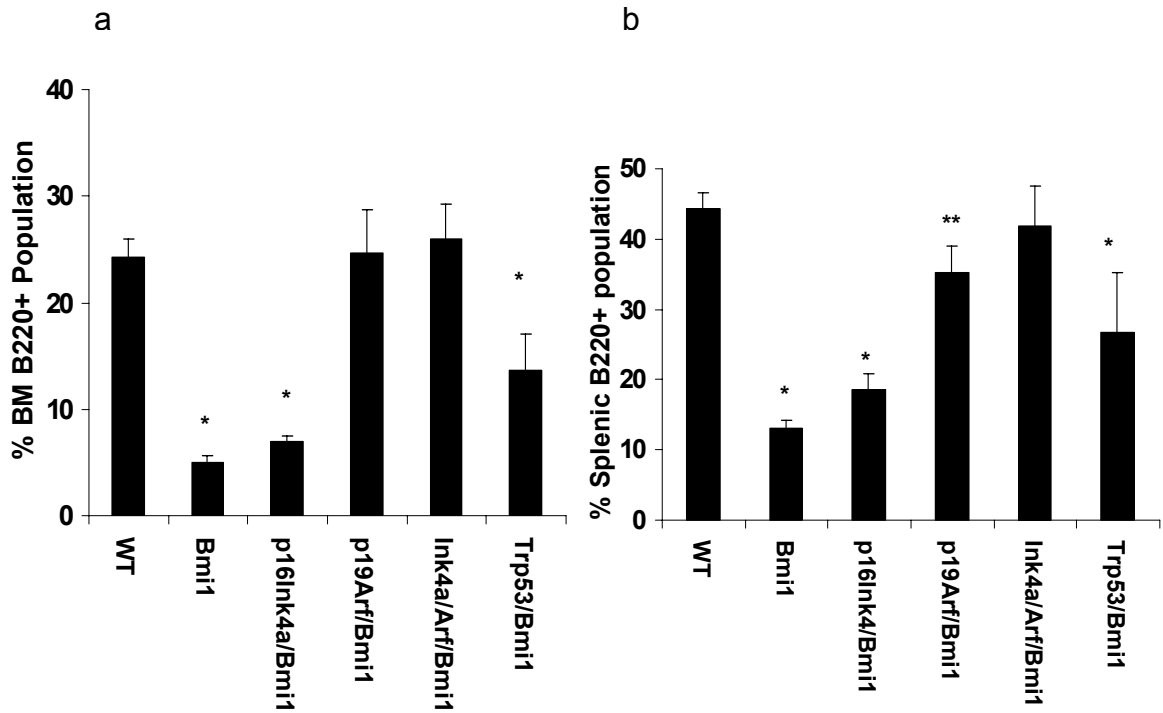
Figure 3.2 Analysis of B lymphocytes in hematolymphoid organs. Bone marrow cells (a), and splenocytes (b), were stained and analyzed for B220$^+$ by flow cytometry. Averages from 22 wild-type, 19 *Bmi1*$^{-/-}$, 11 *Bmi1*$^{-/-}$*p16*$^{Ink4a-/-}$, 10 *Bmi1*$^{-/-}$*p19*$^{Arf-/-}$, 12 *Bmi1*$^{-/-}$*p16*$^{Ink4a-/-}$*p19*$^{Arf-/-}$, 5 *Bmi1*$^{-/-}$*Trp53*$^{-/-}$ mice are shown; these differences were statistically significant;*, P < 0.01; **, P < 0.05 (Student's t-test). Error bars denote s.e.m.
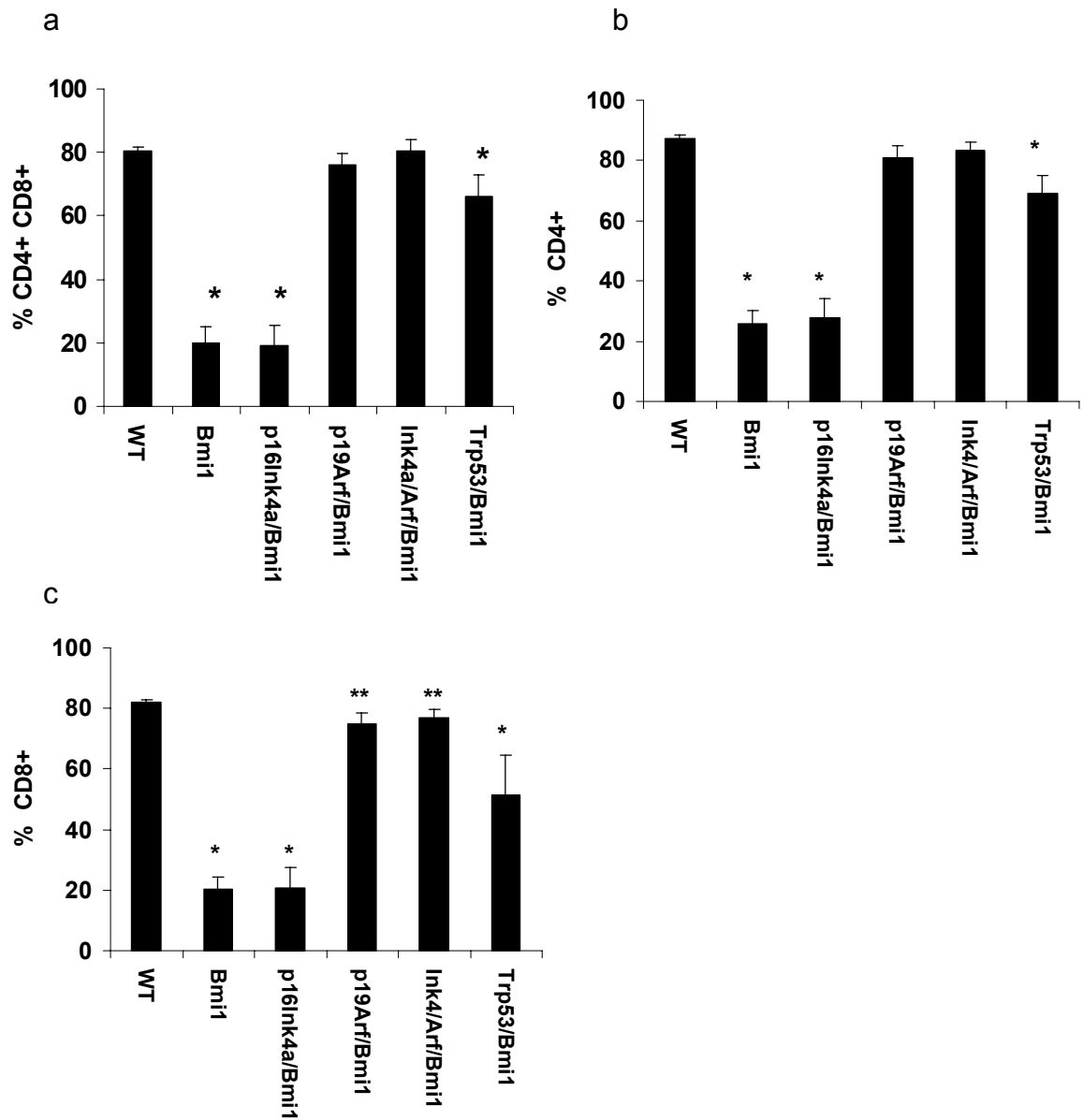
Figure 3.3 Analysis of T lymphocytes in the Thymus. CD4^{+}CD8^{+} Thymocytes (a), CD4^{+} Thymocytes (b), and CD8^{+} Thymocytes (c), were stained and analyzed for by flow cytometry. Averages from 22 wild-type, 19 *Bmi1^{-/-}*, 11 *Bmi1^{-/-}p16[Ink4a-/-]*, 10 *Bmi1^{-/-}p19[Arf-/-]*, 12 *Bmi1^{-/-}p16[Ink4a-/-]p19[Arf-/-]*, 5 *Bmi1^{-/-}Trp53^{-/-}* mice are shown; these differences were statistically significant;*, P < 0.01; **, P < 0.05 (Student's t-test). Error bars denote s.e.m.
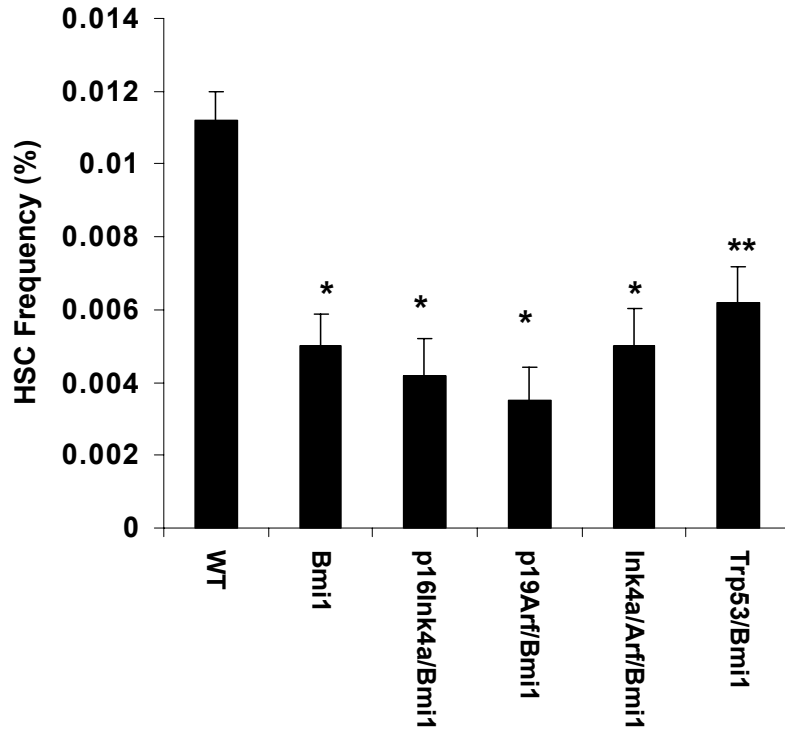
Figure 3.4 Analysis of HSC frequency by flow cytometry. Bone marrow cells were stained for HSCs (Lin⁻CD135⁻Thy-1.1^{lo}Sca-1⁺c-kit⁺) and analyzed by flow cytometry. Averages from wild-type (n=22), *Bmi1$^{-/-}$* (n=21), *Bmi1$^{-/-}$p16$^{Ink4a-/-}$* (n=11), *Bmi1$^{-/-}$p19$^{Arf-/-}$* (n=11) *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* (n=12), and *Bmi1$^{-/-}$Trp53$^{-/-}$* (n=6) mice are shown; these differences were statistically significant;*, P < 0.0001; **, P < 0.01 (Student's t-test). Error bars denote s.e.m**.
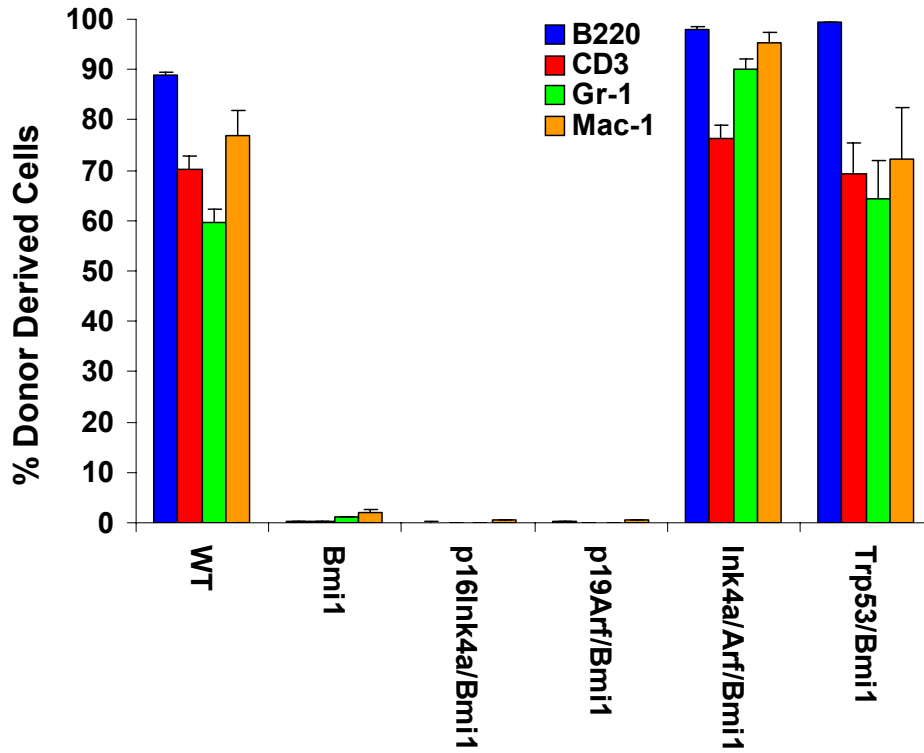
Figure 3.5 Primary transplants showing rescue of HSC activity of mutant mice in *Bmi1* deficient background. For primary transplants lethally irradiated mice were competitively reconstituted with 5×10^5 whole bone marrow cells mixed together with 2×10^5 un-irradiated recipient bone marrow cells. Donor derived cells were monitored at 4 week intervals for contribution to mature hematopoietic cells, the analysis at 20 weeks post-transplantation is shown. The averages of 10 recipient mice transplanted with wild-type bone marrow, 5 recipient mice transplanted with Bmi1$^{-/-}$ bone marrow, 4 recipient mice transplanted with *Bmi1$^{-/-}$p16$^{Ink4a-/-}$* bone marrow, 5 recipient mice transplanted with *Bmi1$^{-/-}$p19$^{Arf-/-}$* bone marrow, 9 recipient mice transplanted with *Bmi1$^{-/-}$p16$^{Ink4a-/-}$p19$^{Arf-/-}$* , and 7 recipient mice transplanted with *Bmi1$^{-/-}$Trp53$^{-/-}$* bone marrow mice are shown.
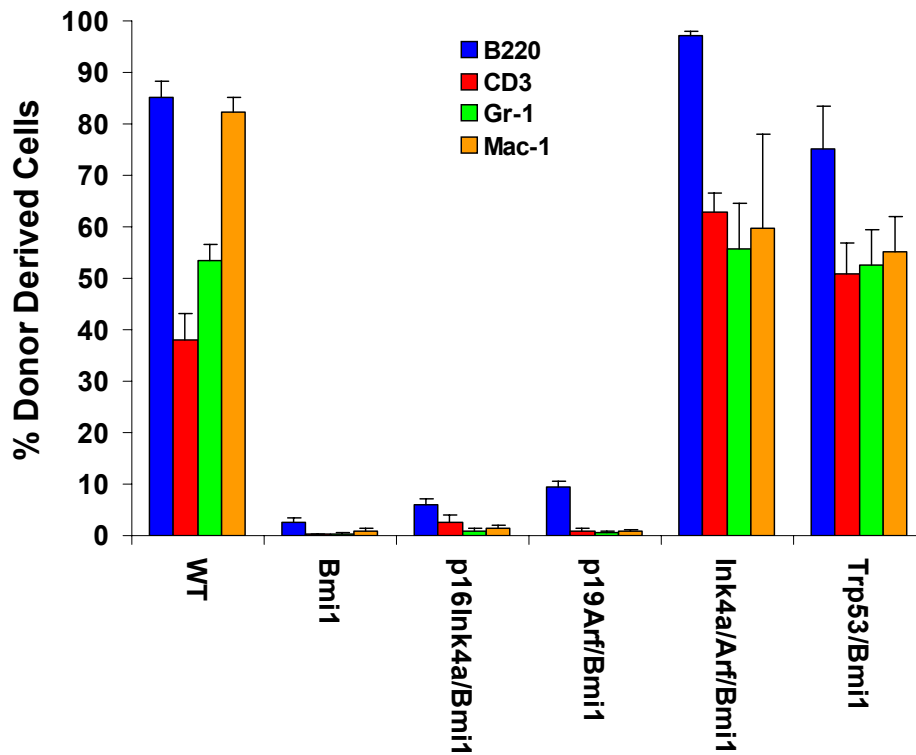
Figure 3.6 Secondary transplants showing rescue of HSC activity of mutant mice in
Bmi1 deficient background. For secondary transplants 1×10^6 whole bone marrow cells
from primary recipient mice at 12 week post-transplantation were used to inject lethally
irradiated secondary recipient mice. 8 week post-transplantation data is shown. The
averages of 8 recipient mice transplanted with wild-type bone marrow, 7 recipient mice
transplanted with $Bmi1^{-/-}$ bone marrow, 3 recipient mice transplanted with $Bmi1^{-/-}p16^{Ink4a-/-}$ bone marrow, 6 recipient mice transplanted with $Bmi1^{-/-}p19^{Arf-/-}$ bone marrow, 4
recipient mice transplanted with $Bmi1^{-/-}p16^{Ink4a-/-}p19^{Arf-/-}$, and 13 recipient mice
transplanted with $Bmi1^{-/-}Trp53^{-/-}$ bone marrow mice are shown. Error bars denote s.e.m.
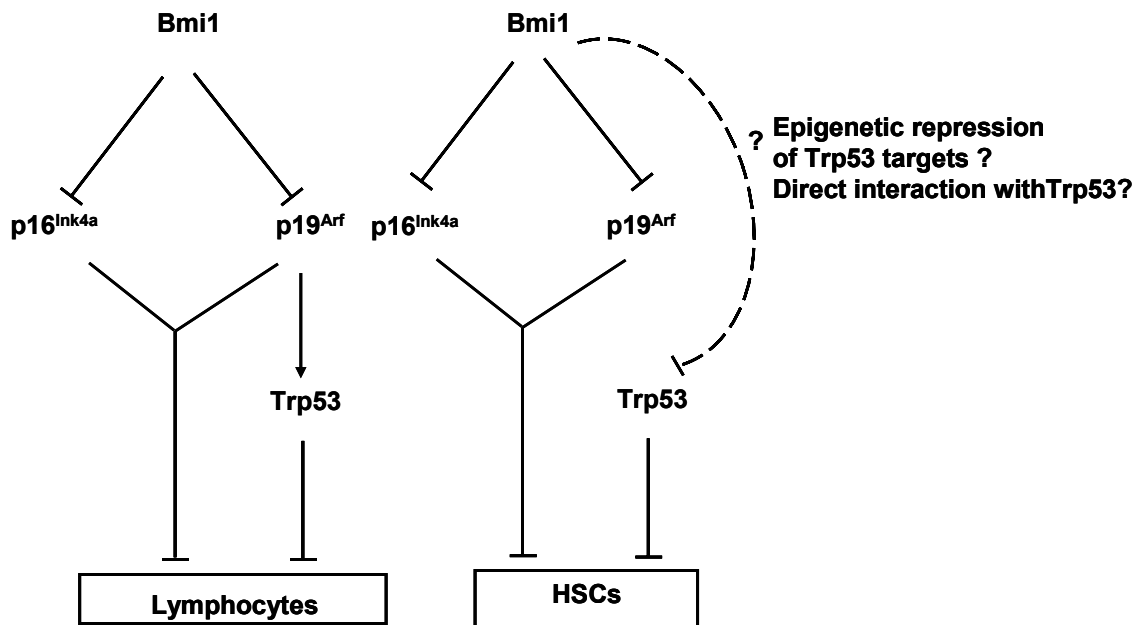
Figure 3.7 Bmi1 targets in hematopoiesis. Deletion of the *Ink4a/Arf* locus genes differentially affects hematopoietic cells. Individually *p19^Arf* functions in lymphocyte maintenance and development, but not in HSC maintenance, whereas individually *p16^Ink4a*, did not restore any of the phenotypes displayed by *Bmi1^-/-* mice. Deletion of both, *Ink4a/Arf* locus shows that in concert their repression allows for HSC maintenance. Trp53 plays a role in both lymphocyte development and HSC maintenance. In lymphocytes, Bmi1 might suppress Trp53 through the p19^Arf-Mdm2 pathway, whereas in HSCs Bmi1 might regulate Trp53 by HSC specific epigenetic mechanism, repressing *Trp53* or its targets, or by regulating Trp53 turnover.

Table 3.1 Rescue of HSC activity of *Bmi1$^{-/-}$* mice by deletion of *Ink4a/Arf* and *Trp53*.

	WT	*Bmi1$^{-/-}$p16$^{Ink4a-/-}$* p19$^{Arf-/-}$	*Bmi1$^{-/-}$* Trp53$^{-/-}$
Primary Transplants	12/12	9/19	15/19
Secondary Transplants	15/15	5/7	17/22

5 x 10^5 whole bone marrow cells for primary transplants and 1 x 10^6 whole bone marrow cells for secondary transplants were used to competitively reconstitute lethally irradiated congenic mice 2 x 10^5 un-irradiated recipient bone marrow cells. Peripheral blood was analyzed for donor derived cells by flow cytometry at 4 week intervals. 20 week post-transplantation data for the primary transplants and 8 week post-transplantation data for the secondary transplants were used to create the table. Numbers of multi-lineage reconstituted mice per number of injections are shown.

CHAPTER FOUR

Acquisition of long-term hematopoietic reconstitution ability by multipotent progenitors in mice deficient of the *Trp53* and *Ink4a/Arf* loci

ABSTRACT

Hematopoiesis is maintained by a hierarchical system where hematopoietic stem cells (HSCs) give rise to multipotent progenitors (MPPs), which in turn differentiate into all types of mature blood cells (Morrison and Weissman, 1994). HSCs maintain themselves for the lifetime of the organism because of their ability to self-renew. However, MPPs lack the ability to self-renew, therefore their mitotic capacity and expansion potential are limited and they are destined to eventually stop proliferating after a finite number of cell divisions (Clarke and Fuller, 2006; Morrison and Weissman, 1994). The molecular mechanisms that limit the proliferation capacity of MPPs and other more mature progenitors are not fully understood (Clarke and Fuller, 2006; Morrison et al., 2002). Here we show that bone marrow cells from mice deficient in three genes genetically downstream of Bmi1 - $p16^{Ink4a}$, $p19^{Arf}$, and *Trp53* (triple mutant mice; $p16^{Ink4a}$ and $p19^{Arf}$ are alternative reading frames of the same gene (also called *Cdkn2a*) that encode different proteins) - have an approximately 10 fold increase in cells able to reconstitute the blood long-term. This increase is associated with the acquisition of long-term reconstitution capacity by cells whose phenotype is c-kit$^+$Sca-1$^+$Flt3$^+$CD150$^-$CD48$^-$Lineage$^-$, which

defines (MPPs) in wild-type mice (Adolfsson et al., 2001; Christensen and Weissman, 2001; Kiel et al., 2005b) . The pattern of triple mutant MPP response to growth factors resembles that of wild-type MPPs but not wild-type HSCs. These results demonstrate that Ink4a/Arf and Trp53 play a central role in limiting the expansion potential of MPPs. These pathways are commonly repressed in cancer, suggesting a mechanism by which early progenitor cells could gain the ability to self-renew and become malignant with further oncogenic mutations.

INTRODUCTION

The replication and expansion of stem cells must be strictly regulated to ensure

proper tissue function and prevent cancer. In the bone marrow, both HSCs and MPPs can

give rise to the different types of mature blood cells. However, only HSCs self-renew

and are therefore tasked with the life-long maintenance of the hematopoietic system.

Although MPPs share multipotent differentiation potential with HSCs, they cannot self-

renew and thus, unlike HSCs, transplanted MPPs only transiently reconstitute the

recipient's hematopoietic system in mice (Morrison and Weissman, 1994). Therefore, the

reconstitution potential of MPPs is restricted as upon division they give rise to daughter

cells with a reduced capacity to proliferate. The molecular mechanisms that limit the

proliferation capacity of progenitor cells are not known (Clarke and Fuller, 2006;

Morrison et al., 2002).

Owing to the requirement of Bmi1 for the maintenance of adult HSCs and neural

stem cells of the central and peripheral nervous system (Molofsky et al., 2003; Park et al.,

2003) as well as the proliferative potential of leukemic stem cells (Lessard and

Sauvageau, 2003); coupled with the results of rescue experiments involving concurrent

deletion of its targets in a *Bmi1* deficient mouse background detailed in chapter 3, we

sought to examine the impact of deleting its targets on hematopoiesis.

RESULTS

Deletion of $p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}/Trp53$ does not lead to abnormal output of mature cells in the hematopoietic system.

Since the deletion of either the *Ink4a/Arf* locus (Figure 3.5, 3.6 and Table 3.1)

(Oguro et al., 2006) or *Trp53* seems to play a role in the rescue of HSCs in Bmi1

deficient mice (Figure 3.5, 3.6 and Table 3.1) , we examined the impact of deleting

$p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}/Trp53$ on the mature cells of the

hematopoietic system. Analysis of peripheral blood from the mutant mice showed that

there were no defects in the output of mature cells. Neutrophil, lymphocyte, and

monocyte counts from mutant mice were normal (Figure 4.1), as were hematocrit and

platelet counts (data not shown). We performed analysis by flow cytometry on bone

marrow, spleen, and thymus cells of the mutant mice using antibodies against markers

found on mature lineage cells (Figure 4.2-4.4). In the bone marrow of mutant mice, levels

of B220$^+$ cells, Ter119$^+$ cells, Gr-1$^+$ cells, and Mac-1$^+$ cells were normal (Figure 4.2).

Analysis of spleen and thymus also showed normal levels of mature hematopoietic cells

(Figure 4.3, 4.4). Therefore, the deletion of $p19^{Arf}$, $Trp53$, $p16^{Ink4a}/p19^{Arf}$, and

$p16^{Ink4a}/p19^{Arf}/Trp53$ does not appear to compromise hematopoietic differentiation.

Limiting dilution analysis of long-term reconstitution potential of $p19^{Arf}$, $Trp53$, $p16^{Ink4a}/p19^{Arf}$, and $p16^{Ink4a}/p19^{Arf}/Trp53$ deficient bone marrow.

In light of the inability of the deletions of $p19^{Arf}$, $Trp53$, $p16^{Ink4a}/p19^{Arf}$, and

$p16^{Ink4a}/p19^{Arf}/Trp53$ to perturb production of mature hematopoietic cells, we decided to

assay for the ability of whole bone marrow from these mutant mice to reconstitute the

hematopoietic system of lethally irradiated recipients. 2 x 10^5 bone marrow cells from

approximately 8 week old mutant mice were injected into lethally irradiated recipient

mice along with a radioprotective dose of recipient-type bone marrow and contribution to

hematopoiesis was monitored by flow cytometry using lineage markers to analyze

peripheral blood. Analysis of the peripheral blood of recipient mice at 20 weeks showed

bone marrow cells from all of the mutant mice are capable of long-term reconstitution of

the hematopoietic system (Figure 4.6). This indicates that the deletions of $p19^{Arf}$, $Trp53$, $p16^{Ink4a}/p19^{Arf}$, and $p16^{Ink4a}/p19^{Arf}/Trp53$ do not compromise the ability to reconstitute whole bone marrow.

We then went on to test if these mutations perturbed the frequencies of the cells responsible for long-term hematopoietic reconstitution in mutant mice by performing limiting dilution analysis. Whole bone marrow cells from approximately 8 week old wild-type and mutant mice were injected at different doses into lethally irradiated recipient mice along with a radioprotective dose of recipient bone marrow (Table 4.2). In $p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}$ deficient bone marrow, the frequency of cells responsible for long-term reconstitution were not significantly different from that of wild-type mice (Table 4.1). The minimum cell dose of wild-type bone marrow capable of long-term multi-lineage reconstitution (two of four mice) was 5×10^4 cells (Table 4.2).The minimum cell dose of $p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}$ mutant bone marrow was 2.5×10^4 cells (four of five mice, two of five mice, and two of four mice, respectively) (Table 4.2). Also, the immunophenotypic frequency of HSCs (Lin$^-$ Thy1.1^{lo}c-Kit$^+$Sca-1$^+$Flt3$^-$) from $p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}$ deficient mice was not significantly different from that of wild-type mice (Figure 4.5).These results agree with previous studies that show that in young mice deficiency of $p16^{Ink4a}$, $p19^{Arf}$, $Trp53$ and both $p16^{Ink4a}$ and $p19^{Arf}$ does not significantly affect the long-term reconstitution frequency of HSCs and has more of an effect in the context of stem cell aging (Dumble et al., 2007; Janzen et al., 2006; Stepanova and Sorrentino, 2005). Interestingly, the frequency of long-term reconstituting cells in triple mutant bone marrow was at least 10-

fold higher than that of wild-type bone marrow (Table 4.1), with reconstitution at cell doses as low as 2×10^3 and 5×10^3 (one of four mice in each case) (Table 4.2).

Long-term reconstitution by *$p16^{Ink4a}$/$p19^{Arf}$/Trp53* deficient multipotent progenitors.
Our results suggest *$p16^{Ink4a}$/$p19^{Arf}$/Trp53* deletion caused a striking increase in the frequency of long-term blood repopulating cells. We therefore decided to further examine the bone marrow of triple mutant mice. Since there was only modest increase in the frequency of bone marrow cells with a phenotype of HSCs in triple mutant mice (Figure 4.7a, 4.7b), we concluded that the increased repopulating activity in the triple mutant mice was not likely due to the expansion of the overall numbers of HSCs, but rather to acquisition of stem cell like properties by other cells. Therefore, we compared the long-term reconstitution capacity of both HSCs (defined as CD150$^+$Sca-1$^+$c-kit$^+$CD48$^-$Lin$^-$) and MPPs (defined as Sca-1$^+$c-kit$^+$CD150$^-$CD48$^-$Lin$^-$) from wild-type and triple mutant mice. We performed two independent experiments where we injected either 100 or 500 HSCs or MPPs from 4 different triple mutant mice into lethally irradiated recipients along with a radioprotective dose of recipient-type bone marrow. Contribution to the generation of lymphocytes, monocytes and granulocytes after 12 weeks is an indicator of long-term reconstitution (Morrison and Weissman, 1994). Consistent with previous reports (Kiel et al., 2005b; Kim et al., 2006; Yilmaz et al., 2006a), wild-type HSCs, but not MPPs contribute to the long-term generation of monocytes and granulocytes (Figure 4.8, 4.9). Recipient mice injected with HSCs from the bone marrow of triple mutant mice were long-term multi-lineage reconstituted (Figure 4.8 and table 4.3). Surprisingly, recipient mice injected with MPPs from each of the 4 triple mutant mice were also long-

term multi-lineage reconstituted (Figure 4.9 and table 4.3). Contribution to hematopoiesis was observed at 14 to 20 weeks (Figure 4.8, 4.9, 4.12, and table 4.3). Observing the kinetics of engraftment clearly shows a decline in the percentage of donor cells contributed by wild-type MPPs indicative of short-term reconstitution activity, as opposed to the triple mutant MPPs that exhibit long-term engraftment potential comparable to that of wild-type HSCs and triple mutant HSCs (Figure 4.10 and 4.11). These results taking together suggest that the greater frequency of cells capable of long-term reconstituting ability observed during limiting dilution analysis of triple mutant bone marrow was due to both the HSC and MPP populations of the mutant mice. An examination of H&E sections of liver, spleen, and bone marrow from triple mutant HSC and MPP reconstituted recipient mice at 20 weeks shows that the hematolymphoid organs of the recipient mice display normal histology at the time of examination suggesting that the triple mutant HSCs and MPPs are not in this case contributing to significantly extramedullary hematopoiesis at this time point (Figure 4.16). Upon secondary transplantation of approximately 2×10^6 whole bone marrow cells from recipient mice engrafted for 20 weeks with 100 triple mutant HSCs or MPPs, we observed contribution to donor $CD3^+$ lymphocytes and $Gr-1^+$ myeloid cells in lethally irradiated recipients (Figure 4.15 and Table 4.3) 12 weeks post-transplantation, indicating cells with the phenotype of MPPs in triple mutant mice can generate mature blood cells of both lymphoid and myeloid lineages for extended periods of time.

Immunophenotypic analysis by flow cytometry showed that there was at most twice the frequency of HSCs by phenotype ($CD150^+Sca-1^+c-kit^+CD48^-$ Lin^-) and a relatively identical frequency of MPPs by phenotype ($Sca-1^+c-kit^+CD150^-CD48^-Lin^-$) in

p16^{Ink4a}/p19^{Arf}/Trp53 deficient bone marrow compared to wild-type marrow (Figure 4.7, 4.8). We also used Flt3, which is expressed by normal MPPs but not HSCs, as an alternative marker to distinguish HSCs from MPPs (Figure 4.17) (Adolfsson et al., 2001; Christensen and Weissman, 2001). We found that the wild-type and triple mutant Sca-1$^+$c-kit$^+$CD150$^-$CD48$^-$Lin$^-$ cells expressed higher levels of this marker than did cells with a stem cell phenotype of CD150$^+$Sca-1$^+$c-kit$^+$ CD48$^-$ Lin$^-$ (Figure 4.7). This further suggests that the phenotype of HSCs and MPPs is not altered in the triple mutant mice.

In vitro proliferation and colony formation of wild-type and *p16^{Ink4a}/p19^{Arf}/Trp53* HSCs and multipotent progenitors

Next, we performed 3 independent *in vitro* proliferation experiments to provide evidence that triple mutant CD150$^+$Sca-1$^+$c-kit$^+$Flt3$^+$CD48$^-$Lin$^-$ cells were indeed MPPs and to understand the mechanisms by which their lifespan was extended. We used culture conditions that allow HSCs to expand and self-renew for a period *in vitro* (Zhang et al., 2006a; Zhang and Lodish, 2004; Zhang and Lodish, 2005). In each experiment we examined the expansion potential of HSCs and MPPs by double sorting 1 or 5 cells into U-bottom 96 well plates with 48 replicates per cell type (Table 4.4). When wild-type MPPs were cultured in StemSpan serum-free media containing the growth factors SCF, TPO, IGF-2, FGF-1, and Angptl3 (STIFA media) they proliferated 5 fold less than wild-type HSCs (Figure 4.18) and the addition of Flt3 ligand to the media doubled their proliferative rate (Figure 4.18). Similarly, in STIFA media triple mutant MPPs proliferate about 5 fold less than triple mutant HSCs and still respond to Flt3 ligand (Figure 4.18). This suggests that they maintain their phenotype and are a cell population that is biologically different than HSCs. In two independent experiments, we also determined

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the percentage of cells undergoing apoptosis in STIFA media in the case of HSCs and STIFA media plus Flt3 ligand for MPPs, measured via annexin V staining. Triple mutant HSCs and MPPs have 3 fold and 2 fold decreases respectively in the percentage of cells undergoing apoptosis (Figure 4.19). The proliferation advantage of triple mutant HSCs and MPPs could be partly due to a decrease in rate of apoptosis in the triple mutants compared to their wild-type counterparts.

To further examine the proliferative capacity of wild-type and triple mutant HSCs and MPPs, we observed the colony forming capacity of single cells double sorted into 96 well plates in Methocult GF M3434 media (Methocult) and Methocult GF M3434 media supplemented with IL-11, GM-CSF, TPO, and Flt3 ligand (Methocult plus) (Table 4.5 and Figure 4.20) in 3 independent experiments. Triple mutant HSCs form more secondary colonies than wild-type HSCs in Methocult plus media, while the triple mutant MPPs form significantly more colonies than wild-type MPPs in both Methocult plus and Methocult medias (Figure 4.20). This clearly shows that triple mutant MPPs have an enhanced proliferative capacity and in the Methocult plus media, they proliferate and form secondary colonies more effectively than even wild-type HSCs (Figure 4.20). When a colony was isolated from secondary colonies that had formed from each cell type and replated in Methocult and Methocult plus media, the proliferative advantage of Triple mutant HSCs and MPPs could clearly be seen (Figure 4.22)

Loss of the *Ink4a/Arf* and *Trp53* loci does not remove the constraints limiting the self-renewal capacity of myeloid progenitors

We then examined the immunophenotypic frequency of the myeloid progenitor compartment. We did not observe a significant difference in the immunophenotypic frequency of the myeloid progenitor compartment in $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ mice compared to wild-type mice (Figure 4.24).We next asked whether loss of the *Ink4a/Arf* and *Trp53* loci conferred long-term reconstituting ability on all proliferating blood cells. To do this, we transplanted 3 recipient mice with 100 c-kit$^+$Sca-1$^-$Lin$^-$ myeloid progenitors, a more differentiated progenitor population. We could not detect contribution to mature lineages at 4 weeks, 10 weeks, and stopped analysis at 16 weeks (Figure 4.23). Next, we transplanted 8 recipient mice with 500 or 1,000 triple mutant common myeloid progenitors (CD34$^+$FcγR^{lo}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ (CMPs) and another 8 recipient mice with 500 or 1,000 granulocyte/macrophage progenitors (CD34$^+$FcγR^{hi}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ (GMPs) (Akashi et al., 2000). After 3 weeks, we were unable to detect mature myeloid cells that were descended from the transplanted CMPs or GMPs (Table 4.3). These results suggest that as progenitor cells mature the constraints on self-renewal increase (Figure 4.25).

DISCUSSION

The cellular hierarchy seen in many tissues with self-renewing stem cells and short lived progenitors likely evolved to limit the number of cells that can accumulate oncogenic mutations. These data demonstrate that loss of both *Ink4a/Arf and Trp53* loci results in substantial expansion of self-renewing cells. Our results suggest that triple mutant MPPs have acquired long-term reconstituting ability, implying that the compound

deletion of $p16^{Ink4}$, $p19^{Arf}$, and *Trp53* removes constraints limiting self-renewal in early

progenitors (Clarke and Fuller, 2006). However, triple mutant CMPs and GMPs, more

differentiated progenitor cells, still have limited life-spans (Figure 4.25), suggesting that

there must be other genetic pathways that also prevent self-renewal in mature

progenitors. This further supports the notion that there are multiple genetic determinants

that regulate the frequency of self-renewing hematopoietic cells in vivo (Morrison et al.,

2002). It suggests that further mutations in pathways restricting self-renewal are

necessary for leukemias that are maintained by transformed CMPs and GMPs (Cozzio et

al., 2003; Huntly et al., 2004; Jamieson et al., 2004; Krivtsov et al., 2006).This expansion

of long-lived cells potentially reveals a cellular mechanism by which these genes

contribute to oncogenesis and may explain why they, or other components of their

regulatory pathways, are so commonly mutated or dysregulated in multiple tumor types

(Berggren et al., 2003; Burke et al., 2005; Esteller et al., 2000; Weber et al., 2002; Zolota

et al., 2007). The regulation of self-renewal in wild-type HSCs by epigenetically

repressing the *Ink4a/Arf* locus and *Trp53* pathway in contrast to that of triple mutant

MPPs achieved by genetic deficiency of *Ink4a/Arf* locus and *Trp53* , hints at differences

in the regulation of self-renewal in normal cells and self-renewing cancer cells that

harbour mutations in these genes. Such differences could be exploited as therapeutic

targets.

MATERIALS AND METHODS

Mice. $Trp53^{-/-}$ mice (B6.129S2-$Trp53^{tm1Tyj}$) were purchased from the Jackson

Laboratory, $p16^{Ink4a-/-}$ mice (FVB/N.129-$Cdkn2a^{tm2Rdp}$) and $p16^{Ink4a-/-}p19^{Arf-/-}$ mice

(B6.129-$Cdkn2a^{tm1Rdp}$) were obtained from Mouse Models of Human Cancers

Consortium (NCI-Frederick). $p19^{Arf-/-}$ mice were obtained from Dr. Charles Sherr (St.

Jude Children's Research Hospital, TN). All mice were bred with BA mice (C57Bl/Ka-

CD45.2/Thy1.1) at least 5 times, and verified for Thy1.1, H-2^b, and CD45.2

homozygosity by peripheral blood analysis using flow cytometry. Recipient mice in

transplant assays were adult C57Bl/Ka-CD45.1/Thy1.2 mice. All mice used for this study

were maintained at the University of Michigan Animal Facility or Stanford Animal

Facility in accordance with the guidelines of both Institutional Animal Care Use

Committees.

Flow cytometry. Antibodies used for sorting bone marrow cells and analysis were

lineage markers (CD3, CD5, CD8, Gr-1, B220, and Ter119), Sca-1, c-kit, CD135 (Flt3),

FcγR(CD16/CD32), CD34, IL-7R, CD150, and CD48. Cells were analyzed or sorted

using a Vantage fluorescence-activated cell sorter (BD) or FACSAria cell sorter (BD).

For earlier analysis of HSC frequency in wild-type and mutant mice, we used Lin$^-$CD135$^-$

Thy-1.1^{lo}Sca-1$^+$c-kit$^+$ HSCs (Christensen and Weissman, 2001). For analysis and sorting

of wild-type and triple mutant HSCs and MPPs we used CD150$^+$CD48$^-$Sca-1$^+$Lin$^-$c-kit$^+$

HSCs and CD150$^-$CD48$^-$Sca-1$^+$Lin$^-$c-kit$^+$ MPPs as previously described (Kiel et al.,

2005b; Kim et al., 2006; Yilmaz et al., 2006a). For sorting of wild-type and triple mutant

myeloid progenitors we used c-kit$^+$Sca-1$^-$Lin$^-$ myeloid progenitors, while for further

fractionation we used CD34$^+$FcγR^{lo}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ CMPs, CD34$^+$FcγR^{hi}IL-7R$^-$

Sca-1$^-$ Lin$^-$ c-kit$^+$ GMPs and CD34$^-$FcγR^{lo}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ MEPs (Akashi et al., 2000). For lineage analysis, both bone marrow cells and splenocytes were stained with antibodies against Gr-1, Mac-1, CD3, B220, and Ter119, and thymocytes with antibodies against CD3, CD4, and CD8. For peripheral blood analysis, red blood cells were lysed with hypotonic buffer, and nucleated cells were stained with antibodies against CD45.2, Gr-1, Mac-1, CD3, and B220. Antibodies were directly conjugated or biotinylated and purchased from e-Bioscience, BD Biosciences, or Biolegend.

Long-term competitive reconstitution. C57Bl/Ka-CD45.1/Thy1.2 congenic mice were lethally irradiated (1140 rads) at a dose rate of approximately 3 Gy/min, delivered in two doses 4 hrs apart. The next day, mice were competitively reconstituted by retro-orbital venous sinus injection of whole bone marrow cells from donor mice mixed with radioprotective dose of 2 x 10^5 bone marrow cells from un-irradiated C57Bl/Ka-CD45.1/Thy1.2 mice. Peripheral blood was drawn monthly up to 20 weeks to monitor reconstitution by donor type (CD45.2) myeloid and lymphoid cells as described above. Mice that had more than 1% donor-derived (Ly5.1$^+$) cells in both lymphoid (CD3$^+$ and B220$^+$) and myeloid (Gr-1$^+$ and Mac-1$^+$) subpopulations were considered to be repopulated by donor cells. The secondary bone marrow transplant was performed using 1 x 10^6 or 2 x 10^6 whole bone marrow cells. Frequency of long-term reconstituting cells from limiting dilution experiments was calculated using L-Calc software (StemCell Technologies).

In vitro **proliferation, colony formation, and Annexin V analysis.** We double sorted 1 or 5 wild-type or triple mutant HSCs or MPPs into U-bottom 96 well plates containing 200μl of StemSpan serum-free media (StemCell technologies) supplemented with 10μg/ml of heparin (sigma), 10ng/ml mouse SCF, 20ng/ml mouse TPO, 20ng/ml mouse

IGF-2, 100ng/ml mouse Angptl3 (all from R&D), and 10ng/ml human FGF-1 as previously described (Zhang et al., 2006a; Zhang and Lodish, 2004; Zhang and Lodish, 2005). In the case of both wild-type and triple mutant MPPs we also clone sorted 1 or 5 cells into the StemSpan serum-free media supplemented as above with the addition of 30ng/ml mouse Flt3 ligand (R&D). The cell counts were performed on day 12.

For colony formation we double sorted single wild-type or triple mutant HSCs or MPPs into U-bottom 96 well plates containing 100μl of Methocult GF M3434 media (StemCell Technologies) or 100μl of Methocult GF M3434 media supplemented with 10ng/ml mouse IL-11, 50ng/ml mouse GM-CSF, 50ng/ml mouse TPO, and 10ng/ml mouse Flt3 ligand (all from R&D) as previously described (Jamieson et al., 2004). Colonies were scored on day 10 of culture.

For Annexin V analysis of apoptotic cells we sorted 1000 wild-type or triple mutant HSCs into StemSpan serum-free media supplemented with the cytokines SCF, TPO, IGF-2, FGF-1, and Angptl3 or 1000 wild-type or triple mutant MPPs sorted into StemSpan serum-free media supplemented with the cytokines SCF, TPO ,IGF-2, FGF-1, Angptl3, and Flt3 ligand. The cells were cultured for 12 days and stained with Annexin V-Fitc (BD Biosciences) and the viability marker 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes).

Statistics. Statistical analysis was performed using the unpaired Student's t-test.

FIGURES
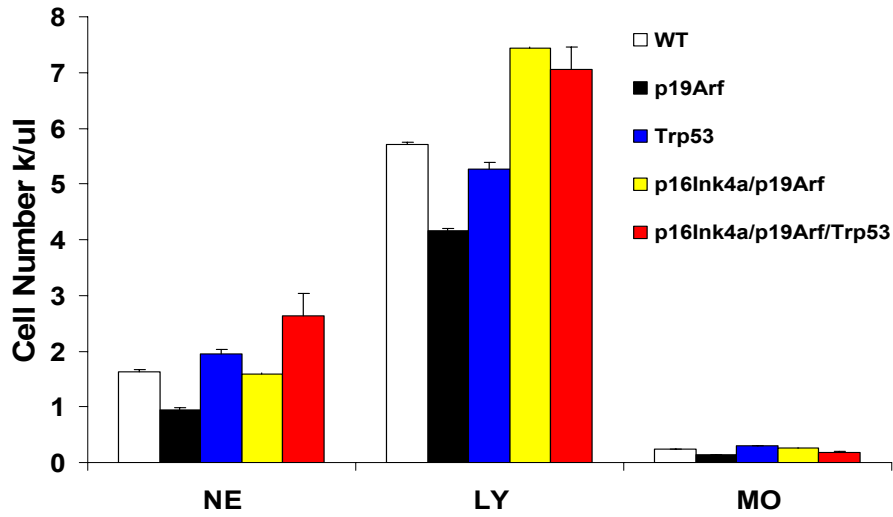
Figure 4.1. Peripheral blood analysis. Peripheral blood was analyzed for complete blood count and differential. Averages of neutrophil (NE), lymphocyte (LY) and monocyte (MO) counts from wild-type (n=31), $p19^{Arf-/-}$ (n=10), $Trp53^{-/-}$ (n=17), $p16^{Ink4a-/-}$ $p19^{Arf-/-}$ (n=12) and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ (n=4) mice are shown.
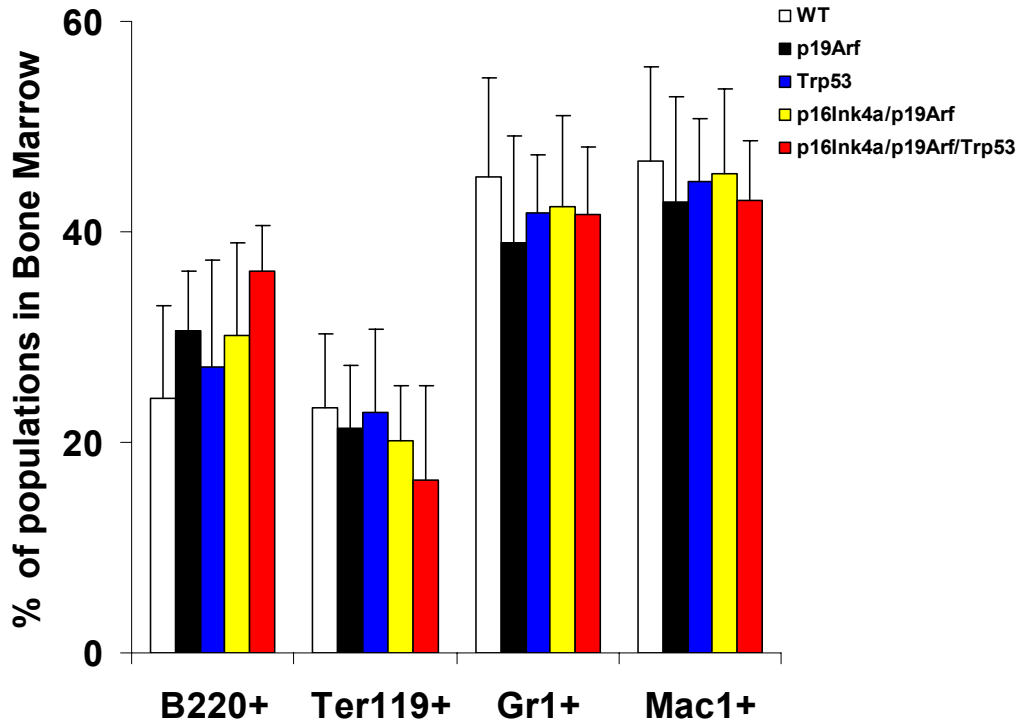
Figure 4.2 Analysis of mature hematopoietic cells in mutant mice bone marrow. The populations of B220$^+$, Ter119$^+$, Gr-1$^+$, and Mac-1$^+$ cells in the bone marrow were normal in the different mice analyzed from wild-type (n=22), *p19$^{Arf-/-}$* (n=8), *Trp53$^{-/-}$* (n=7), *p16$^{Ink4a-/-}$p19$^{Arf-/-}$* (n=11) and *p16$^{Ink4a-/-}$p19$^{Arf-/-}$Trp53$^{-/-}$* (n=3).
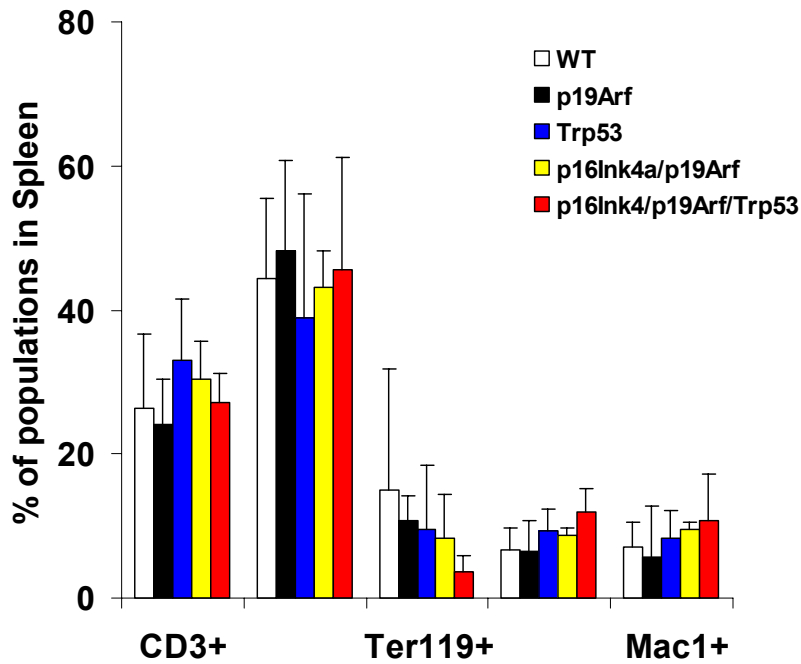
Figure 4.3 Analysis of mature hematopoietic cells in mutant mice spleen. The populations of $CD3^+$, $B220^+$, $Ter119^+$, $Gr-1^+$, and $Mac-1^+$ cells in the bone marrow were normal in the different mice analyzed from wild-type (n=22), $p19^{Arf-/-}$ (n=8), $Trp53^{-/-}$ (n=7), $p16^{Ink4a-/-}p19^{Arf-/-}$ (n=11) and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ (n=3).
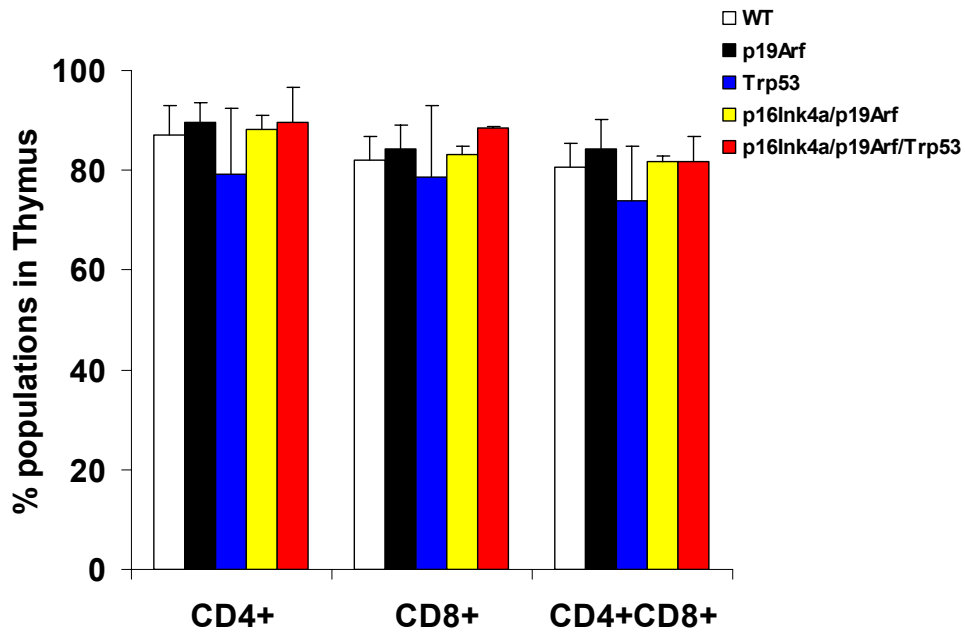
Figure 4.4 Analysis of double positive and single positive thymocytes in mutant mice thymus. The populations of CD4$^+$, CD8$^+$, CD4$^+$CD8$^+$ cells in the thymus were normal in the different mice analyzed from wild-type (n=22), $p19^{Arf-/-}$ (n=8), $Trp53^{-/-}$ (n=7), $p16^{Ink4a-/-}p19^{Arf-/-}$ (n=11) and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ (n=3).
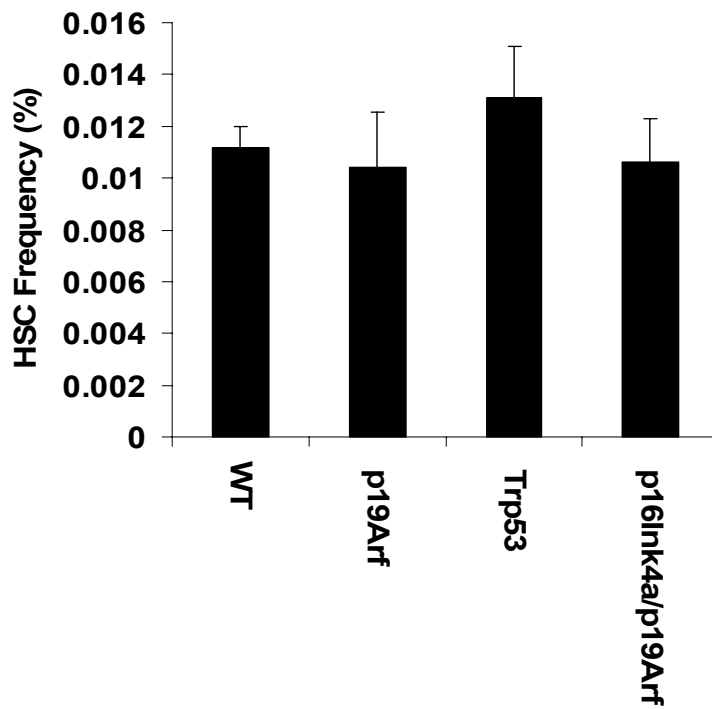
Figure 4.5 Immunophenotypic frequency of HSCs from wild-type, *p19^{Arf-/-}*, *Trp53^{-/-}*, and *p16^{Ink4a-/-}p19^{Arf-/-}* mice. HSCs were defined with the markers, Lin⁻Thy1.1^{lo}c-Kit⁺Sca-1⁺Flt3⁻.Wild-type (n=22), *p19^{Arf-/-}* (n=8), *Trp53^{-/-}* (n=9), *p16^{Ink4a-/-}p19^{Arf-/-}* (n=8). Error bars denote s.e.m.
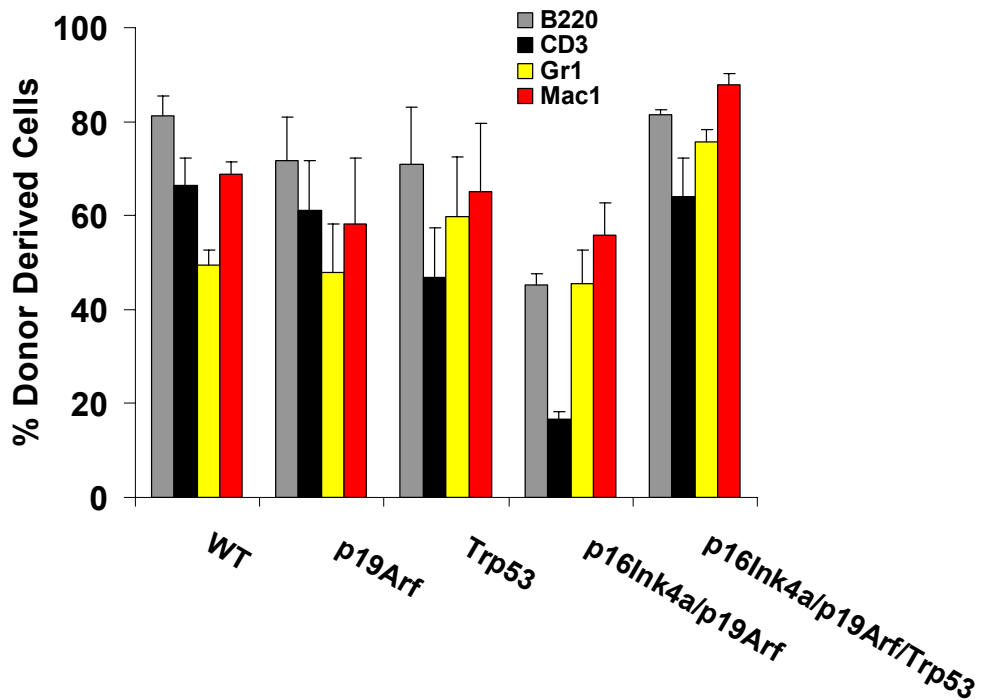
Figure 4.6 Analysis of long-term reconstitution by mutant bone marrow. 2×10^5 whole bone marrow cells from wild-type, $p19^{Arf-/-}$, $Trp53^{-/-}$, $p16^{Ink4a-/-}p19^{Arf-/-}$ and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ mice were used to competitively reconstitute lethally irradiated congenic mice along with 2×10^5 un-irradiated recipient bone marrow cells. Peripheral blood analysis at 20 weeks of lymphocytes (B220$^+$ and CD3$^+$ cells) and myeloid cells (Gr-1$^+$ and Mac-1$^+$ cells) shows that in each case the donor marrow is capable of contributing to long-term hematopoietic reconstitution.
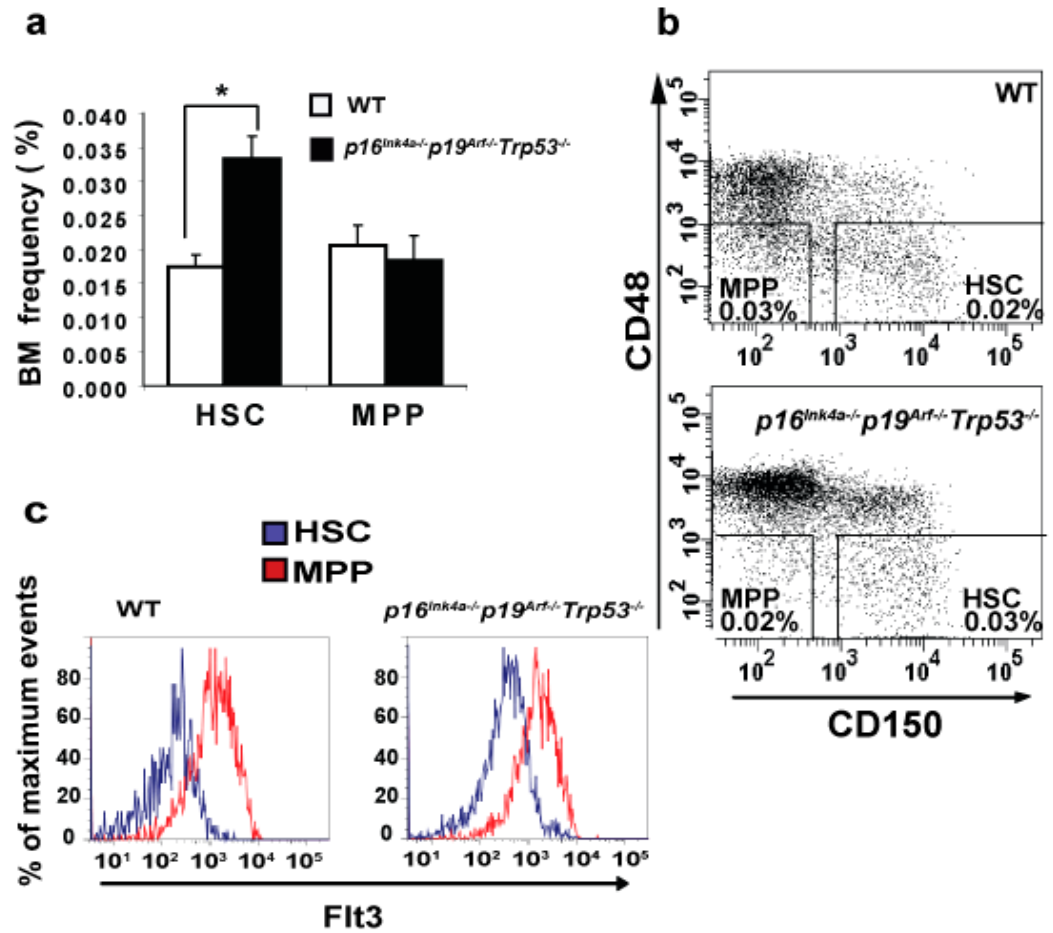
**Figure 4.7 Immunophenotypic frequency of HSCs and MPPs in wild-type and
$p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ mice.** a, The immunophenotypic frequency of HSCs
(CD150$^+$Sca-1$^+$c-kit$^+$CD48$^-$Lin$^-$) and MPPs (Sca-1$^+$c-kit$^+$CD150$^-$CD48$^-$Lin$^-$) was
assessed by flow cytometry. There was a modest increase in HSC frequency in $p16^{Ink4a-/-}$
$p19^{Arf-/-}Trp53^{-/-}$ mice compared to wild-type and no significant difference in the MPP
frequency. The averages of wild-type (n=7) and triple mutant mice (n=5) are shown. b,
A representative FACS plot gated on Sca-1$^+$c-kit$^+$Lin$^-$ bone marrow cells depicting HSC
and MPP frequency. c, Mean intensity fluorescence of Flt3 expression of wild-type (n=9)
and triple mutant (n=2) HSCs and MPPs. *, P<0.005 (Student's t-test). Error bars denote
s.e.m.
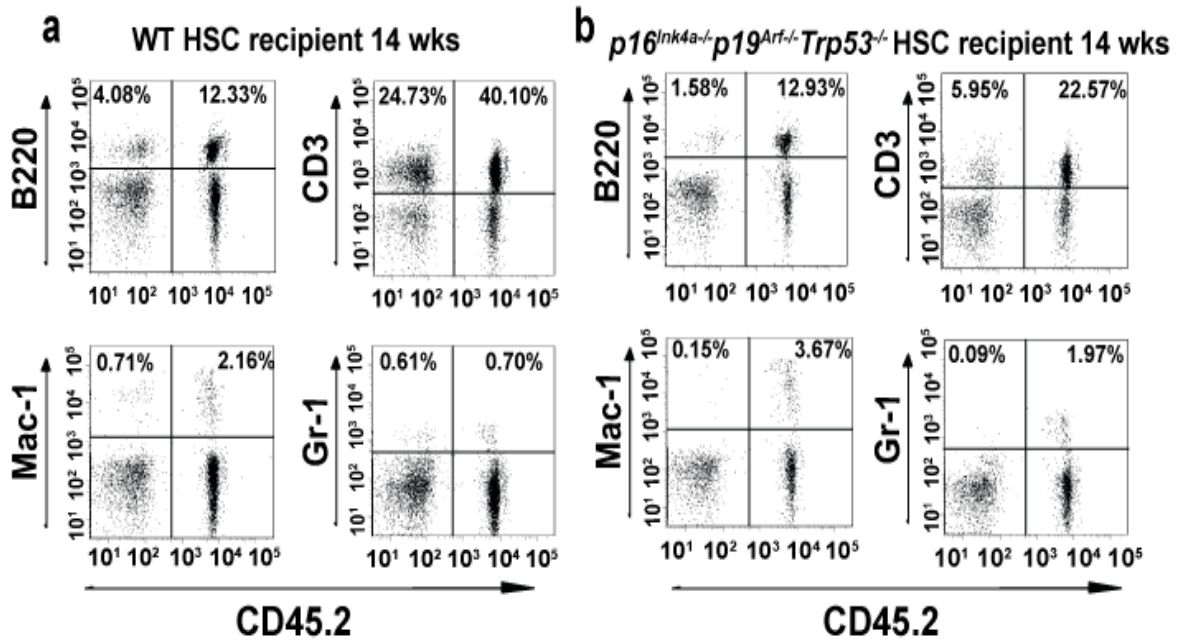
Figure 4.8 Long-term multi-lineage reconstitution by HSCs from *p16^Ink4a-/-^p19^Arf-/-^Trp53^-/-^* mice. Representative FACS plots a, Long-term reconstitution by double sorted HSCs (CD150^+^Sca-1^+^c-kit^+^CD48^-^Lin^-^) from wild-type mice. b, Transplantation of HSCs from *p16^Ink4a-/-^p19^Arf-/-^Trp53^-/-^* mice result in long-term multi-lineage reconstitution of recipient mice when analyzed 14 to 20 weeks post-transplantation (four of four mice).
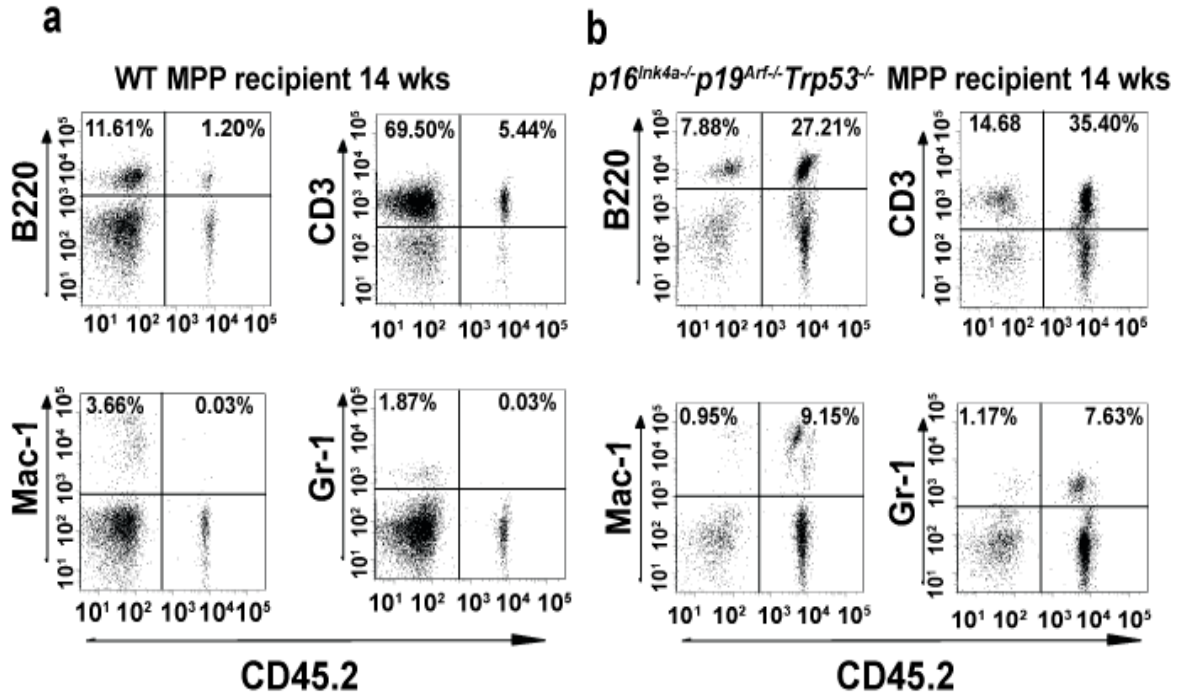
Figure 4.9 Long-term multi-lineage reconstitution by MPPs from *p16^Ink4a-/-^p19^Arf-/-^Trp53^-/-^* mice. Representative FACS plots a, double sorted MPPs (Sca-1$^+$c-kit$^+$CD150$^-$CD48$^-$Lin$^-$) from wild-type mice are only capable of contributing to long-lived lymphocyte B220$^+$ and CD3$^+$ populations and not to short lived myeloid Mac-1$^+$ and Gr-1$^+$ populations. b, surprisingly, transplanted double sorted MPPs from triple mutant mice also resulted in long-term multi-lineage reconstitution of recipient mice when analyzed 14 to 20 weeks post-transplantation (four of four mice).
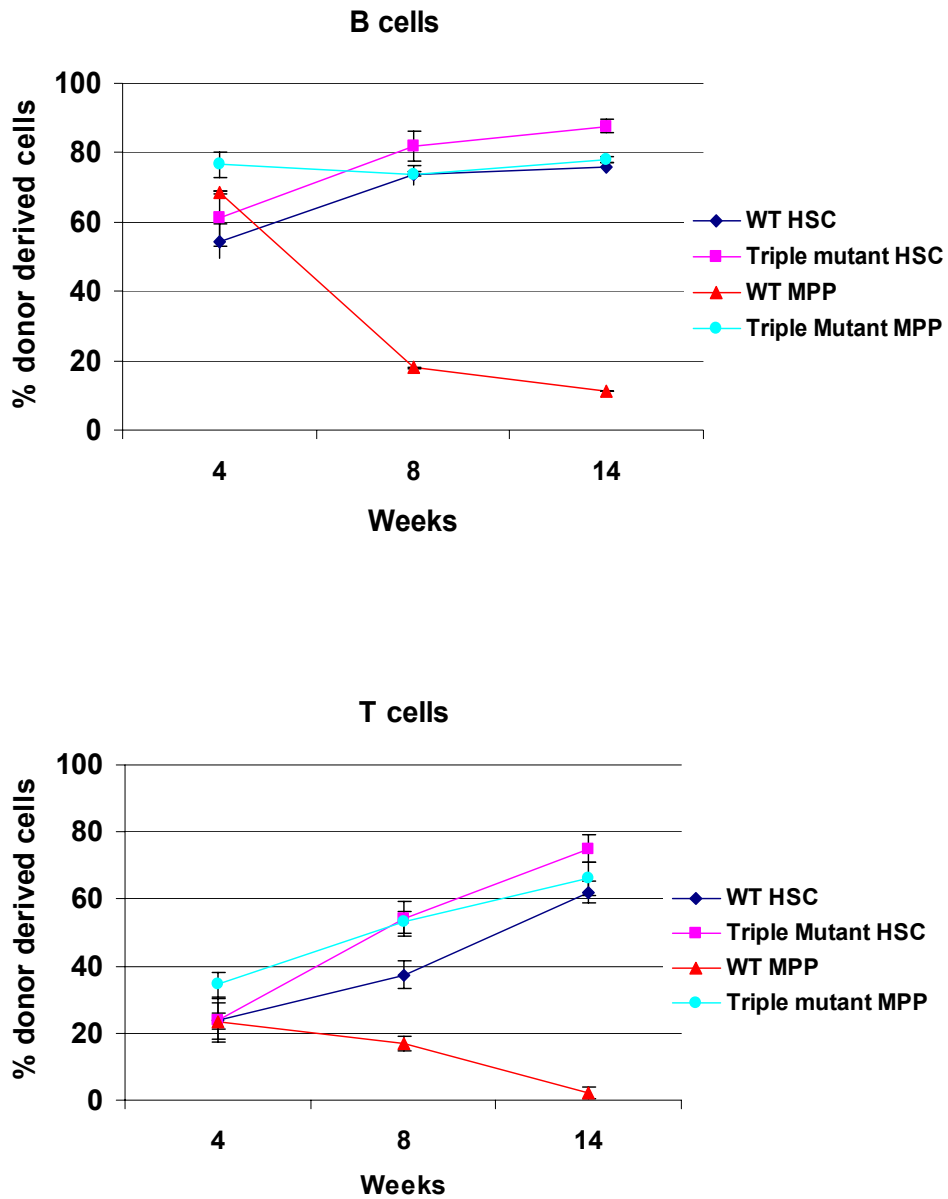
Figure 4.10 Kinetics of lymphoid engraftment for injections with 500 wild-type HSCs, 500 wild-type MPPs, 500 triple mutant HSCs and 500 triple mutant MPPs. There is a decline in the percentage of donor cells contributed by wild-type MPPs indicative of short-term reconstitution activity, as opposed to the triple mutant MPPs that exhibit long-term engraftment potential comparable to that of wild-type HSCs and triple mutant HSCs.

Monocytes

Granulocytes
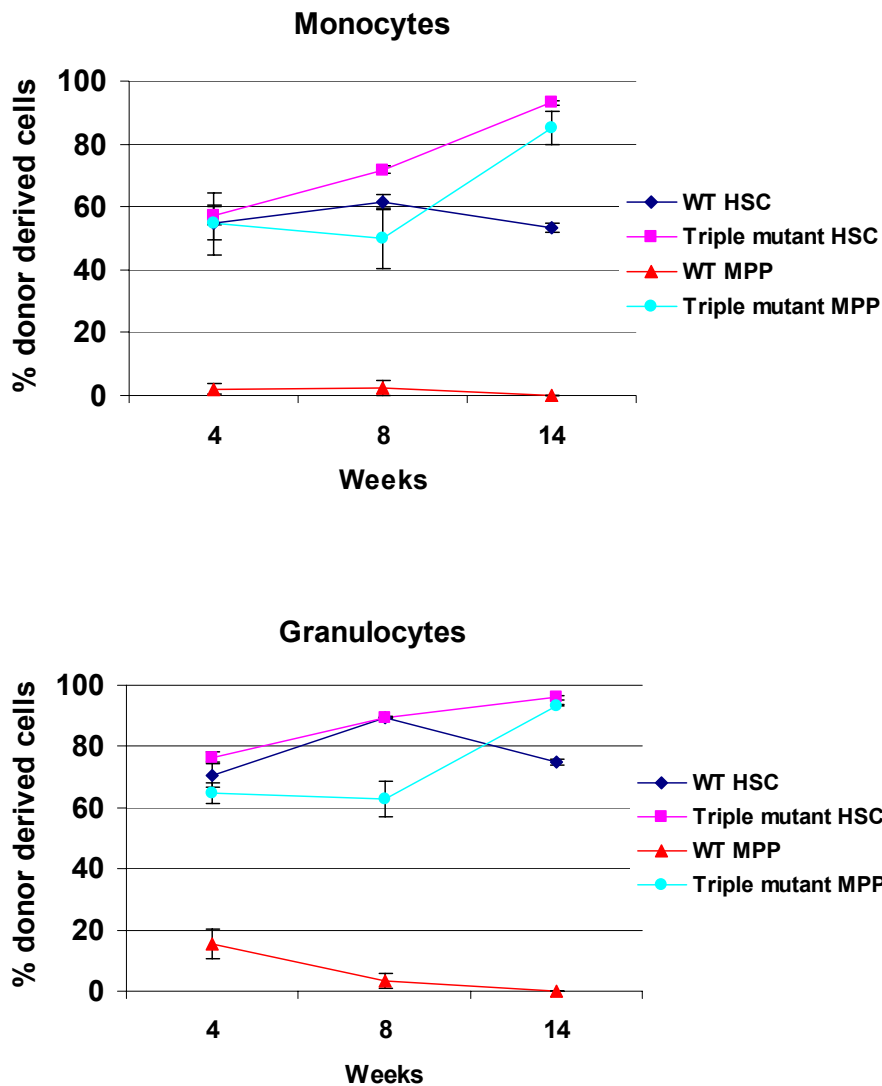
Figure 4.11 Kinetics of myeloid engraftment for injections with 500 wild-type HSCs, 500 wild-type MPPs, 500 triple mutant HSCs and 500 triple mutant MPPs. There is a decline in the percentage of donor cells contributed by wild-type MPPs indicative of short-term reconstitution activity, as opposed to the triple mutant MPPs that exhibit long-term engraftment potential comparable to that of wild-type HSCs and triple mutant HSCs.
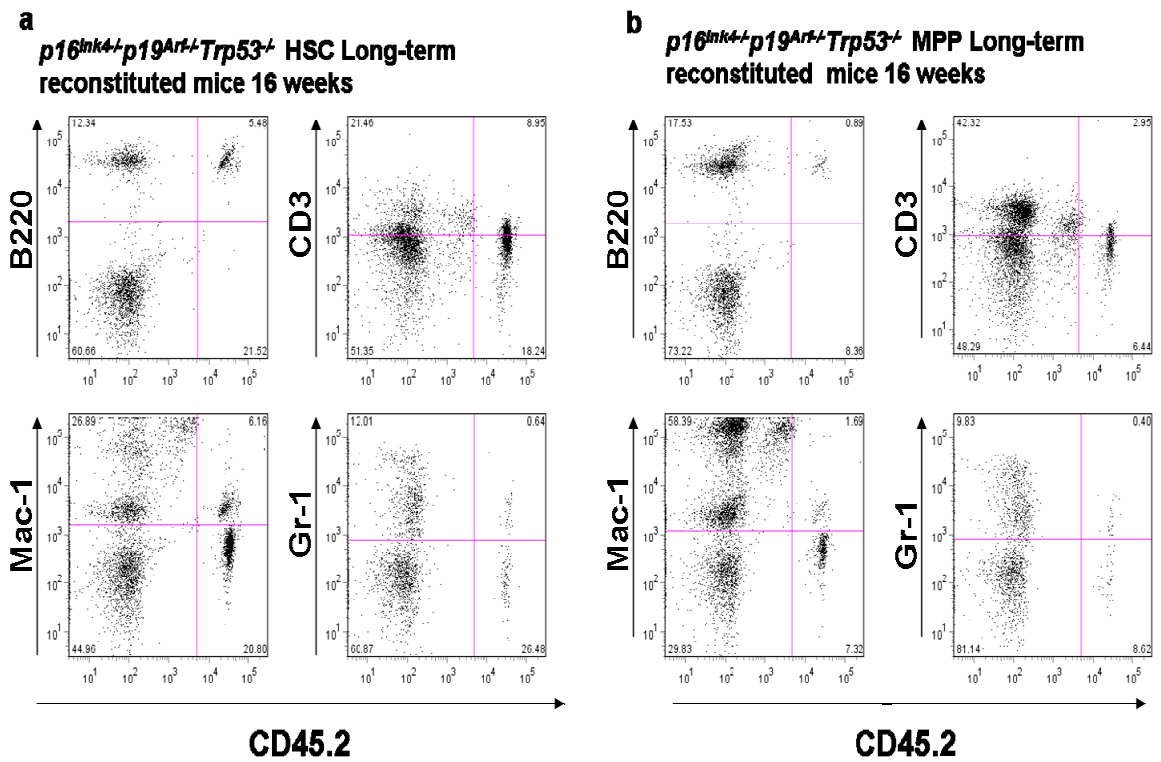
Figure 4.12 Long-term multi-lineage reconstitution at 16 weeks. Representative FACS plots a, Long-term reconstitution at 16 weeks by 100 $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ HSCs, b, Long-term reconstitution at 16 weeks by 100 $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ MPPs.
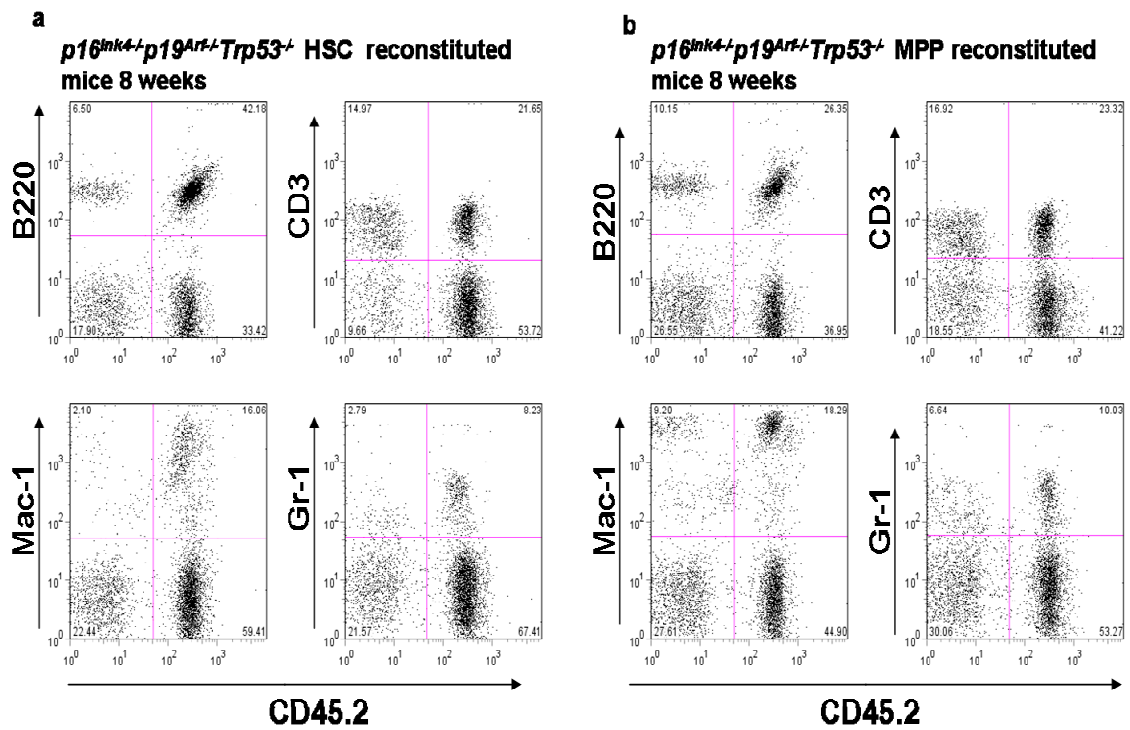
Figure 4.13 Triple mutant reconstitution at 8 weeks. Representative FACS plots a, Contribution of donor cells by 500 $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ HSCs to myeloid and lymphoid lineages 8 weeks post-transplantation. b, Contribution of donor cells by 500 $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ MPPs to myeloid and lymphoid lineages 8 weeks post-transplantation.
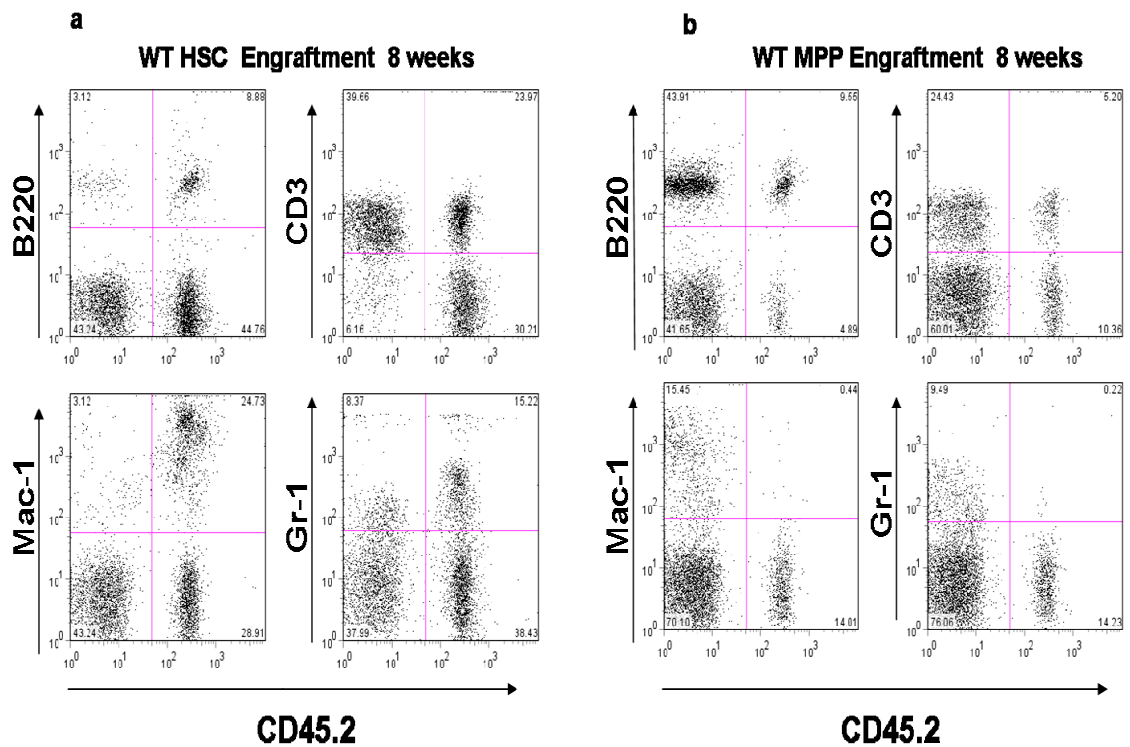
Figure 4.14 Wild-type reconstitution at 8 weeks. Representative FACS plots a, Contribution of donor cells by 500 wild-type HSCs to myeloid and lymphoid lineages 8 weeks post-transplantation. b, Contribution of donor cells by 500 wild-type MPPs to mainly B and T lymphocytes but not to myeloid cells 8 weeks post-transplantation.

a

*p16^Ink4a-/-^p19^Arf-/-^Trp53^-/-^* HSC reconstituted
marrow secondary transplant 12 weeks

b

*p16 ^Ink4a-/-^p19 ^Arf-/-^Trp53 ^-/-^* MPP reconstituted
marrow secondary transplant 12 weeks
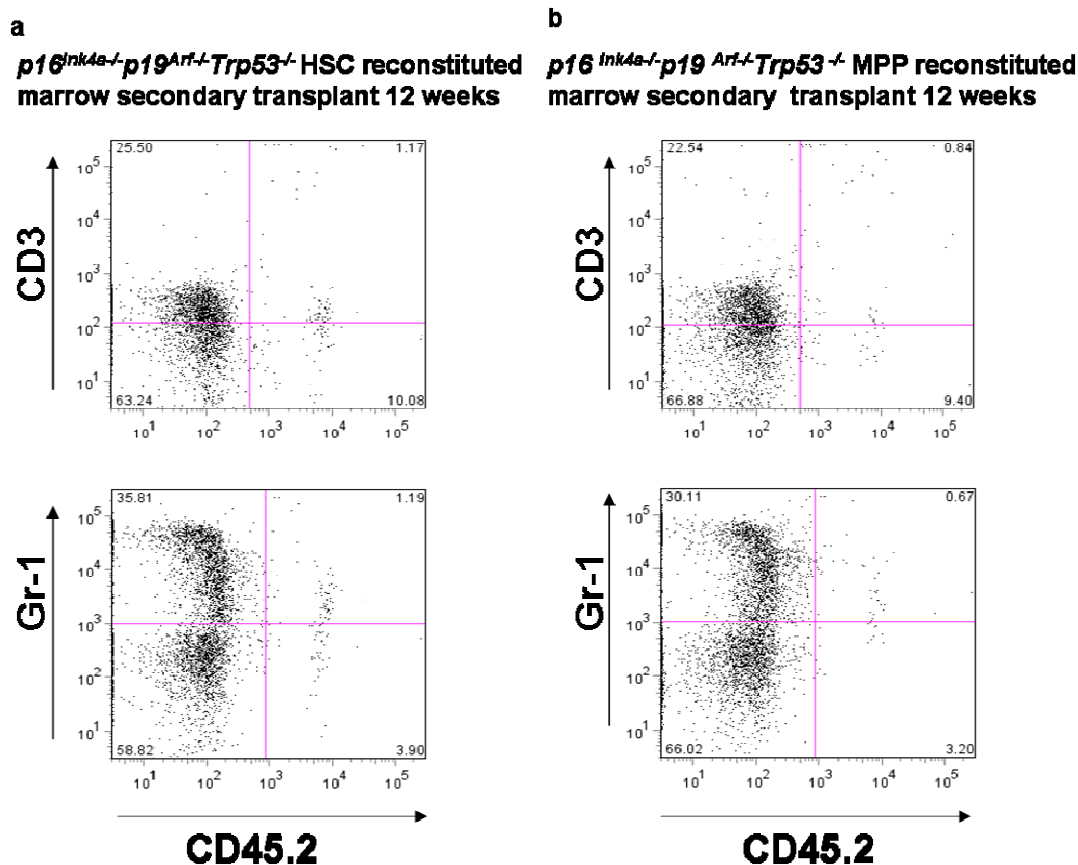
Figure 4.15 Secondary transplant engraftment of recipient mice transplanted with triple mutant reconstituted marrow. Representative FACS plots a, Engraftment at 12 weeks of triple mutant HSC reconstituted marrow showing contribution to the production of donor CD3$^+$ lymphocytes and Gr-1$^+$ myeloid cells. b, Engraftment at 12 weeks of triple mutant MPP reconstituted marrow showing contribution to the production of donor CD3$^+$ lymphocytes and Gr-1$^+$ myeloid cells. Engrafted mice had greater than 1% CD45.2 contribution to myeloid and lymphoid cells. The production of short-lived granulocytes at 12 weeks post-transplantation is an indicator of long-term engraftment.

Triple mutant MPP reconstistuted recipient

Triple mutant HSC reconstituted recipient
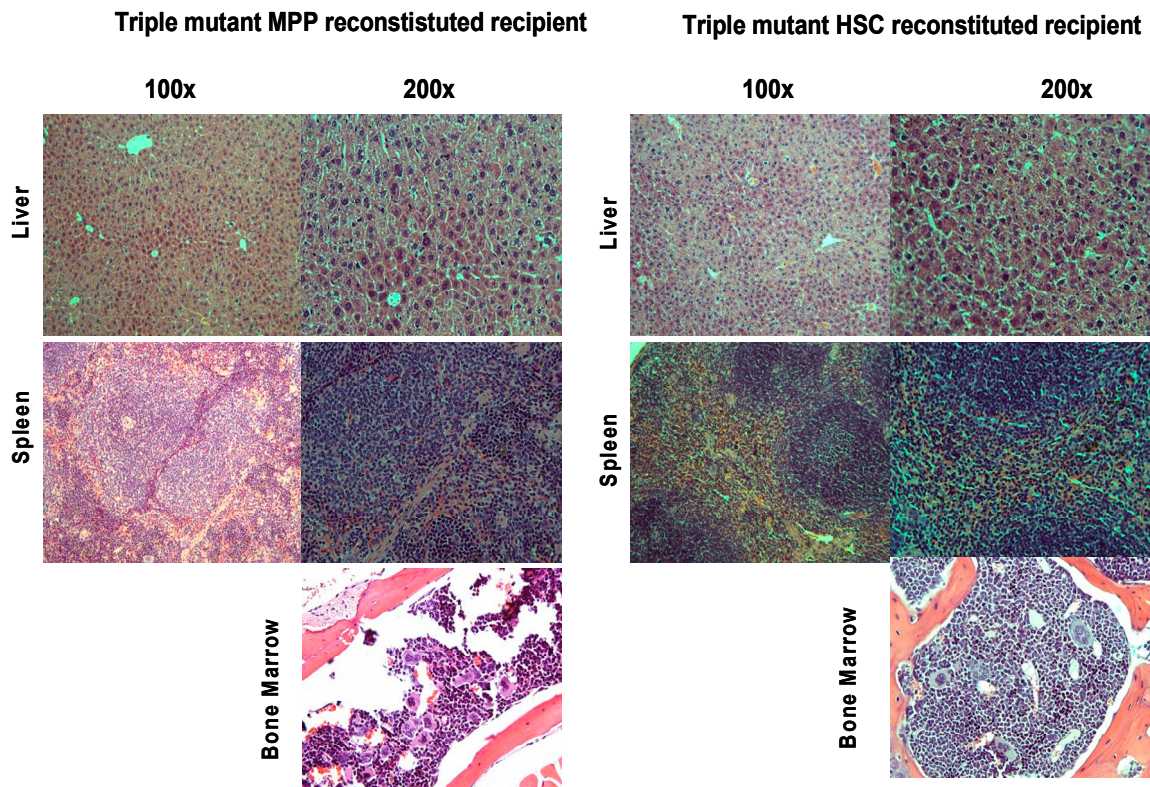
Figure 4.16 Histology of liver, spleen, and bone marrow from recipient mice transplanted with triple mutant HSCs and MPPs. HE sections from 20 week old recipient mice show that the tissues are normal. Slides were examined by a clinical pathologist.
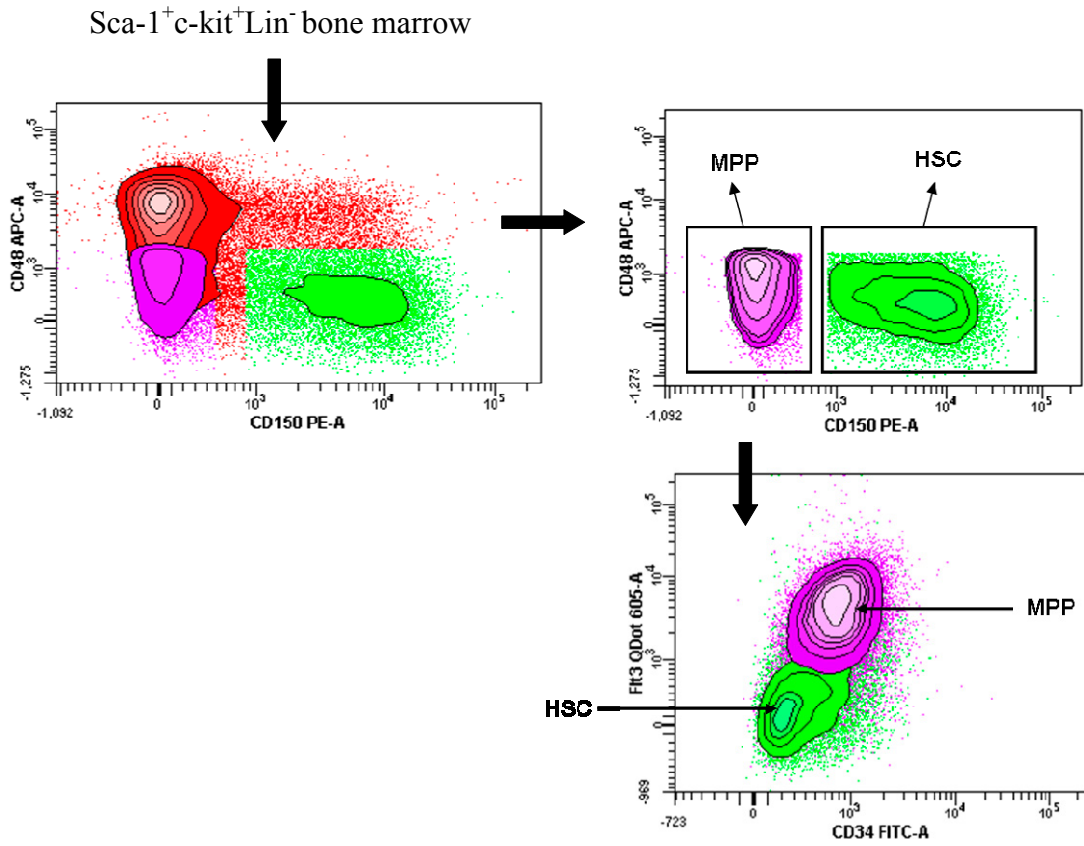
Figure 4.17 Flt3 receptor and CD34 status of wild-type HSCs and MPPs.
Representative FACS plot showing differential expression of Flt3 receptor between wild-type HSCs and wild-type MPPs.
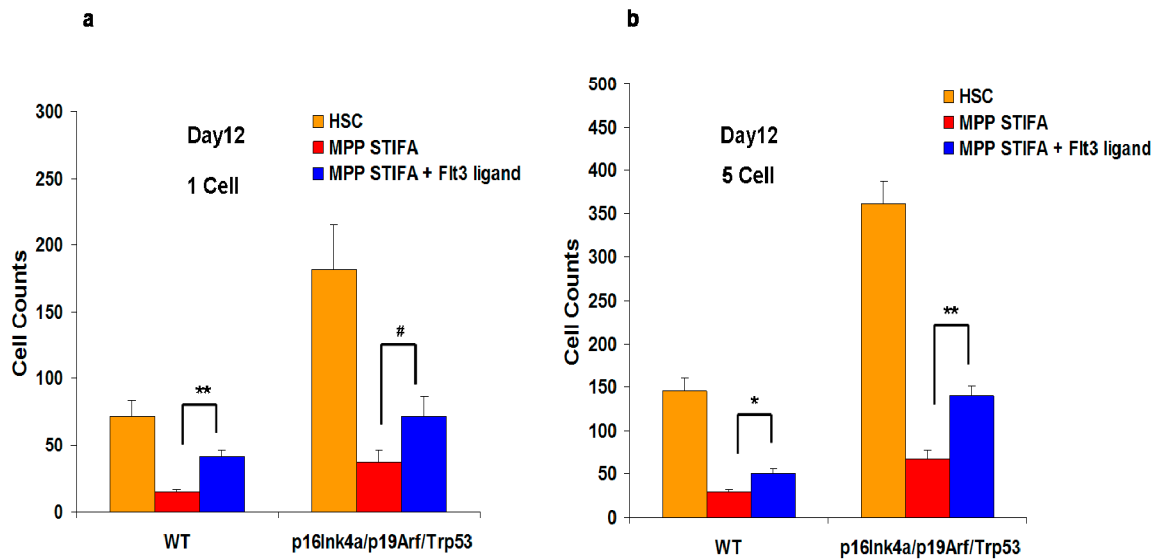
Figure 4.18 *In Vitro* proliferation of wild-type and Triple mutant HSCs and MPPs.
Proliferation of wild-type and triple mutant HSCs and MPPs in expansion media. Wild-type and triple mutant HSCs are responsive to cytokines that promote their expansion and proliferate considerably better than wild-type and triple mutant MPPs in serum free stemspan media containing SCF, TPO, IGF2, FGF1, and Angptl3 (STIFA) media. Wild-type and triple mutant MPPs show a doubling in their proliferation rates with the addition of Flt3 ligand to the STIFA media. 1 cell or 5 cells were double sorted into U-bottom 96 well plates. For 1 cell, replicates of at least 10 wells for each experimental group were counted. For 5 cells, replicates of at least 21 wells for each experimental group were counted. [#], P=0.0601; *, P<0.005; **, P<0.0003(Student's t-test). Error bars denote s.e.m.
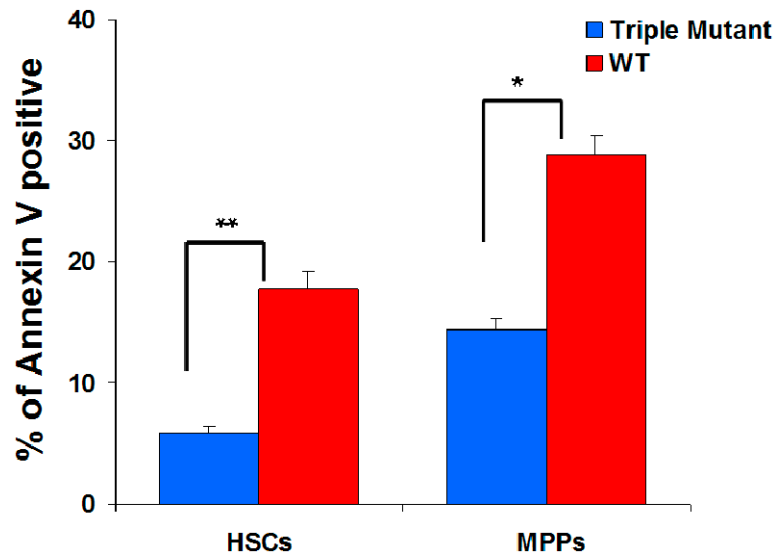
Figure 4.19 Annexin V analysis of *in vitro* cultured wild-type and triple mutant HSCs and MPPs. 1000 wild-type or triple mutant HSCs and MPPs were sorted into STIFA media and STIFA media plus Flt3 ligand respectively. The cells were analyzed on day 12 and apoptotic cells were annexin V positive and DAPI negative. Triple mutant HSCs and MPPs have 3 fold and 2 fold decreases respectively in the percentage of cells undergoing apoptosis compared to their wild-type counterparts. Replicates of at least 4 wells were used in each experiment. *, P<0.0005; **, P<0.0001(Student's t-test). Error bars denote s.e.m.
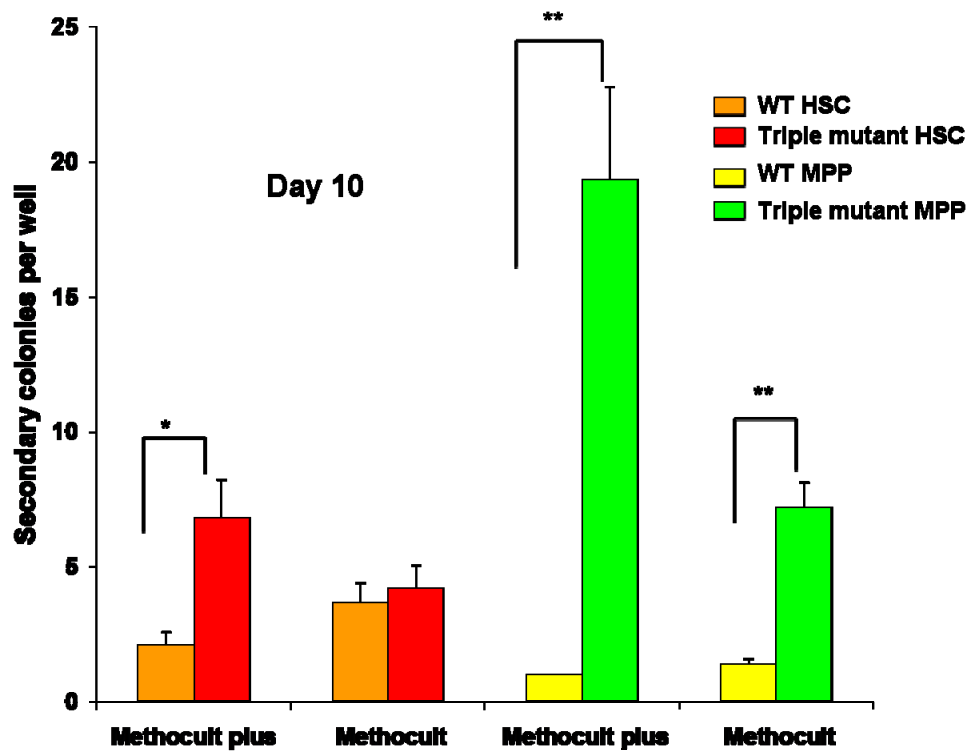
Figure 4.20 Colony formation of wild-type and Triple mutant HSCs and MPPs.
Triple mutant MPPs show an increase in the number of secondary colonies that they form
compared to wild-type MPPs in Methocult and Methocult plus media demonstrating their
proliferative advantage. Triple mutant MPPs are also very responsive to the additional
cytokines IL-11, GM-CSF, Flt3 ligand, and TPO added to the methocult media and can
form secondary colonies even better than wild-type HSCs. Single cells were double
sorted into U-bottom 96 well plates for the colony formation experiments. Replicates of
at least 20 wells were scored in the methylcellulose colony formation experiments. *,
$P<0.005$; **, $P<0.0001$(Student's t-test). Error bars denote s.e.m.

WT HSC

WT MPP

Triple Mutant HSC
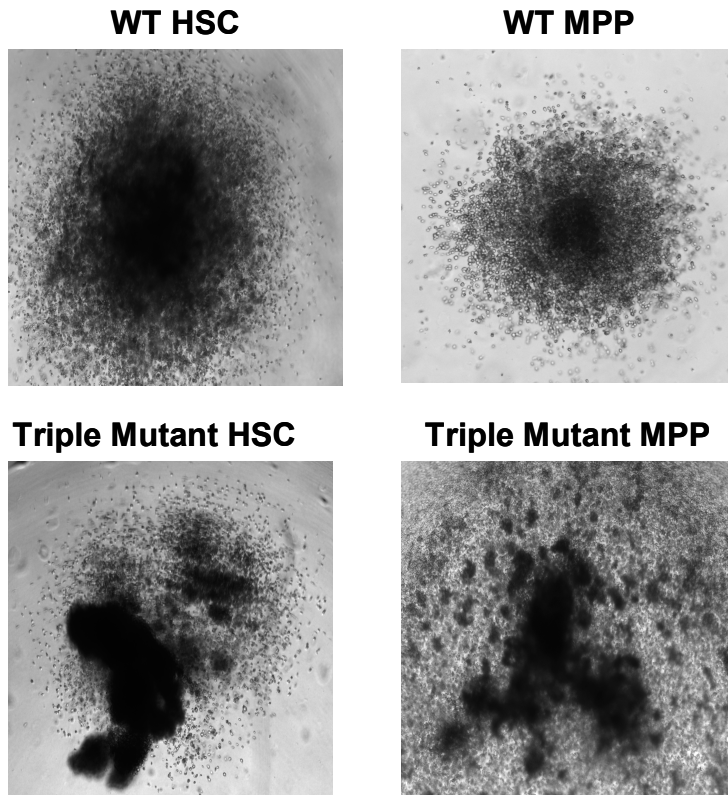
Triple Mutant MPP

Figure 4.21 Images of colonies from single cells. Representative images of wild-type and triple mutant colonies from single double sorted HSCs and MPPs taken at 25x magnification.
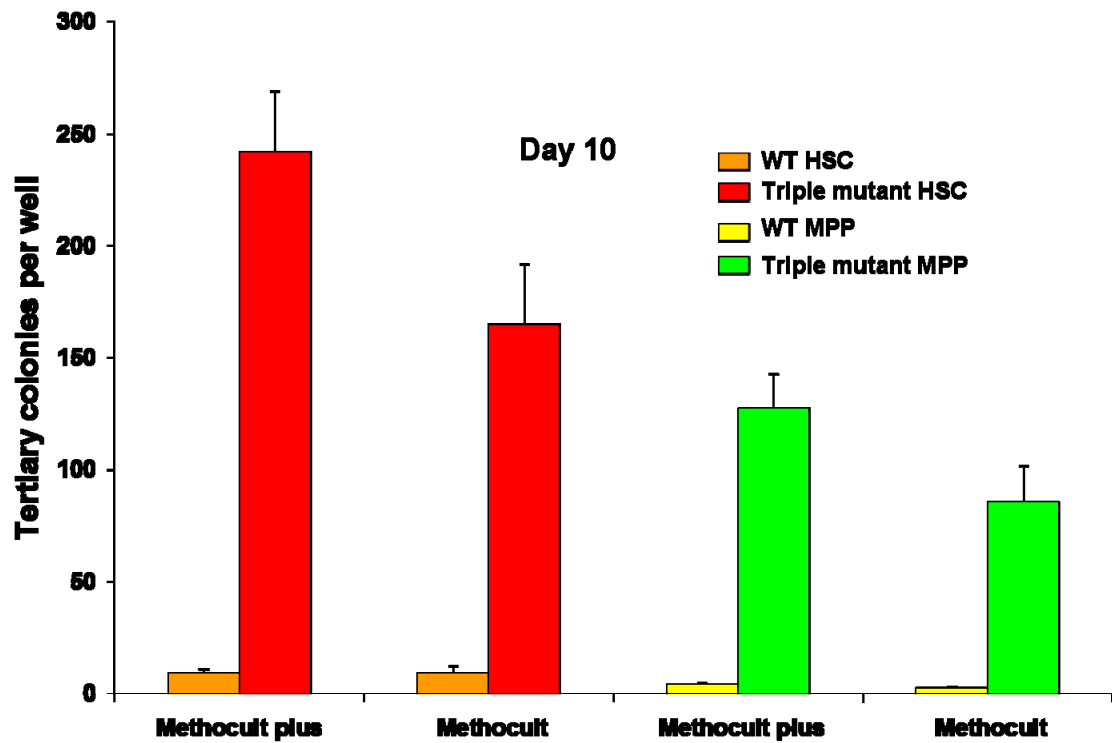
Figure 4.22 Colony formation of wild-type and triple mutant HSCs and MPPs after replating. A single colony was picked from the secondary colonies that formed after depositing single cells into Methocult and Methocult plus media. The enhanced proliferative capacity of Triple mutant HSCs and MPPs is evident. Also, it is clear that the triple mutant HSCs replate at a greater efficiency than triple mutant MPPs underscoring that they are indeed different cell types.

Figure 4.23 Lack of long-term myeloid engraftment by *p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}* myeloid progenitors. Double sorted *p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}* c-kit$^+$Sca-1$^-$Lin$^-$ myeloid progenitors do not contribute to short lived myeloid Mac-1$^+$ and Gr-1$^+$ populations in recipient mice 16 weeks after transplantation.
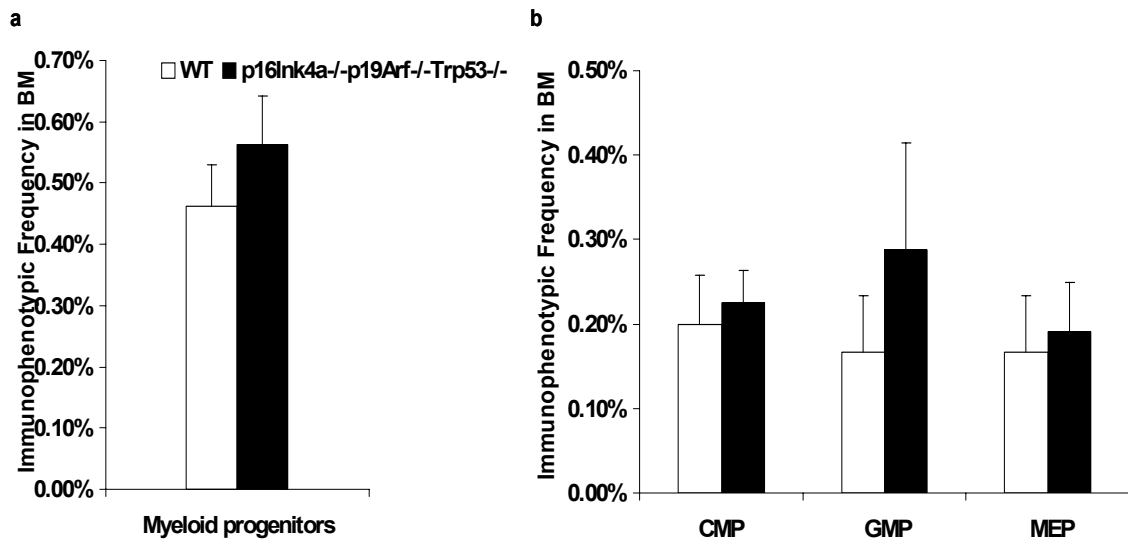
Figure 4.24 Immunophenotypic frequency of myeloid progenitors in wild-type and *p16$^{Ink4a-/-}$p19$^{Arf-/-}$Trp53$^{-/-}$* **mice.** a, Immunophenotypic frequency of c-kit$^+$Sca-1$^-$Lin$^-$ myeloid progenitors in the bone marrow assessed by flow cytometry. There was a modest but not significant increase in myeloid progenitor frequency in *p16$^{Ink4a-/-}$p19$^{Arf-/-}$Trp53$^{-/-}$* mice compared to wild-type. The averages of wild-type (n=8) and triple mutant mice (n=8) are shown. b, Further detailed analysis of the immunophenotypic frequency of defined constituent populations of myeloid progenitors;CD34$^+$FcγR^{lo}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ common myeloid progenitors (CMPs), CD34$^+$FcγR^{hi}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ granulocyte/monocyte progenitors (GMPs), and CD34$^-$FcγR^{lo}IL-7R$^-$ Sca-1$^-$ Lin$^-$ c-kit$^+$ megakaryocyte-erythroid progenitors (MEPs), show that there is a slight increase in the frequency of CMPs and MEPs in *p16$^{Ink4a-/-}$p19$^{Arf-/-}$Trp53$^{-/-}$* mice compared to wild-type mice, while there is a greater, albeit also not significant increase in the frequency of GMPs. The averages of wild-type (n=3) and triple mutant mice (n=3) are shown. Error bars denote s.e.m.

81
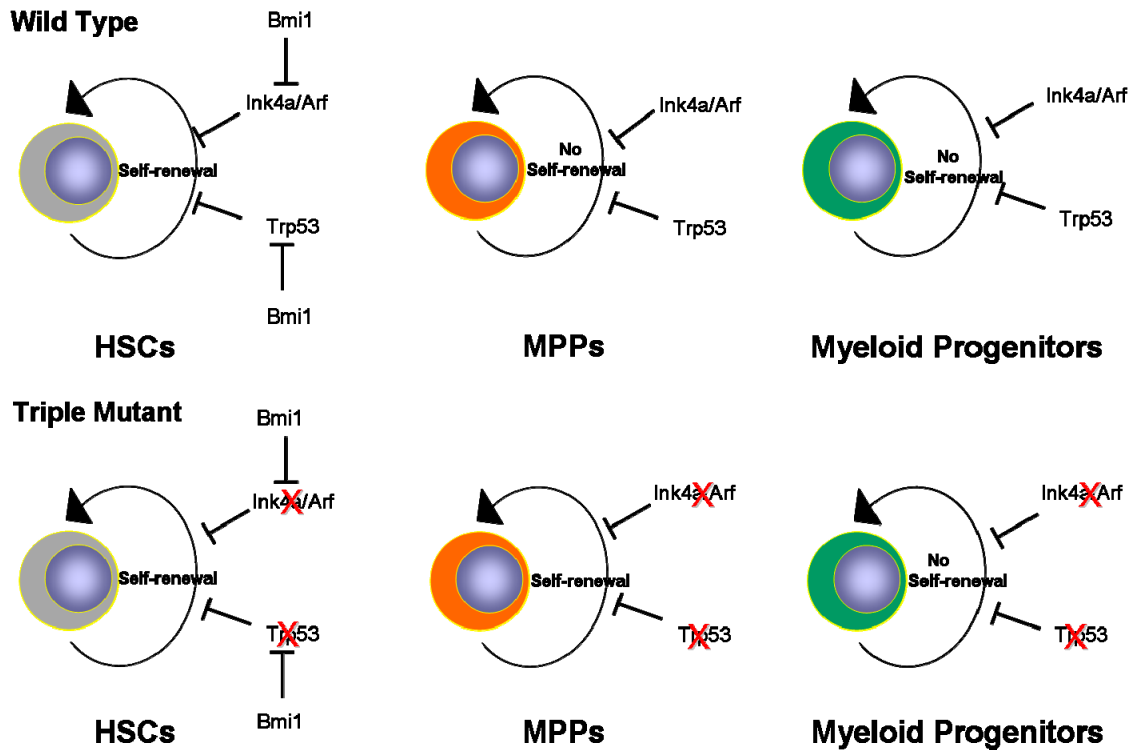
Figure 4.25 Deletion of *Ink4a/Arf* and *Trp53* removes the constraints limiting the self-renewal of early progenitors, but is not sufficient to remove the constraints limiting the self-renewal of mature progenitors. In wild-type HSCs Bmi1 epigenetically represses the *Ink4a/Arf* and *Trp53* pathways allowing HSCs to self-renew. Repression of these genes by Bmi1 in wild-type MPPs and Myeloid progenitors is not sufficient for self-renewal. In triple mutant MPPs the compound deletion of the *Ink4a/Arf* and *Trp53* loci removes the constraints limiting their capability to self-renew, however this is not sufficient in myeloid progenitors to enable them to self-renew. This suggests as progenitors differentiate and mature there are further constraints that limit their ability to self-renew.

Table 4.1 Frequency of long-term reconstituting cells determined by limiting dilution analysis

	1 long-term reconstituting cell in	Range	Frequency (%)
WT	92049	70278 - 120564	0.0011
$p19^{Arf\ -/-}$	55072	43857 - 69154	0.0018
$Trp53^{-/-}$	50317	35056 - 72222	0.0020
$p16^{Ink4a-/-}\ p19^{Arf-/-}$	53002	41344 - 67948	0.0019
$p16^{Ink4a-/-}\ p19^{Arf-/-}Trp53^{-/-}$	7657	5221 - 11230	**0.0131***

Using the limiting dilution analysis from Table 4.2, the frequency of long-term reconstituting cells was calculated according to Poisson statistics using L-calc software. $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ bone marrow showed a 10 fold increase in the frequency of cells capable of long-term reconstitution when measured from 20 weeks after transplantation compared to wild-type cells (two-tailed t-test; *$P<0.0005$). The other mutant bone marrow did not show a marked difference in the frequency of long-term reconstituting cells.

Table 4.2 Limiting dilution analysis of engraftment of WT, $p19^{Arf\ -/-}$, $Trp53^{-/-}$, $p16^{Ink4a-/-}p19^{Arf-/-}$ and $p16^{Ink4a-/-}p19^{Arf-/-}$ $Trp53^{-/-}$ bone marrow

Number of cells Injected	WT	$p19^{Arf\ -/-}$	$Trp53^{-/-}$	$p16^{Ink4a-/-}$ $p19^{Arf-/-}$	$p16^{Ink4a-/-}$ $p19^{Arf-/-}$ $Trp53^{-/-}$
2×10^3	-	-	-	-	1/4
5×10^3	-	0/2	-	-	1/4
1×10^4	0/9	0/9	-	0/4	5/6
2×10^4	-	0/4	-	-	-
2.5×10^4	-	4/5	2/5	2/4	-
5×10^4	2/4	7/11	3/4	5/10	4/4
1×10^5	8/11	8/10	3/4	10/11	4/4
2×10^5	5/6	6/6	3/3	4/4	4/4
5×10^5	7/7	4/4	-	5/5	3/3
1×10^6	5/5	-	-	-	-

In order to determine the frequency of cells capable of long-term reconstitution ability we injected wild-type, $p19^{Arf-/-}$, $Trp53^{-/-}$, $p16^{Ink4a-/-}p19^{Arf-/-}$ and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ bone marrow cells at differing doses into lethally irradiated congenic mice with 2×10^5 un-irradiated recipient bone marrow cells. Engrafted mice had greater than 1% CD45.2 contribution to myeloid and lymphoid blood cells 20 weeks post-transplantation.

Table 4.3 Engraftment of $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ HSC, MPP, and myeloid progenitor transplants.

Immunophenotype of $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ injected cells	Engrafted mice with long-term multi-lineage reconstitution	Engrafted mice from Secondary Transplant
CD150$^+$CD48$^-$Sca-1$^+$Lin$^-$c-kit$^+$ (HSCs)	4/4	yes
CD150$^-$CD48$^-$Sca-1$^+$Lin$^-$c-kit$^+$ (MPPs)	4/4	yes
Sca-1$^-$Lin$^-$c-kit$^+$ (myeloid progenitors)	0/3	-
CD34$^+$FcγR^{lo}IL-7R$^-$Sca-1$^-$Lin$^-$c-kit$^+$ (CMPs)	0/8	-
CD34$^+$FcγR^{hi}IL-7R$^-$Sca-1$^-$Lin$^-$c-kit$^+$ (GMPs)	0/8	-

Four out of four mice injected with double sorted 100 or 500 HSCs show long-term multi-lineage reconstitution 14 weeks post-transplantation. Surprisingly four out of four mice injected with double sorted 100 or 500 MPPs also show long-term multi-lineage reconstitution at 14 weeks post-transplantation. Secondary transplants of whole bone marrow from recipient mice that were long-term reconstituted by $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ HSCs and $p16^{Ink4a-/-}p19^{Arf-/-}Trp53^{-/-}$ MPPs show engraftment at 12 weeks of Gr-1$^+$ myeloid cells and CD3$^+$ lymphocytes cells. The production of short-lived granulocytes at 12 weeks post-transplantation is an indicator of the ability to sustain hematopoietic reconstitution on a long-term basis (Morrison and Weissman, 1994). There is no contribution to mature Gr-1$^+$ or Mac-1$^+$ cells by triple mutant myeloid progenitors.

Table 4.4 *In Vitro* proliferation assay of wild-type and $p16^{Ink4a-/-}p19^{Arf\ -/-}$ $Trp53^{-/-}$ HSCs and MPPs.

Cell Number	WT HSC STIFA	WT MPP STIFA	WT MPP STIFA + Flt3 Ligand	$p16^{Ink4a-/-}$ $p19^{Arf-/-}$ $Trp53^{-/-}$ HSC STIFA	$p16^{Ink4a-/-}$ $p19^{Arf-/-}$ $Trp53^{-/-}$ MPP STIFA	$p16^{Ink4a-/-}$ $p19^{Arf-/-}$ $Trp53^{-/-}$ MPP STIFA + Flt3 Ligand
1 cell	17/48	10/48	14/48	24/48	12/48	12/48
5 cell	41/48	21/48	31/48	47/48	33/48	45/48

A table from a representative *In vitro* proliferation experiment. There are more wells with cells that survive after 12 days of culture when 5 wild-type or mutant HSCs or MPPs are initially sorted into STIFA media or STIFA media plus Flt3 ligand.

Table 4.5 Colony formation assay for wild-type and *p16^Ink4a-/-^ p19^Arf -/-^ Trp53^-/-^* HSCs and MPPs.

Media	WT HSC	WT MPP	*p16^Ink4a-/-^ p19^Arf-/-^ Trp53^-/-^* HSC	*p16^Ink4a-/-^ p19^Arf-/-^ Trp53^-/-^* MPP
Methocult	27/48	20/48	32/48	21/48
Methocult Plus	28/48	30/48	32/48	27/48

A table from a representative colony formation experiment. There are more wells with colonies from both triple mutant and wild-type MPPs when additional cytokines IL-11, GM-CSF, Flt3 ligand, and TPO added to the methocult media.

CHAPTER FIVE

Conclusions and Future Directions

In this thesis, we attempt to further understand the mechanism by which Bmi1 plays a role in the maintenance of adult hematopoietic stem cells by examining the implications of its downstream targets on hematopoiesis. Chapter 2 is a review on the recurring theme of the thesis, hematopoietic stem cell self-renewal. In chapter 3, we examined the impact of deleting $p16^{Ink4a}$, $p19^{Arf}$, and $Trp53$ in a $Bmi1$ null background in order to ascertain whether they are capable of rescuing the hematopoietic defects seen in $Bmi1$ mutant mice. In chapter 4, we examined the impact of deleting $p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}$, $Trp53$, and $p16^{Ink4a}/p19^{Arf}/Trp53$ on the mature cells of the hematopoietic system.

The goal of my published review of hematopoietic stem cell self-renewal, Akala and Clarke 2006, in chapter 2 was to stress the importance to the field for the need to begin integrating the data on the different regulators of adult HSC maintenance in order to understand self-renewal at a molecular level. Can the different requirements of survival, cell cycle and proliferation control, and the maintenance of an undifferentiated state be executed simultaneously? If so, how is this achieved? Studies geared towards understanding the molecular control of pluripotency in embryonic stem cells serve as a model that can be applied to the hematopoietic system and other adult tissues that rely on a self-renewing compartment for homeostasis. These studies have begun to build a regulatory network linking transcription factors, external signaling pathways, polycomb

group proteins and microRNA genes (Boyer et al., 2005; Boyer et al., 2006; Chen et al., 2008; Kim et al., 2008; Lee et al., 2006; Marson et al., 2008). Historically, a major limitation of the biochemical and molecular characterization of HSCs is their rarity. However technological platforms allowing single cell digital RT-PCR (Warren et al., 2006), miniChIP and ChIP sequencing (Attema et al., 2007; Marson et al., 2008), and the Firefly protein analysis system (O'Neill et al., 2006) will allow analysis of a small number of cells. This approach coupled with Tet-on and Tet-off inducible retroviral expression and knockdown of regulators of HSC maintenance will allow for the building of the self-renewal network operating in HSCs. This network can then be further refined with functional experiments.

The rescue experiments in chapter 3, published in the supplementary section of Akala et al., 2008, uncover the role that downstream targets of Bmi1 play in the maintenance of the two long-lived compartments in the hematopoietic system, HSCs and lymphocytes. The deletion of $p16^{Ink4a}$ in a *Bmi1* deficient background could not rescue either the lymphocytic or hematopoietic stem cell defects of $Bmi1^{-/-}$ mice. This result was quite different from that seen in neural stem cells, where deletion of $p16^{Ink4a}$ partially rescued self-renewal, stem cell frequency, forebrain proliferation and gut neurogenesis of $Bmi1^{-/-}$ mice (Molofsky et al., 2005). However, the deletion of $p19^{Arf}$ in a *Bmi1* deficient background partially rescued lymphocytic, but not hematopoietic stem cell defects of $Bmi1^{-/-}$ mice. Bone marrow B220^{+} lymphocytes seemed to be completely rescued, while splenic B220^{+} lymphocytes were 80% of wild-type. Double positive CD4^{+}CD8^{+} thymocytes and single positive CD4^{+} thymocytes were also completely rescued, while single positive CD8^{+} thymocytes were 91% of wild-type. The differential ability of $p19^{Arf}$ compared to $p16^{Ink4a}$ to rescue lymphocytes, suggests that of the two, $p19^{Arf}$ is the main target of Bmi1 repressed in order to ensure lymphocyte maintenance. This has actually been confirmed in other work with more detailed thymocyte analysis, showing that

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thymocyte proliferation during the transition from the double negative CD4$^-$CD8$^-$ to the double positive CD4$^+$CD8$^+$ stage is dependent on Bmi1 and is affected through p19^{Arf} and not p16^{Ink4a} (Miyazaki et al., 2008). The fact that there is not a complete rescue of lymphocytes by *p19^{Arf}* in a *Bmi1* null background implies that in certain lymphoid populations there are Bmi1 targets other than p19^{Arf}. This indeed is the case in CD4$^+$ T helper (Th1/Th2) cells where the repressed target of Bmi1 is *Noxa* (Yamashita et al., 2008).

However, as in the case of *p16^{Ink4a}*, the deletion of *p19^{Arf}* in a *Bmi1* deficient background could not rescue the hematopoietic stem cell defects of *Bmi1$^{-/-}$* mice. Once again as in the case of *p16^{Ink4a}*, this result was different from that seen in neural stem cells, where deletion of *p19^{Arf}* partially rescued self-renewal, stem cell frequency, forebrain proliferation, and gut neurogenesis, as well as cerebellum development of *Bmi1$^{-/-}$* mice (Bruggeman, 2005; Molofsky et al., 2005). These data point to the differences in the tissue specificity of Bmi1 targets in the regulation of self-renewal.

The deletion of the entire *Ink4a/Arf* locus in a *Bmi1* deficient background to partially rescue hematopoietic stem cell defects of *Bmi1$^{-/-}$* mice. This has also been reported by other groups in the hematopoietic system (Oguro et al., 2006) and the same is seen in the mammary gland (Pietersen et al., 2008). Notably, this is in clear contrast to the neural system where either deletion of *p16^{Ink4a}* or *p19^{Arf}* can lead to a partial rescue of the defects of neural stem cells in *Bmi1$^{-/-}$* mice.

The more intriguing result we discovered is the difference between the phenotype resulting from the deletion of *Trp53* versus *p19^{Arf}* in a *Bmi1* deficient background. We expected the phenotypes to be similar since p19^{Arf} has been shown to be a positive regulator of Trp53 via its binding and sequestration of Trp53 inhibitor Mdm2 (Moll and Petrenko, 2003), and that overexpression of p19^{Arf} and the results in Trp53-dependent HSC apoptosis (Park et al., 2003). In the lymphocyte compartment, the rescue of B220$^+$

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B lymphocyte numbers in the bone marrow and spleen of *Bmi1$^{-/-}$Trp53$^{-/-}$* mice is 55% and 75% less than the rescue of the same populations in *Bmi1$^{-/-}$p19$^{Arf-/-}$* mice. A similar trend can be seen in the thymus where the rescue of cell number in double-positive CD4^{+}CD8^{+}, single-positive CD4^{+} thymocytes, and single-positive CD8^{+} thymocytes was 86%, 85%, and 68% of control in *Bmi1$^{-/-}$Trp53$^{-/-}$* versus *Bmi1$^{-/-}$p19$^{Arf-/-}$* mice. These results suggest that in the lymphocytic compartment, there are targets other than Trp53 which are critical for maintenance that are suppressed by Bmi1 via *p19^{Arf}*-dependent processes.

Of greater interest, we discovered that deletion of *Trp53* in a *Bmi1* deficient background is capable of partially rescuing the hematopoietic stem cell defects of *Bmi1$^{-/-}$* mice. As opposed to *Bmi1$^{-/-}$p19$^{Arf-/-}$* bone marrow, transplantation of bone marrow from *Bmi1$^{-/-}$Trp53$^{-/-}$* mice in competitive reconstitution experiments resulted in long-term multi-lineage engraftment and subsequent secondary engraftment upon serial transplantation. However, this was not complete since engraftment occurred in 15 out of 19 mice in primary transplant experiments and 17 out of 22 mice in secondary transplant experiments. Also, the rescue of the immunophenotypic frequency of HSCs in *Bmi1$^{-/-}$Trp53$^{-/-}$* mice was not complete. These results suggest that in HSCs, Bmi1 may bypass p19^{Arf} for regulation of Trp53, and directly regulate Trp53 turnover or mark Trp53 transcriptional targets for epigenetic silencing.

Recent data showing that the PRC1 complex can function as a E3 ubiquitin ligase that is capable of ubiquinating histone H2A and geminin (Ohtsubo et al., 2008; Wang et al., 2004) potentially provides a mechanism to explain how Bmi1 might bypass p19^{Arf} for regulation of Trp53. Future experiments exploring whether the transcriptional targets of Trp53 or *Trp53* itself have monoubiquinated H2A histone marks indicative of PRC1 silencing activity should be undertaken. These experiements could provide new evidence that Bmi1, as well as the PRC1 complex employ epigenetic silencing mechanisms to

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repress the Trp53 pathway in regulating HSC self-renewal. Regulation of Trp53 by Bmi1 and the PRC1 complex could also be achieved by regulating its proteosomal mediated degradation. The turnover of Trp53 is mediated by its polyubiquitination by Mdm2 and subsequent targeting to the proteasome (Moll and Petrenko, 2003). An examination of whether the PRC1 E3 ubiquitin ligase is capable of polyubiquinating Trp53 and targeting it to the proteasome, as it does in the case of geminin (Ohtsubo et al., 2008), would provide a mechanism linking Bmi1 and the PRC1 complex to the direct stabilization of Trp53 in the maintenance of HSCs. It is quite likely that both of these mechanisms will be employed and provide an   example of dual epigenetic transcriptional silencing and protein stability regulation by the PRC1 complex.

In chapter 4, published in Akala et al., 2008, we examined the impact of the deletion of the Bmi1 targets that were able to rescue a subset of the phenotypes observed in $Bmi1^{-/-}$ mice. We found that deletion of $p19^{Arf}$, $p16^{Ink4a}/p19^{Arf}$, and $Trp53$ did not have any effect on hematopoietic differentiation, the ability of bone marrow to long-term engraft in lethal irradiated mice, or on the frequency of long-term reconstituting cells in the bone marrow. Previous studies support our conclusions that in young mice, the deficiency of $p16^{Ink4a}$, $p19^{Arf}$, $Trp53$ and both $p16^{Ink4a}$ and $p19^{Arf}$ do not significantly affect the long-term reconstitution frequency of HSCs, while exerting a greater effect within the context of stem cell aging (Dumble et al., 2007; Janzen et al., 2006; Stepanova and Sorrentino, 2005).

As the regulation of stem cells is controlled by multiple genetic loci (Morrison et al., 2002), we opted to examine the impact of the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, $and\ Trp53$ on the hematopoietic system. As in the case of the other mutants, we found that hematopoietic differentiation in $p16^{Ink4a}/p19^{Arf}/Trp53$ (triple mutant) mice was not compromised and that triple mutant bone marrow was functional in long-term multi-lineage reconstitution experiments, indicative of normal stem cell activity. The more

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striking result we uncovered while performing limiting dilution experiments that assess the frequency of cells capable of long-term reconstitution, was the fact that there was approximately a 10-fold increase in the frequency of these cells in triple mutant mice compared to wild-type. We assumed that this would correspond with a concomitant increase in the immunophenotypic frequency of HSCs. However, to our surprise, we discovered only a modest increase in the immunophenotypic frequency of HSCs (defined by $CD150^+Sca-1^+c-kit^+CD48^-Lin^-$) in triple mutant mice relative to wild-type controls. We therefore decided to further examine the bone marrow of the triple mutant mice to assay whether other populations that do not normally contribute to long-term reconstitution were now doing so in the context of the compound deletions of $p16^{Ink4}$, $p19^{Arf}$, and $Trp53$. In these studies we found that the immediate progeny of HSCs, multipotent progenitors (MPPs) (defined by $Sca-1^+c-kit^+CD150^-CD48^-Lin^-$), had acquired the ability of long-term multi-lineage reconstitution. The question arose as to whether these cells were indeed MPPs or HSCs with an aberrant loss of the positive marker CD150. Experiments showed that the Flt3 receptor, a marker of multipotent progenitors (Adolfsson et al., 2001; Christensen and Weissman, 2001), was differentially expressed between triple mutant HSCs and triple mutant MPPs in the same manner seen in their wild-type counterparts. *In vitro* proliferation and colony forming experiments further showed that the pattern of triple mutant response to growth factors resembles that of wild-type MPPs, but not wild-type HSCs. Thus, the deletion of $p16^{Ink4}$, $p19^{Arf}$, and $Trp53$ in multipotent early progenitors removes the molecular constraints that limit their capacity to self-renew. We then examined further differentiated myeloid progenitors to determine whether the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, and $Trp53$ would also enable them to contribute to long-term myeloid engraftment. However, in triple mutant myeloid progenitors these deletions were not sufficient to increase their longevity. The results seen in triple mutant myeloid progenitors suggest that there must be other genetic

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pathways that prevent self-renewal in more mature progenitors, hinting at the fact that as cells differentiate, the constraints on self-renewal increase (Clarke and Fuller, 2006).

Our findings in chapter 4 give us insight into the regulation of self-renewal in HSCs and their immediate progeny, MPPs. In order for HSCs to self-renew, Bmi1 is required to epigenetically repress the *Ink4a/Arf* locus and *Trp53*. These pathways are then required in early progenitors to restrict their ability to self-renew. This suggests that the actions of the *Ink4a/Arf* locus and *Trp53* are essential for the regulation of the counting mechanisms that have evolved to limit the number of mitoses that these normally short lived progenitors can undergo in order to maintain tissue homeostasis. The next question that arises is what exactly happens when triple mutant MPPs acquire long-term reconstitution ability? To address this issue, we will need to do single cell transplants of triple mutant MPPs and analyze their clonal progeny. There is one possibility depicted in Figure 5.1, where the triple mutant MPP acquires full self-renewal capacity comparable to that of HSCs and functions as a life-long autonomous self-renewing unit in the hematopoietic system. In this case, it will go on to differentiate appropriately and result in the presence of two self-renewing multipotent cell populations in the bone marrow. The second possibility shown in Figure 5.2 is where the triple mutant MPP actually phenotypically reverts or de-differentiates into HSCs in order to execute the self-renewal program. In this scenario, we expect to find immunophenotypic HSCs included with the other more differentiated populations has clonogenic progeny of the transplanted single triple mutant MPP. Another possibility that we will test as shown in Figure 5.3 is whether the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, and *Trp53* only results in an enhanced regenerative potential in triple mutant MPPs, rather than the acquisition of life-long self-renewal capacity. In this case, we would have to subject the single triple mutant MPP reconstituted bone marrow to a series of serial transplantation experiments comparing it to the HSCs to assess if there is a decrease of its regenerative capacity.

Regardless of the exact mechanism by which the compound deletion of $p16^{Ink4}$, $p19^{Arf}$, and $Trp53$ expands the population of long-lived cells capable of long-term reconstitution in the bone marrow, it represents a substantial increase in the number of cells that can accumulate the complex set of additional mutations needed for malignant transformation (Sjoblom et al., 2006). This unveils a potential role that these genes play in oncogenesis, where mutations or dysregulation of components in these regulatory pathways expand progenitor compartments by allowing them to escape from the fail-safe mechanisms that normally restrict their longevity, potentially making them fertile ground for the acquisition of further mutations that result in malignant transformation (Clarke and Fuller, 2006). In this regard, further studies are needed to define the implications of potential oncogenic transformation in triple mutant HSCs and MPPs. These experiments should help determine whether the acquired longevity of triple mutant MPPs enhances their potential for transformation. More importantly, we plan to explore the type of malignancies that arise from triple mutant HSCs and MPPs to determine if the differences in the means by which they self-renew dictate the progression of the ensuing malignancy.
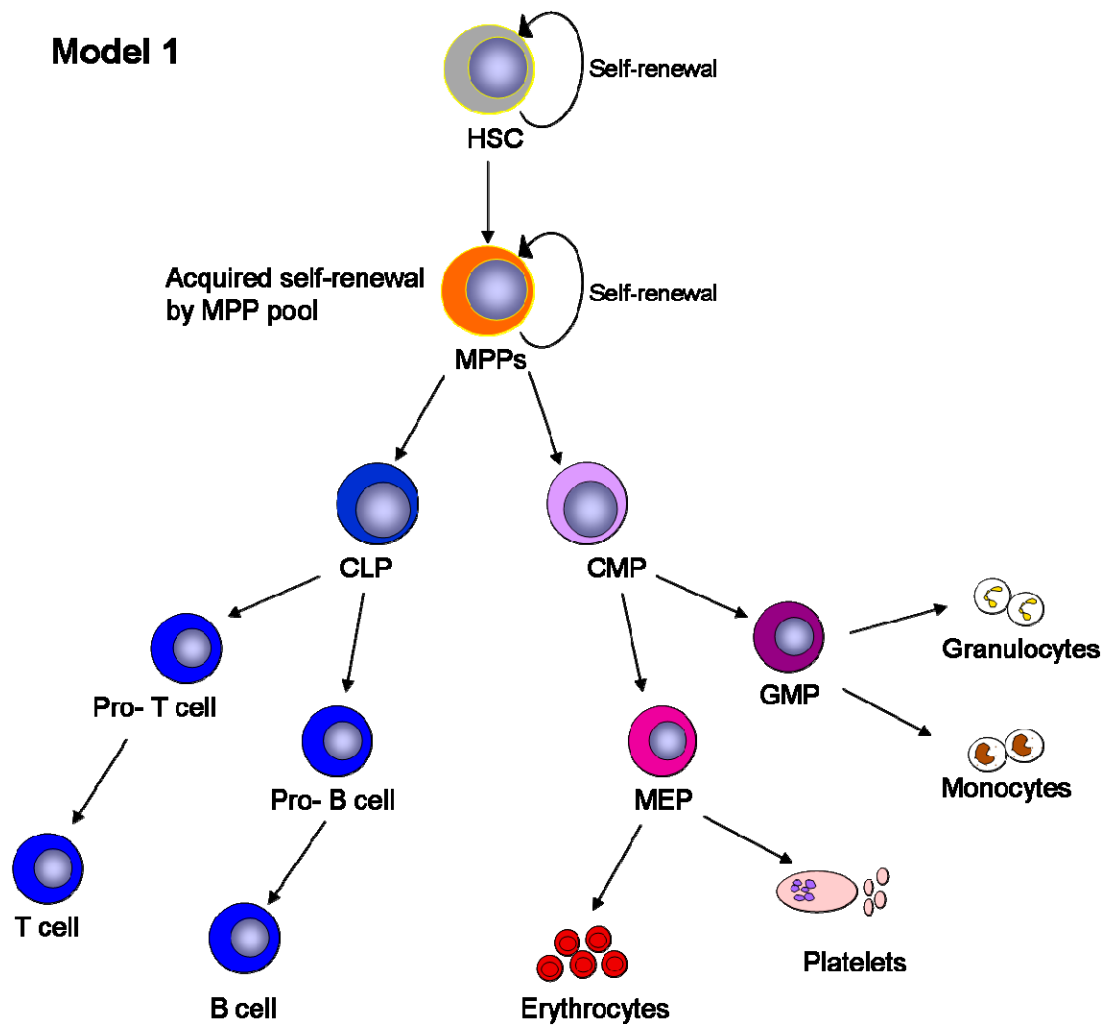
Figure 5.1 Triple mutant MPPs acquire life-long self-renewal. In this scenario triple mutant MPPs acquire full self-renewal capability and are an independent pool of long-lived cells in the bone marrow.
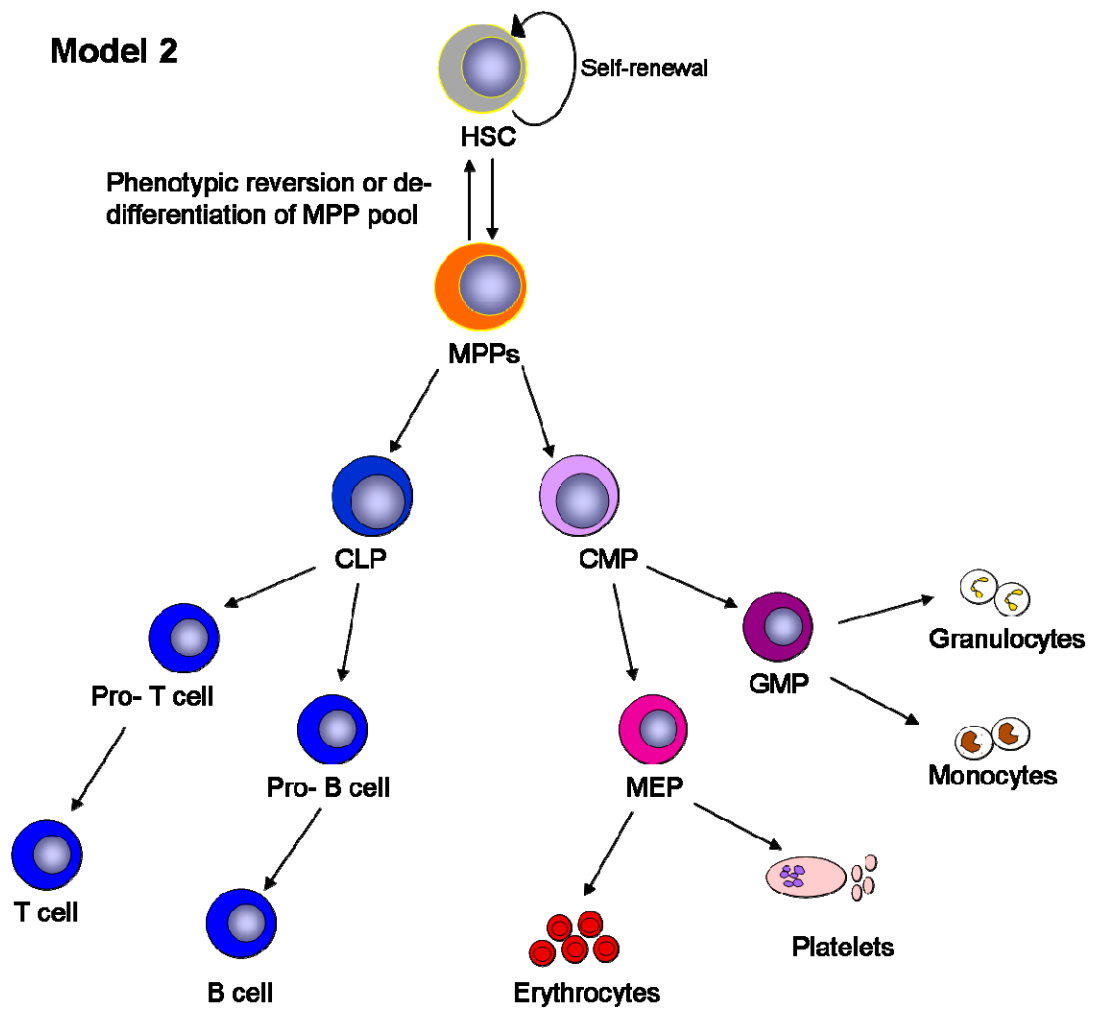
Figure 5.2 Triple mutant MPPs de-differentiate into HSCs. In order to execute the self-renewal program triple mutant MPPs are capable of returning to an earlier immature state.
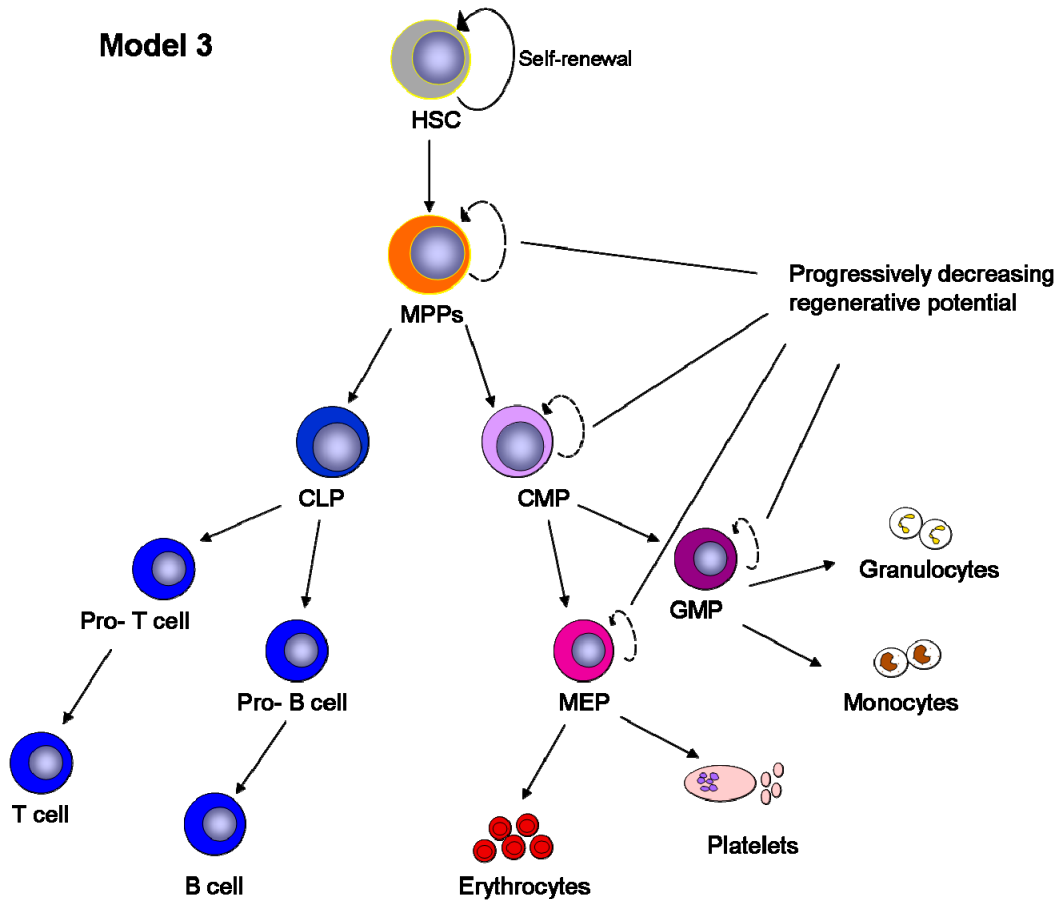
Figure 5.3 Triple mutant MPPs have extended regenerative capacity but cannot self-renew. In this scenario triple mutant MPPs have an extended life span thereby increasing their regenerative potential, but eventually exhaust, because they do not acquire life-long self-renewal capacity.

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