MUTANT P53 IN PRE-LEUKEMIC HEMATOPOIETIC STEM CELLS AND THE PATHOGENESIS OF MYELODYSPLASTIC SYNDROME

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Sisi Chen

MUTANT P53 IN PRE-LEUKEMIC HEMATOPOIETIC STEM CELLS AND THE PATHOGENESIS OF MYELODYSPLASTIC SYNDROME

Myelodysplastic syndrome (MDS) is a clonal disease arising from mutated hematopoietic stem cells (HSCs). MDS stem cells originate from pre-leukemic HSCs, which have enhanced competitive advantage over wild-type (WT) HSCs but normal differentiation capacity. Recently, acquired somatic gain-of-function (GOF) *TP53* mutations were identified in the blood of aged healthy individuals as well as in patients with MDS. However, the role of GOF *TP53* mutations in clonal hematopoiesis and the pathogenesis of MDS is largely unknown.

Based upon our previous studies and clinical findings, I hypothesized that GOF mutant p53 drives the development of pre-leukemic HSCs with enhanced competitive advantage, leading to clonal expansion and the pathogenesis of MDS. To test my hypothesis, I examined HSC behaviors in young $p53^{+/+}$ and $p53^{R248W/+}$ mice. I discovered that $p53^{R248W}$ enhances the repopulating potential of HSCs without affecting terminal differentiation. I also found that GOF mutant p53 protects HSCs from genotoxic stress and promotes their expansion. To investigate the role of mutant p53 in the pathogenesis of hematological malignancies, I monitored disease development in $p53^{+/+}$ and $p53^{R248W/+}$ mice and observed that some mutant p53 mice develop MDS during aging. Therefore, I demonstrated that GOF mutant p53 enhances the repopulating potential of

HSCs and drives the development of pre-leukemic HSCs, predisposing aged mutant p53 mice to MDS development.

Mechanistically, I found that mutant p53 increases the chromatin accessibility to genes important for HSC maintenance, including pluripotent gene *Sox2* and chemokine gene *Cxcl9*. By performing biochemical experiments, I discovered that GOF mutant p53, but not WT p53, interacts with histone methyltransferase EZH2 and enhances histone H3 lysine 27 trimethylation (H3K27me3) at genes, including *Mef/Elf4* and *Gadd45g*, that negatively regulate HSC self-renewal.

Collectively, these findings demonstrated that GOF mutant p53 drives pre-leukemic HSC development through modulating epigenetic pathways. Thus, our studies have uncovered novel mechanistic and functional links between GOF mutant p53 and epigenetic regulators in pre-leukemic HSCs. This research may identify epigenetic regulator EZH2 as a novel target for the prevention and treatment of MDS patients with *TP53* mutations.

Yan Liu, Ph.D.

TABLE OF CONTENTS

LIST OF TABLES xiii

LIST OF FIGURES xiv

LIST OF ABBREVIATIONS xvii

CHAPTER ONE 1

Introduction 1

Hematopoiesis 1

1. Hematopoietic hierarchy 1

2. Hematopoietic cell identification 3

3. Molecular mechanisms controlling adult hematopoietic stem cell fate 6

3.1 Transcription factors 8

3.2 Epigenetic modifications 9

3.3 Polycomb Repressive Complex 9

3.4 Enhancer of Zeste Homologue 2 10

Pre-leukemic hematopoietic stem cells 12

Pathogenesis of Myelodysplastic Syndrome 16

p53 in hematopoiesis 20

1. Role of p53 in steady state hematopoiesis 20

2. Role of p53 in stress hematopoiesis 22

TP53 mutations 24

1. Mechanisms of *TP53* mutations 24

2. *TP53* mutations in hematological malignancies 27

Summary and Specific Aims 30

Significance 33
CHAPTER TWO 35
Materials and methods 35
Materials 35
1. Mice 35
1.1 HUPKI (*p53*$^{+/+}$) and *p53*$^{R248W/+}$ mice 35
1.2 C57BL/6 mice 35
1.3 BoyJ mice 35
1.4 F1 C57BL/6/ BoyJ mice 35
2. Primers 36
3. Antibodies 37
4. Commercial Kits 37
5. Reagents 38
Methods 38
1. Stem and progenitor cell assays 38
2. Flow cytometry 38
3. Ki-67 staining 39
4. AnnexinV staining 40
5. Transplantation 40
5.1 Competitive bone marrow transplantation 40
5.2 Competitive HSC transplantation 40
5.3 Whole bone marrow transplantation 41
6. Limiting dilution assay 41

7. Homing assays 41
8. Histology 42
9. Quantitative Real-Time PCR (qRT-PCR) 42
10. Quantification of γ-H2Ax by ImageStream Flow Cytometry 42
11. Quantification of H3K27me3 by ImageStream Flow Cytometry 43
12. Co-IP 43
13. RNA sequencing 43
14. Expression microarray analysis 44
15. Chromatin immunoprecipitation sequencing (ChIP-seq) 44
16. ATAC sequencing 46
17. Statistical analysis 46
CHAPTER THREE 48
GOF mutant p53 enhances the repopulating potential of hematopoietic stem cells 48
Introduction 48
Results 54
1. GOF mutant p53 does not affect HSC frequency 54
2. GOF mutant p53 does not affect HSC survival in steady state hematopoiesis 58
3. GOF mutant p53 does not affect HSC quiescence in steady state 60
4. GOF mutant p53 enhances the repopulating potential of HSCs without affecting terminal differentiation 62
5. GOF mutant p53 does not affect HSC Homing 72

Conclusion and Discussion (Chapter Three) 74

CHAPTER FOUR 76

GOF mutant p53 protects HSCs from genotoxic stress and promotes their clonal expansion 76

Introduction 76

Results 79

1. Mutant p53 HSCs are not sensitive to irradiation 79

2. Mutant p53 maintains HSC function following irradiation 82

3. Mutant p53 HSCs are not sensitive to chemotherapy 85

4. Mutant p53 promotes clonal expansion of hematopoietic cells following genotoxic stress 89

Conclusion and Discussion (Chapter Four) 91

CHAPTER FIVE 93

Aged heterozygous mutant p53 mice develop MDS 93

Introduction 93

Results 96

1. Mutant p53 alters terminal differentiation during aging 96

2. Characterize aged heterozygous mutant p53 mice 98

3. Some aged heterozygous mutant p53 mice developed MDS 100

4. Mutant p53-driven MDS is transplantable 106

Conclusion and Discussion (Chapter Five) 109

CHAPTER SIX 112

GOF mutant p53 drives pre-leukemic stem cell development through

regulating gene expression..... 112
Introduction..... 112
Results 115
1. GOF mutant p53 HSCs show gene expression signatures important for stem cell maintenance and leukemia development..... 115
2. GOF mutant p53 alters chromatin accessibility in HSPCs..... 121
3. GOF mutant p53 interacts with transcription factors to regulate chromatin accessibility 124
4. GOF mutant p53 increases the chromatin accessibility to genes important for HSC maintenance..... 126
Conclusion and Discussion (Chapter Six) 128
CHAPTER SEVEN 133
GOF mutant p53 interacts with EZH2 to drive pre-leukemic stem cell development..... 133
Introduction..... 133
Results 136
1. GOF mutant p53 proteins enhance EZH2 activity in HSCs..... 136
2. Pharmacological inhibition of EZH2 activity decreased the replating potential of mutant HSPCs..... 138
3. GOF mutant p53 proteins enhances H3K27me3 in genes important for HSC self-renewal..... 140
4. GOF mutant p53 proteins interact with EZH2..... 142
Conclusion and Discussion (Chapter Seven) 145

CHAPTER EIGHT 149

Summary and Future Directions 149

REFERENCES 155

CURRICULUM VITAE

LIST OF TABLES

Table 1.1 Cell surface markers for hematopoietic populations 5

Table 2.1 Primers 36

Table 2.2 Antibodies 37

Table 2.3 Commercial Kits 37

Table 2.4 Reagents 38

LIST OF FIGURES

Figure 1.1 Hematopoietic hierarchy in adult bone marrow 2

Figure 1.2 Molecular mechanisms involved in adult HSC self-renewal and differentiation 7

Figure 1.3 Model of evolution of leukemia 13

Figure 1.4 Hot-spot p53 mutations identified in human cancer 25

Figure 3.1 Functional screening of mutant p53 in hematopoietic stem cells 51

Figure 3.2 Mutant p53 does not affect the frequency of hematopoietic stem and progenitor cells 56

Figure 3.3 Mutant p53 does not affect survival of hematopoietic stem cells 59

Figure 3.4 Mutant p53 does not affect quiescence of hematopoietic stem cells 61

Figure 3.5 Mutant p53 enhances the replating potential of hematopoietic progenitor cells 63

Figure 3.6 Mutant p53 enhances repopulating potential of hematopoietic stem cells in serial competitive bone marrow transplantations 65

Figure 3.7 Mutant p53 enhances the repopulating potential of hematopoietic stem cells in competitive HSC transplantation 69

Figure 3.8 Limiting dilution assays show that mutant p53 increases the number of functional HSCs *in vivo* 71

Figure 3.9 Mutant p53 does not affect HSC homing 73

Figure 4.1 HSCs expressing mutant p53 are less sensitive to irradiation 80

Figure 4.2 Mutant p53 maintains the repopulating potential of irradiated hematopoietic stem cells in serial competitive bone marrow transplantation assays 83
Figure 4.3 HSCs expressing mutant p53 are less sensitive to 5-FU administration 87
Figure 4.4 Mutant p53 promotes clonal evolution of HSCs under genotoxic stress *in vivo* 90
Figure 5.1 Mutant p53 impairs multi-lineage differentiation during ageing 97
Figure 5.2 Survival rate and disease spectrum of aged *p53*$^{R248W/+}$ mice 99
Figure 5.3 Aged *p53*$^{R248W/+}$ mice show hypercellular bone marrow and extramedullary hematopoiesis in spleen. 101
Figure 5.4 Aged heterozygous mutant p53 mice developed MDS 104
Figure 5.5 Mutant p53 driven MDS is transplantable 107
Figure 6.1 *p53*$^{R248W/+}$ HSCs show gene expression signatures important for stem cell maintenance and leukemia development 117
Figure 6.2 *p53*$^{R248W/R248W}$ HSCs show gene expression signatures important for stem cell maintenance and leukemia development 120
Figure 6.3 Enriched biological processes of genes associated with upregulated ATAC-seq peaks in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSKs. 122
Figure 6.4 Enrichment of transcription factor motifs in peaks that gain open accessibility in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs determined by HOMER *de novo* or Known Motif Analysis 125
Figure 6.5 Expression of *Cxcl9* and *Sox2* are increased in *p53*$^{R248W/+}$ HSCs ... 127

Figure 7.1 GOF mutant p53 enhances H3K27me3 in HSCs 137

Figure 7.2 Inhibiting EZH2 decreased replating potential of mutant HSPCs *in vitro* 139

Figure 7.3 Several mutant p53 proteins, but not wild type p53, interact with EZH2 141

Figure 7.4 GOF mutant p53 regulates genes important for HSC self-renewal through enhancing H3K27me3 143

LIST OF ABBREVIATIONS

5-FU	Fluorouracil
AML	Acute myeloid leukemia
ATAC-seq	Assay for Transposase-Accessible Chromatin with high-throughput sequencing
BM	Bone marrow
CH	Clonal hematopoiesis
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphoid leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
Co-IP	Co-Immunoprecipitation
CRU	competitive repopulation units
DBA	Diamond blackfan anemia
DBD	DNA-binding domain
DN	dominant-negative
EMH	extramedullary hematopoiesis
ESC	embryonic stem cells
EZH2	Enhancer of Zeste Homologue 2
Gfi-1	growth factor independent 1
GOF	gain-of-function
GSEA	Gene Set Enrichment Analysis
Hb	Hemoglobin
H3K9ac	acetylation of histone 3 at lysine 9
H3K4me3	the tri-methylation of histone 3 at lysine 4
H3K27me3	the tri-methylation of histone 27 at lysine 4
HSC	Hematopoietic stem cell
HUPKIN	humanized p53 knock-in
IUCAC	Institutional Animal Care and Use Committee
JMML	Juvenile myelomonocytic leukemia
Lin-	Lineage negative
LOF	loss-of-function
LSK	Lin-Sca1+c-kit+
LSC	Leukemic stem cell
LT-HSC	Long-term hematopoietic stem cell
MCV	Mean corpuscular volume
MDS	Myelodysplastic stem cell
MEF	Myeloid Elf-1-Like Factor
MM	multiple myeloma
MPP	Multipotent progenitor cell
MRN	Mre11/Rad50/Nbs1
MSigDB	Molecular Signatures Database
PcG	Polycomb group proteins

PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2
RBC	Red blood cell
ROS	reactive oxygen species
RNA Pol II	RNA polymerase II
SCF	Stem cell receptor
SEM	Standard error of the mean
SLAM	Signaling lymphocytic activation molecule
ST-HSC	Short-term hematopoietic stem cell
SV-40	simian virus 40
TBI	total body irradiation
TF	transcription factor
WBC	White blood cell

CHAPTER ONE

Introduction

Hematopoiesis

1. Hematopoietic hierarchy

Hematopoiesis is the process of blood cell production in an organism. It is initiated by a rare population of hematopoietic stem cells (HSCs) residing in the bone marrow (Kondo, et al., 2003; Attar et al., 2004). The frequency of HSCs is less than 0.01% of total bone marrow cells (Orkin, 2008; Zon, 2008). HSCs are the only population within the hematopoietic compartment that can self-renewal life-long and undergo multi-lineage differentiation to produce all types of mature blood cells (Akala et al., 2006, Zon, 2008). The majority of HSCs are in the quiescent state in order to maintain the integrity and function of HSC pool. In order to replenish blood system and maintain HSC pool throughout the life of an organism, HSCs have to keep the balance of self-renewal and differentiation (Orkin, 2008; Zon, 2008; Walasek et al., 2012). Disruption of the balance may cause blood disorders, such as anemia, bone marrow failure, and leukemia.

Hematopoiesis has a hierarchical structure and HSCs are at the top of the hierarchy as illustrated in Figure 1.1 (Wang et al., 2011). HSCs can be divided into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs have unlimited self-renewal potential, whereas the self-renewal potential ST-HSCs is limited. ST-HSCs tend to enter the cell cycle and give rise to

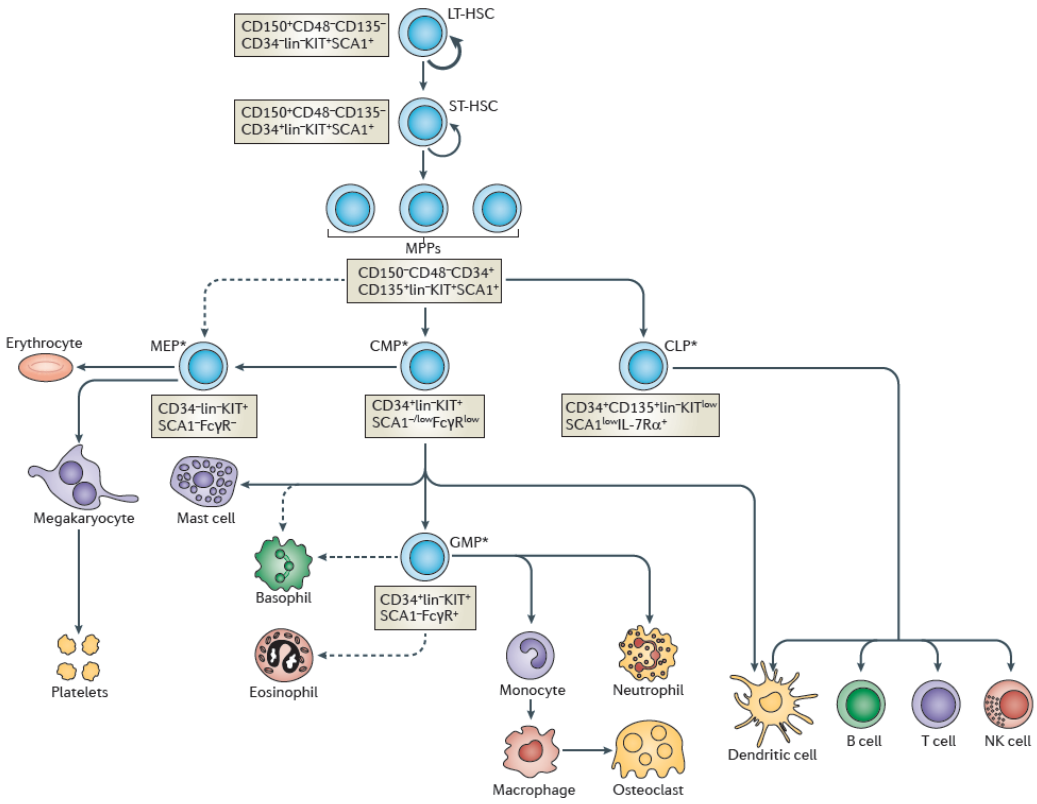


Figure 1.1 Hematopoietic hierarchy in adult bone marrow.

(Adapted from Wang et al., 2011)

multi-potent progenitor cells (MPPs), which then generate several types of lineage-committed progenitors, including common myeloid progenitors (CMPs), megakaryocyte-erythrocyte progenitors (MEPs), granulocyte-macrophage progenitors (GMPs) and common lymphocyte progenitors (CLPs). These lineage-committed progenitors have excessive proliferating capability and eventually differentiate into all mature blood cells, such including erythrocytes, neutrophils, macrophage, platelet, granulocyte, B cells and T cells. Diverse mature blood cells have distinct functions that sustain the life of an organism. For example, red blood cells (RBCs) carry oxygen and remove carbon dioxide, B cells and T cells build adaptive immune system, and platelets involve in clotting to stop bleeding.

2. Hematopoietic cell identification

Different subsets of hematopoietic stem and progenitor cell populations can be functionally and immuno-phenotypically identified. Functional LT-HSCs can be determined by *in vivo* transplantation experiments. LT-HSCs can mediate multi-lineage reconstitution in lethally irradiated recipient mice for at least 4 months, and these HSCs can serially repopulate the blood systems in secondary transplantation and even tertiary transplantation assays (Akala et al., 2006; Orkin, 2008; Zon, 2008). However, multipotent progenitor cells can only contribute to hematopoietic reconstitution for up to 3 months. Hence, serial bone marrow transplantation assay is the gold standard for examining LT-HSC function and self-renewal capacity.

HSCs can be purified from the bone marrow immuno-phenotypically by using cell surface markers and flow cytometry analysis. In the murine blood

system, mature blood cells express distinct surface markers, including B220, CD3e, CD11b, Gr1, and Ter119, shown in Table 1. However, hematopoietic stem and progenitor cells do not express these lineage cell surface markers and therefore designated as lineage negative (Lin-) cells. In the past two decades, significant progress has been made to isolate the most primitive murine hematopoietic stem cells. For example, stem cell factor (SCF) receptor c-Kit was identified in 1990s to be exclusively expressed in mouse hematopoietic progenitor cells that have multi-lineage reconstitution capability (Ogawa et al., 1991). Lin-Sca1+c-Kit+ (LSKs) are still heterogeneous and only 4% LSKs are functional HSCs (Morrison et al., 1994; Challen et al., 2009). The identification of the signaling lymphocytic activation molecule (SLAM) family proteins has led to further enrichment of more primitive HSCs (Kiel et al., 2005; Challen et al., 2009). LSKs can be separated into several functionally distinct sub-populations by using the SLAM markers. LT-HSCs highly express the SLAM marker CD150, but not CD48. The expression of CD48 increases as HSCs differentiate into less primitive populations, including ST-HSCs and MPPs. LT-HSCs can be purified as $Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD48^{-}CD150^{+}$ cells from the bone marrow (Challen et al., 2009). In this study, I utilized the SLAM surface markers to isolate LT-HSCs as shown in Table 1.

Table 1.1 Cell surface markers for hematopoietic populations

Hematopoietic Populations	Surface Marker
Long-term hematopoietic stem cells (LT-HSC)	$Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD48^{-}CD150^{+}$
Short-term hematopoietic stem cells (ST-HSC)	$Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD48^{-}CD150^{+}$
Multipotent progenitors (MPP)	$Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD48^{+}CD150^{-}$
LSK	$Lin^{-}Sca1^{+}c\text{-}Kit^{+}$
Common lymphocyte progenitors (CLP)	$Lin^{-}Sca^{-}1^{Low}c\text{-}Kit^{Low}IL\text{-}7R\alpha^{+}$
Common myeloid progenitors (CMP)	$Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{low}CD34^{high}$
Granulocytic monocytic progenitors (GMP)	$Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{high}CD34^{high}$
Megakaryocyte erythroid progenitors (MEP)	$Lin^{-}Sca1^{-}Kit^{+}FcγRII/III^{low}CD34^{low}$
Granulocytes and monocytes	$Gr1^{+}Mac1^{+}$
B cells	$B220^{+}$
T cells	$CD3^{+}$
Erythroid cells	Ter119+

3. Molecular mechanisms controlling adult hematopoietic stem cell fate

Elucidating the molecular mechanisms underlying HSC fate determination has been a focus of HSC research. Adult HSCs mainly reside within the bone marrow, which consists of various hematopoietic and non-hematopoietic cells. The complexity of the bone marrow microenvironment indicate that HSC fate may be controlled by both stem cell intrinsic factors and extrinsic factors (Akala et al., 2006, Zon, 2008; Wang et al., 2011).

The regulation of HSC behavior demands a complex crosstalk between the stem cell-intrinsic regulators and extrinsic regulatory signals from the microenvironment, illustrated in Figure 1.2 (Zon 2008). In this session, I will briefly describe how HSC functions are tightly regulated by transcription factors and epigenetic regulators in a cell-autonomous manner.

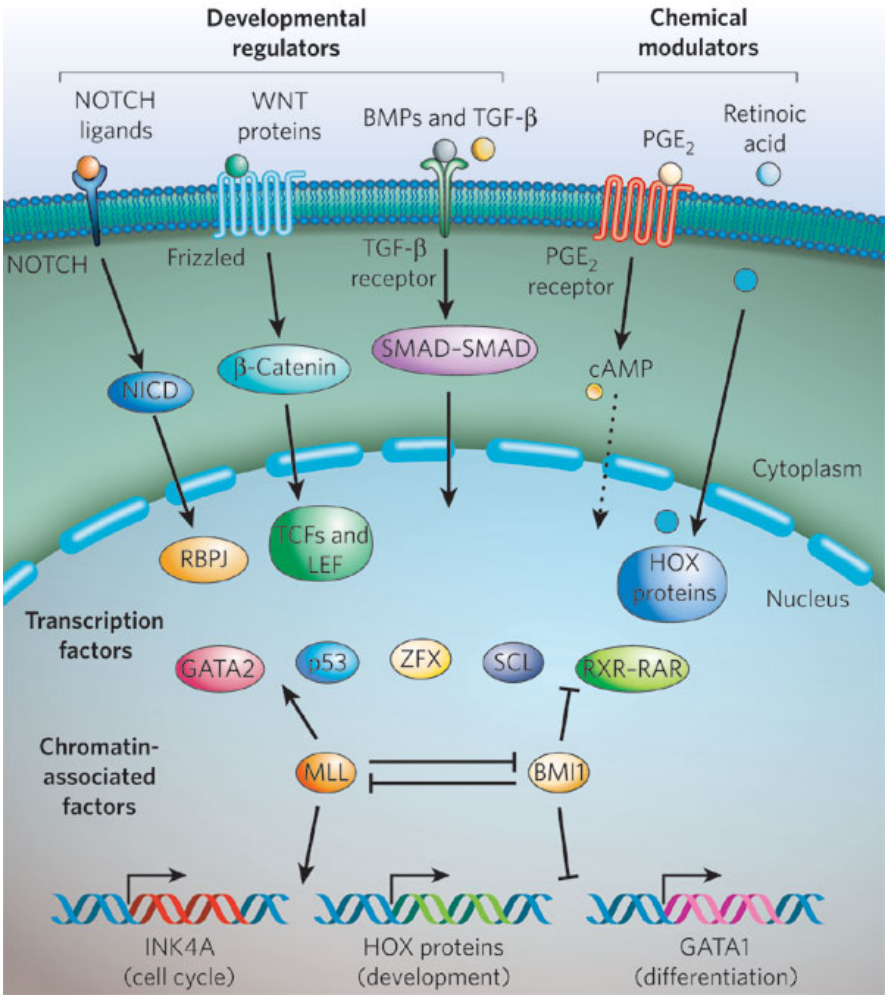


Figure 1.2 Molecular mechanisms involved in adult HSC self-renewal and differentiation.

(Adapted from Zon, 2008)

3.1 Transcription factors

A large number of transcription factors play essential roles in controlling HSC fates. For example, the cluster of Hox proteins, an evolutionary conserved family of proteins by a DNA-binding motif, have been implicated in the regulation of HSCs as well as leukemogenesis. Enforced expression or silencing of certain Hox genes in mouse model or human blood cells has delineated their impacts on hematopoiesis. Overexpression of Hoxb4 in bone marrow cells promotes expansion of HSC populations *in vivo* and *ex vivo*, enhancing HSC stemness without affecting normal bone marrow development (Sauvageau, et al., 1995). Ectopic Hoxa10 expression increases HSC self-renewal but impairs megakaryocyte and erythroid cell differentiation (Magnusson et al., 2007). By contrast, overexpression of Hoxa9 promotes myeloid differentiation and leukemia development (Thorsteinsdottir et al., 2002).

Members of the ETS (‘E twenty-six’) family of transcription factors govern the gene regulatory networks important for hematopoiesis (Ciau-Uitz et al., 2013). While all ETS transcription factors share similar binding motif, their functions are context-dependent and each ETS transcription factor plays distinct roles in different contexts. For instance, PU.1 is one of the most important ETS family proteins in hematopoiesis, playing indispensable roles supporting HSC commitment (Will et al., 2015). Its expression is low in HSCs but increases as HSCs undergo lineage commitment. Recently, findings by Steidl and colleagues suggested that reduction of PU.1 at 35% promotes the development of pre-leukemic HSCs and subsequently leads to leukemic transformation (Will et al.,

2015). MEF, also known as ELF4, is another a key ETS factor in regulating HSC quiescence (Lacorazza et al., 2006). Mef null HSCs are more quiescent and thus are resistant to chemotherapy or irradiation treatment (Lacorazza et al., 2006). ERG maintains HSC self-renewal during stress hematopoiesis and regulates megakaryopoiesis in steady state (Min et al., 2008). The ETS transcription factor ETV7 forces quiescent HSCs to enter the cell cycle and exhausts HSCs by promoting proliferation (Geltink et al., 2013).

3.2 Epigenetic modifications

Epigenetic modifications, including histone modifications and DNA methylation, impact important biological processes through modulating unique gene expression patterns in a context-dependent manner (Beerman et al., 2015). Epigenetic modulations play essential roles at different stages of HSC development and stem cell fate determinations (Goldberg et al., 2007; Buenrostro et al., 2013; Sun et al., 2014). Here, I will use Polycomb group (PcG) proteins as examples to illustrate this aspect.

3.3 Polycomb Repressive Complex

The PcG proteins are epigenetic gene silencers that have been implicated in stem cell maintenance and cancer development (Bernstein et al., 2006; Goldberg et al., 2007; Bracken et al., 2009; Margueron et al., 2011). Genetic and biochemical studies indicate that Polycomb group proteins exist in at least two protein complexes, Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), that work in concert to initiate and maintain stable gene repression (Bernstein et al., 2006; Bracken et al., 2009). In stem cells, the

PcG proteins repress the transcription of lineage-specific differentiation genes to maintain stemness. In response to differentiation signals, the PcG proteins are displaced from lineage-specific genes and recruited to stem cell-specific genes to facilitate differentiation (Bernstein et al., 2006; Xie et al., 2014b).

Bmi1 is a critical component of the PRC1 complex in hematopoiesis. Loss of Bmi1 impairs HSC self-renewal and blocks terminal differentiation, whereas forced expression of Bmi1 in HSCs promotes HSC self-renewal by repressing cell-cycle regulator p16INK4A and p19ARF, resulting in symmetrical stem cell division (Molofsky et al., 2003; Park et al., 2003; Gao et al., 2012). The self-renewal defect of $Bmi1^{-/-}$ mice can be rescued by elimination of both p16 and p19 (Iwama et al., 2004). We have been studying the role of Bmi1 in HSC self-renewal and terminal differentiation. We reported that AKT-mediated phosphorylation of Bmi1 impairs its function in HSC self-renewal (Liu et al., 2012), implicating that phosphorylation of Bmi1 can act as a therapeutic strategy to eliminate cancer stem cells. Additionally, we discovered that Bmi1 promotes erythroid development partially through regulating ribosome biogenesis and that Bmi1 deficiency may cause diamond blackfan anemia (DBA) (Gao et al., 2014).

3.4 Enhancer of Zeste Homologue 2

EZH2, Enhancer of Zeste Homologue 2, is a methyltransferase. It is a catalytically active component of the PRC2 complex. EZH2 represses gene expression through di- and tri-methylations lysine 27 of histone H3 (H3K27me2 or H3K27me3) (Margueron et al., 2011; Xie et al., 2014b). Overexpression of EZH2 enhances HSC maintenance, whereas loss of EZH2 decreases the

engraftment of HSCs in repopulation assays (Kamminga et al., 2006; Mochizuki-Kashio et al., 2017). Meanwhile, EZH2 controls normal B cell differentiation (Xie et al., 2014b). These findings suggest that EZH2 regulates both HSC self-renewal and differentiation. Remarkably, gain-of-function EZH2 mutations, have been discovered in many hematological malignancies, like lymphoma, acute myeloid leukemia and multiple myeloma (Shih et al., 2012). Pharmacological inhibition of EZH2 inhibits HSC self-renewal (Campbell et al., 2015), therefore providing a potentially therapeutic strategy to treat patients with high activity of EHZ2. On the other hand, EZH2 loss-of-function mutations also exist to promote myeloid progenitor self-renewal reprogram through inducing *HOXA9* expression (Margueron et al., 2011; Xie et al., 2014b). These findings suggest a dual role of EZH2 as tumor suppressor or onco-protein in a context-dependent manner.

Pre-leukemic hematopoietic stem cells

Acute myeloid leukemia (AML) is an aggressive hematological malignancy in which mutated hematopoietic stem and progenitor cells give rise to clonal blast cells without normal differentiation (Sykes et al., 2015). Previously, it was believed that HSCs acquiring driving mutations will directly transformed to disease initiating cells, so called leukemic stem cells (LSC) (Majeti, 2014; Sykes et al., 2015). However, this concept was challenged by next-generation sequencing of genetic mutations in AML patients. Dr. Dick's and Dr. Majeti's groups recently reported that some recurrent mutations in AML, such as *DNMT3A* mutations, are pre-leukemic mutations (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Most of them are recurrent mutated in genes involved in epigenetic modifications.

Based on their work, people started to appreciate that the transformation of disease initiating cells follows a stepwise manner of acquisition of mutations or epigenetic changes in HSCs. As illustrated in Figure 1.3 (Corces-Zimmerman et al., 2014), HSCs first acquire pre-leukemic mutations and give rise to pre-leukemic HSC. These pre-leukemic mutations confer HSCs with competitive growth advantage, allowing clonal expansion. Pre-leukemic stage may have long latency and achieve clonal dominance over time. Subsequently, when acquiring additional mutations, chromosome aberrations or epigenetic changes, pre-leukemic HSCs progress to fully transformed LSCs, giving rise to overt blood cancer (Corces-Zimmerman et al., 2014; Jan et al., 2014). According to this model, pre-leukemic HSCs and leukemic stem cells are functionally

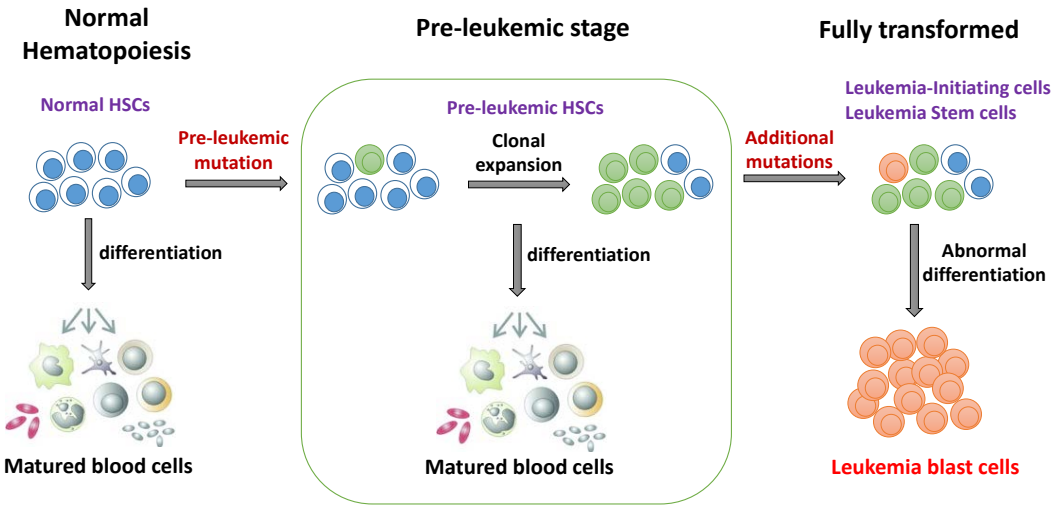


Figure 1.3 Model of evolution of leukemia.

different and can be distinguished by experimental approaches. Transformed leukemic stem cells are defined by their capacity to initiate short-onset disease in irradiated murine model. By contrast, pre-leukemic HSCs show normal multi-lineage differentiation but have competitive growth advantage over wild-type HSPCs in a competitive repopulation assay.

Dick and colleagues also demonstrated that pre-leukemic HSCs are resistant to chemotherapy (Corces-Zimmerman et al., 2014; Shlush et al., 2014). The prolonged pre-leukemic phase may be shortened by genotoxic stress, such as radiation, X-ray and chemotherapy. Genotoxic stress causes lesions on the genome (Majeti, 2014; Sykes et al., 2015), resulting in introduction of secondary hits onto pre-leukemic background. The recognition of pre-leukemic mutations provide substantial understanding of why some patients with solid tumors developed leukemia after chemotherapy or radiation treatment. Additionally, the most common death of patient with AML and MDS is due to relapse (Anderson et al., 2011). Considering that pre-leukemic HSCs is of high risk to LSCs transformation under stress condition, pre-leukemic HSCs serve as a reservoir for relapse (Shlush et al., 2015). Therefore, targeting pre-leukemic HSCs is essential for the cure of blood malignancies.

Current studies suggest that pre-leukemic state exist in many myeloid and lymphoid malignancies, including AML, myelodysplastic syndrome (MDS), chronic lymphoid leukemia (CLL), chronic myeloid leukemia (CML), and etc (Pandolfi et al., 2013; Vasanthakumar et al., 2014). Of note, diverse diseases may carry a same recurrent mutation. For example, *DNMT3a* R882 mutation has

been found in AML, MDS, and juvenile myelomonocytic leukemia (JMML) (Vasanthakumar et al., 2014). Therefore, targeting pre-leukemic HSCs prior to the development of a malignancy is an attractive therapeutic strategy for cancer prevention.

Pre-leukemic stage is not rare, especially in the elderly. Recent deep sequencing analyses have suggested that pre-leukemic somatic mutations are common in older people without an evidence of blood malignancies, leading to hematopoietic clonal expansion (Corces-Zimmerman et al., 2014; Genovese et al., 2014). This phenomenon is termed Clonal Hematopoiesis (CH) (Bejar et al., 2011). The frequency of clonal somatic mutations rises with age: in 10% of people older than 70 years and 20% of individuals older than 90 years (Jaiswal et al., 2014; Jan et al., 2017). The high frequency of pre-leukemic mutations in healthy elder people may due to physiological stress caused by ageing, like chronic inflammation (Xie et al., 2014a). Notably, CH is associated with high risk of overt malignancies with poor prognosis.

The most frequently mutated genes found in large populations with CH were epigenetic regulators, including *DNMT3A*, *TET2*, and *ASXL1*, which are also recurrently mutated in hematological malignancies (Corces-Zimmerman et al., 2014). Emerging studies have identified the biological impact of mutations in *DNMT3A*, *TET2*, and *ASXL1* in HSCs and leukemic transformation (Abdel-Wahab et al., 2012; Sato et al., 2016; Zhang et al,. 2016). Further studies are required to uncover the mechanism by which pre-leukemic HSCs initiate and transform into a fully transformed leukemic state.

Pathogenesis of Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is one of the most common hematological disorders related to ageing. It is a heterogeneous clonal disease driven by mutated HSCs (Sperling, et al., 2017; Woll, et al., 2014). According to the definition from The World Health Organization, MDS is characterized by peripheral cytopenia due to ineffective hematopoiesis, multi-lineage morphological dysplasia in bone marrows and peripheral blood, and the presence of less than 5% blast cells with high risk of progression to acute myeloid leukemia (AML) (Nimer, et al., 2008; Woll, et al., 2014; Sperling, et al., 2017).

MDS mostly occurs in the elderly. The awareness of the disease is improving since the aging population is increasing over time. In the United States, 0.005% to 0.013% of persons were diagnosed with MDS (Bejar et al., 2014; Sperling, et al. 2017). The median age at diagnosis is about 71 to 76-year-old (Cazzola et al., 2013). Patients with MDS have symptoms of fatigue, bleeding and fevers or infections, due to anemia, thrombocytopenia and neutropenia respectively (Lindsley et al., 2013; Sperling, et al. 2017). They often die from cytopenia induced complications or progression to acute myeloid leukemia.

Identifying the cell of origin of MDS is critical to understand the development of the disease and find the cure for the patients. Recently, several groups have demonstrated that MDS is proceeded from rare mutated HSCs (Nimer et al., 2008; Wong et al., 2015; Sperling et al., 2017). Genetic studies of blood cells from large cohorts of healthy individual and MDS patients have

revealed that MDS results from step-wise acquisition of somatic recurrent mutations (Bejar et al., 2011; Kennedy et al., 2017). Clonal hematopoiesis led by the presence of initiating mutations in HSCs has been thought to be the first step in the development of clonal hematological diseases, leading to expansion of pre-leukemic HSCs (Bejar et al., 2011; Genovese et al., 2014; Jan et al., 2017). Further acquisition of cooperating mutations or chromosome aberrations in pre-leukemic stage will eventually cause MDS or even transition to AML (Makishima et al., 2017; Sperling, et al. 2017). Therefore, elimination of MDS stem cells is likely to be essential to curing the disease.

Recurrent mutations identified in the pathogenesis of MDS can be sorted into several categories according their functions, such as splicing factors, epigenetic regulators, tumor suppressors, cohesion complex and etc (Bejar, et al., 2014; Kennedy et al., 2017; Sperling et al., 2017). Genes involved in spliceosome components are the most frequent recurrent lesions in MDS made up to 60% of cases, including *SF3B1*, *SRSF2*, *U2AF1* (Abdel-Wahab et al., 2011; Folco et al., 2011; Lee et al., 2016; Inoue et al., 2016). Interestingly, these mutations are heterozygous missense mutations and occur mutually exclusive, leading to distinct mechanisms of alteration of the splicing machinery (Yoshida et al., 2011; Inoue et al., 2016). *SF3B1* mutations are found to be associated with ring sideroblasts and relatively favorable prognosis (Folco et al., 2011; Obeng et al., 2016; Papaemmanuil et al., 2011). Missense mutations on *SRSF2* has been shown to alter their binding to exonic splice enhancers, leading to mis-splicing of EZH2 (Kim et al., 2015); whereas *U2AF1* mutations enhances exon skipping

(Shirai et al., 2017). MDS patients with *SRSF2* or *U2AF1* mutations are usually associated with poor prognosis (Abdel-Wahab et al., 2011; Shirai et al., 2017). In addition, lesions in genes involved in epigenetic modifications are the second most common category of recurrent mutations in MDS (Bejar et al., 2011; Sperling et al., 2017). For example, *TET2*, gene involved in DNA methylation, is mutated in approximately 30% of MDS cases. These mutations cause hypermethylation of cytosines at enhancer sequences, resulting in downregulation of genes important for myeloid differentiation. *ASXL1* alterations have been found in 20% of patients with MDS (Bejar et al., 2011; Sperling et al., 2017). ASXL1 physically interacts with PRC2 and regulates H3K27me3 (Abdel-Wahab et al., 2012). Loss of function mutations of ASXL1 leads to a decrease of global H3K27me3 and dysregulation of important hematopoietic genes, including the HOXA cluster genes, resulting in ineffective hematopoiesis (Abdel-Wahab et al., 2012; Abdel-Wahab et al., 2013).

Apart from genetic mutations, cytogenetic rearrangements are also commonly found in patients with MDS. Importantly, acquisition of these abnormities during disease development is a predictor of unfavorable prognosis. *Chromosome 5q deletions*, *Chromosome 7 deletions*, *Chromosome 17 deletions* are one of the best understood cytogenetic aberrations in MDS (Bejar et al. 2011; Kennedy et al., 2017).

Although several agents have been approved by FDA to treat MDS patients, such as lenalidominde, 5’-azacitidine and decitabine, the clinical outcome of MDS patients still remains poor (Bejar et al., 2011; Cazzola et al.,

2013; Lindsley et al., 2013). Allogeneic bone marrow transplantation seems to be the most efficient treatment for MDS (Christopeit et al., 2016; Kennedy et al., 2017; Lindsley et al., 2017), but it is far from completely cure the disease. Critically, some mutations, in particularly p53 alterations, are associated with increase relapses after allogeneic bone marrow transplantations, leading to even worse survival (Lindsley et al., 2017). Therefore, it is of great clinical significance to understand the pathogenesis of MDS and find effective therapeutic approaches for MDS patients.

p53 in hematopoiesis

1. Role of p53 in steady state hematopoiesis

The human *TP53* gene is located on chromosome 17p and has 11 exons and 10 introns, encoding p53 protein. p53 was first identified as a partner protein of simian virus 40 (SV-40)-derived tumor antigens in 1979 (Lane et al., 1979). It was proposed as a proto onco-protein due to its low expression in normal cells and prevalent stability in transformed cells. Until decade later, p53 has been demonstrated as a critical tumor suppressor against cancer development (Brown et al., 2009). p53 proteins function as a transcription factor regulating gene expression and involve in different biological events depending on cell contexts (Khoo et al., 2014). In response to stress signals, such as oncogene activation, DNA damage or hypoxia, p53 is activated and trigger cell cycle arrest, DNA damage repair, senescence and apoptosis (Bálint et al., 2001; Brown et al., 2009). Accordingly, p53 proteins inhibit cell proliferation, eliminate abnormal cells, maintain genome integrity and thereby prevent tumor development.

In the hematopoietic system, p53 is preferentially expressed in HSC-enriched LSK cells compared to their downstream hematopoietic progenitor cells (Kastan et al., 1911), implicating its important role in regulating HSC behavior. Indeed, emerging evidences have demonstrated that tumor suppressor p53 is a critical regulator of HSCs, affecting proliferation, differentiation, apoptosis and aging in hematopoietic system. Our group has defined a profound function of p53 in regulating HSC quiescence through analyzing p53 knock-out mouse model. In the loss of p53, we observed more HSCs entering into cell cycle, suggesting that

p53 maintains HSC quiescence (Liu et al., 2009b). Transcript profiling assay of $p53^{+/+}$ and $p53^{-/-}$ HSCs have identified *Gfi-1* and *Necdin* as direct p53 target genes in regulating HSC quiescence (Liu et al., 2009b). Gfi-1 (growth factor independent 1) regulates HSC quiescence and maintain HSC function as Gfi-$1^{-/-}$ HSCs are more proliferative than normal HSCs (Zeng et al., 2004). Necdin is a growth-suppressing protein associated with myeloid leukemia (Asai et al., 2012). Analysis of HSCs behavior in Necdin knock-out mice indicated that Necdin maintains HSC quiescence in steady state and suppresses p53-dependent apoptosis under stress conditions.

Cell competition is a process to select the superior (fittest cells) and eliminate the inferior (damaged cells). Bondar and colleagues discovered that p53 mediates HSPCs competition in hematopoietic system (Bondar, 2010). Cells with higher p53 activity were outcompeted by cells with lower p53 activity. Mechanistically, winner cells reprogram the loser cells and induce p53-related senescence phenotype to the losers (Bondar, 2010; Marusyk et al., 2010). This novel finding highlights the significance of p53 in hematopoietic homeostasis, and implicates a potential mechanism by which oncogenic mutations contribute to clonal expansion. Furthermore, Clarke and colleagues discovered that early progenitors could acquire stem-like features in the absence of *Trp53, $p16^{Ink4a}$* and *$p19^{Arf}$*, as multipotent progenitor cells from *$Trp53^{-/-}$p16Ink4a$^{-/-}$p19Arf$^{-/-}$* triple knock-out mice achieve long-term reconstitution capacity as WT HSCs (Akala, et al., 2008). This study suggested a potential role of p53 in regulating self-renewal

of progenitor cells, implying the importance of p53 pathways in reprogramming of progenitor cells to self-renewal cancer stem cells.

2. Role of p53 in stress hematopoiesis

Stress conditions, such as irradiation, reactive oxygen species (ROS), chemotherapy and X-Ray, perturb hematopoiesis. The Mre11/Rad50/Nbs1 (MRN) complex is sensor protein binding to damaged DNA. MRN complex activates ATM kinase and then phosphorylates p53 and other target proteins, leading to p53 dependent apoptosis on HSCs and progenitor cells (Mihara et al. 2003; Brown et al., 2009; Marusyk et al., 2010). As a consequence, high dose of stress causes bone marrow aplasia, anemia and infections. Elimination of Bmi1 in HSCs impairs its self-renewal capability due to the accumulation of $p19^{Arf}$, which induces p53-dependent cell death. In contrast, inhibition of p53 activity results in cell resistance to the apoptosis, augmenting the effects of genotoxic stress (Bálint et al., 2001; Mihara et al., 2003). These further prove that p53 plays a pivotal role in regulating apoptosis in HSCs.

We worked to gain a further understanding of p53 function in HSCs upon stress. Myeloid Elf-1-Like Factor (MEF), is a ETS Factor. We found that p53 and MEF double knock-out HSCs have more DNA damage indicator r-H2AX foci than MEF-deficient HSCs following irradiation, demonstrated that p53 promotes DNA damage repair in HSCs under condition of genotoxic stress (Liu et al., 2009b). DNA damage repair is critical for preservation of stem cell function, as it reported that decreased DNA damage repair activity attributed to accumulation of damaged DNA, resulting in reduced self-renewal capability of stem cells (Park et

al., 2003). Chemotherapeutic drugs trigger both p53 dependent and p53 independent apoptosis. We reported that HSCs from p53 knock-out mice are more resistance to apoptosis induced by chemotherapeutic agents, and p53-null HSCs show enhanced repopulation potential following chemotherapy (Shounan et al., 1996; Park et al., 2003; Liu et al., 2009b). These studies implicated that activation of p53-regulated gene contributes to the death of leukemic stem cells.

From a molecular perspective, p53 transcriptionally regulates the expression of pro-apoptotic genes, such as Puma, Bax and Noxa. Puma (p53 upregulated mediator of apoptosis) induces apoptosis in HSCs under genotoxic stress (Brosh et al., 2009; Brown et al., 2009). Puma-null HSCs are more quiescent and highly resistant to irradiation (Shao et al., 2010). On the contrary, transcription repressor Slug, also a p53 target, functions as an anti-apoptotic protein in HSPCS (Wu et al., 2005). A study from Look and colleagues suggested that Slug prevents the damaged HSPCs from apoptosis by directly repressing Puma gene expression. The activation of Slug in leukemic cells may attribute to their persistence in response to irradiation or chemotherapy, indicating a therapeutic strategy to inhibit Slug in cancer treatment (Wu et al., 2005).

TP53 mutations

1. Mechanisms of *TP53* mutations

Unlike most tumor suppressor genes, which are typically biallelic deleted during tumorigenesis, *Tp53* is mostly inactivated by monoallelic missense mutations that lead to the formation of a stable full-length protein with the substitution of a single amino acid (Brosh et al., 2009; Freed-Pastor et al., 2012). The majority of the substitutions are located at the DNA-binding domain (DBD) of p53 and alter its sequence-specific DNA-binding activity, as shown in Figure 1.3 (Brosh et al., 2009). As a result, these mutations may lost wild-type p53 activity, which is called “loss-of-function” (LOF) mutant. In addition, given that p53 proteins function as a tetramer, mutant p53 proteins may dampen the activity of wild-type p53 and exert “dominant-negative” (DN) effect. Interestingly, numerous findings suggested some mutant p53 acquire oncogenic functions independent of wild-type p53 function, namely “gain-of-function” (GOF) mutant (Brosh et al., 2009; Patricia et al., 2013). Introduction of mutant p53 into $p53^{-/-}$ cells generates new phenotypes, such as increased metastasis and genomic instability, in comparison to $p53^{+/-}$ and $p53^{-/-}$ cells.

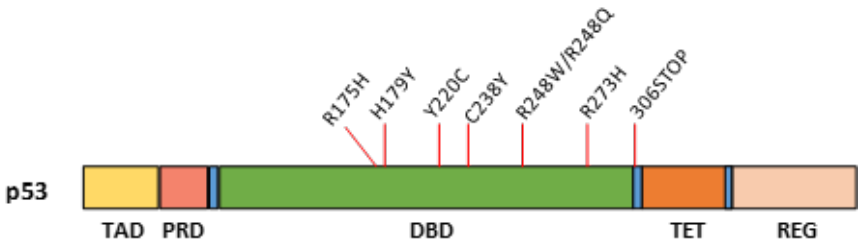


Figure 1.4 Hot-spot p53 mutations identified in human cancer.

Accumulating studies have elucidated molecular mechanisms underlying mutant p53 driven tumorigenesis. Since most mutant p53 proteins still retain the N-terminal transcriptional transactivation domains, studies have been focused on their activity to directly regulate gene expression (Barlow et al., 2010). One amino acid is substituted within the DNA-binding domain of most mutant p53 proteins, thereby the ability to bind WT p53 response elements is impaired. This raises one of the possible mechanism of action in that sequence-specific DNA binding activities are altered, resulting in unique mutant p53 response elements attributing to gain-of-function effect. Recently, it was published that mutant p53 binds to and activates epigenetic regulatory genes, *MLL1*, *MLL2* and *MOZ*, thereby promoting cancer cell proliferation (Zhu et al., 2015). Moreover, mutant p53 can interact with transcription machinery modulating their transcription activity. The interaction of mutant p53 with transcription factors could be simulation or inhibition. For example, in breast cancer cells, mutant p53 cooperates with SWI/SNF chromatin remodeling complex and trans-activates *VEGFR2* expression through remodeling its promoter (Pfister et al., 2015). The other scenario is that mutant p53 directly interacts with some proteins and changes their functions which are not related to transcription. For instance, Xu and colleagues have discovered that mutant p53 directly interacts with MRE11, a DNA nuclease required for DNA repair, prevents MRN complex from phosphorylating ATM, leading to impaired DNA damaged repair and genome instability (Song et al., 2007). Other groups showed that wild-type p53 both promotes and inhibits topoisomerase 1 (Top1), which maintains topology of DNA.

In contrast, mutant p53 binds to Top1 and enhances its activity, resulting in hyper-recombination and loss of genome integrity (Muller et al., 2013).

2. *TP53* mutations in hematological malignancies

The p53 pathway is activated upon a variety of stress conditions, allowing p53 to coordinate transcription programs that ultimately contribute to genome integrity and tumor suppression (Meek, 2009). When the *Tp53* gene is mutated or the p53 pathway is compromised, cells are suspected to acquiring mutations or chromosome aberrations leading to cancer transformation (Meek, 2009; Brown et al., 2009). Indeed, p53 is the most frequently altered tumor suppressor in human cancers. More than 60% of solid tumors exhibit deletions or point mutations in the p53 genes (Hollstein et al., 1991; Hainaut et al., 1998), such as breast cancer, ovarian cancer and prostate cancer.

In contrast to solid tumors, hematological malignancies present a relatively low frequency of alterations in this gene (Rucker et al., 2012; Muller et al., 2013). For example, *TP53* is mutated in 10-20% of chronic lymphocytic leukemia (CLL), around 10% of AML, less than 3% in acute lymphoblastic leukemia (ALL), and 10-12% cases of multiple myeloma (MM) (Peller et al., 2003; Harutyunyan et al., 2011). Of note, humans born with a single allele *TP53* mutation, the Li–Fraumeni syndrome, have an increased tendency to develop many types of cancer, including MDS and AML (Lang et al., 2004; Olive et al., 2004; Talwalkar et al., 2010). Nevertheless, clinical observations indicate that the survival rate of patients with p53 mutations is shorter than those with wild-type p53 in hematological malignancies, mainly due to poor prognosis, complex karyotype,

and drug resistance (Volkert, et al. 2014; Wong et al., 2015; Welch, et al 2016; Welch et al., 2016; Chang, et al. 2017;). TP53 mutated clones are often found to expand following treatment with lenalidomide, leading to resistance of the drug (Kulasekararaj et al., 2013). Moreover, alterations of p53 occur mostly in therapy-related secondary AML and MDS in patients, with many of them carrying a further chromosome abnormities (Tehranchi et al., 2010; Sebaa et al., 2012; Metzgeroth et al., 2016). Furthermore, allogenic bone marrow transplantation, which is likely to be the most efficacy treatment for MDS patients, failed to improve the clinical outcome of those patients with TP53 mutations (Christopeit, et al. 2016; Lindsley, et al. 2017).

Next generation sequencing (NGS) have allowed an understanding of the sequence of mutations in the evolution of primary AML and MDS. Recently, acquired somatic gain-of-function (GOF) p53 mutations were identified in the blood of aged healthy individuals as well as in MDS or/and AML patient (Genovese, et al. 2014; Jaiswal, et al. 2014). Moreover, TP53 are among the genes most common found to be altered in people with CHIP (Bejar et al., 2011). These evidences suggest that p53 mutations may be early events in the pathogenesis of the disease. However, further understanding of the origin of is still lacking.

Apart from *TP53* mutations or deletions, other mechanisms may negatively affect the expression and activity of wild-type p53, resulting in the loss of tumor suppression activity. The most compelling case is the elevated expression of MDM2, a negative regulator of p53 (Zhou et al., 2000). MDM2 is

transcriptionally activated by p53 and then binds to the N-terminal region of p53, thereby preventing p53 from interacting with the transcriptional programs and inducing proteasome-mediated degradation of p53 (Ringshausen et al., 2006).

Although functions of mutant p53 in solid tumor have been well characterized, their role in hematological malignancies is poor understood. The effects of mutant p53 have been shown to be context dependent. Interactions that enhance activity in some circumstances may be inhibitory in others. Therefore, it is critical to decipher the mechanisms by which mutant p53 proteins initiate and drive hematological malignancies in order to gain better knowledge to develop therapeutic options for those dismal diseases with *TP53* mutations.

Summary and Specific Aims

Myelodysplastic syndrome (MDS) is characterized by ineffective hematopoiesis, multi-lineage morphological dysplasia and less than 5% blast cells with high risk to progress to acute myeloid leukemia (AML) (Nimer, 2008; Lindsley et al., 2013; Woll, et al., 2014; Sperling, et al., 2017;). Mutations on tumor suppressor gene *TP53* present approximately 10% in MDS (Bejar et al., 2014; Sperling, et al. 2017), whereas MDS patients with *TP53* mutations are associated with a very dismal prognosis and respond unfavorably to chemotherapy (Chang, et al. 2017; Volkert, et al. 2014; Welch, et al 2016) , leading to shorter survival of these patients. Therefore, a better understanding of the pathogenesis of MDS with *TP53* mutations is of great clinical significance.

MDS is a clonal disease arising from the expansion of mutated hematopoietic stem cells (HSCs) (Nimer et al., 2008; Wong et al., 2015; Sperling et al., 2017). Accumulating studies indicate that the transformation event is initiated from the acquisition of pre-leukemic mutations driving the development of pre-leukemic HSCs, preceding the formation of fully transformed MDS stem cells (Bejar et al., 2011; Genovese et al., 2014; Jan et al., 2017). Pre-leukemic HSCs have competitive advantage compared to normal HSCs without affecting hematopoietic differentiation (Genovese et al., 2014). Recently, acquired somatic gain-of-function (GOF) mutations in *TP53* gene have been identified in the blood of aged healthy individuals as well as in patients with MDS (Genovese et al., 2014), implying the importance of *TP53* mutations in the pre-leukemic phase of MDS development.

We have been investigating the role of tumor suppressor p53 in hematopoiesis and discovered that p53 maintains HSC quiescence and promotes DNA damage repair in response to irradiation (Liu et al., 2009b), suggesting a critical role of p53 in HSC function. Considering that mutations in T*P53* alter the DNA-binding specificity of wild-type p53 (Brosh et al., 2009), mutant p53 proteins may utilize distinct "gain-of-function" mechanisms to regulate HSC behavior. This aspect of mutant p53 is largely unknown and need to be explored.

Based on clinical evidence and our preliminary data, I hypothesized that **GOF mutant p53 drives the development of pre-leukemic HSCs with enhanced competitive advantage, contributing to clonal expansion and the pathogenesis of MDS**. The main objective of this study was to understand the biological impact and molecular basis of GOF mutant p53 in pre-leukemic HSCs and the pathogenesis of MDS. In this project, I specifically addressed the following specific aims:

Specific Aim 1: **Determine the role of GOF mutant p53 in hematopoietic stem cells**. I *hypothesized* that mutant p53 enhances HSC self-renewal without affecting terminal differentiation in order to drive the development of pre-leukemic HSCs. I performed serial competitive bone marrow transplantation, competitive HSC transplantation and limiting dilution assays to determine the role of mutant p53 in HSCs. (Chapter 3)

Specific Aim 2: Determine the role of GOF mutant p53 in HSCs following genotoxic stress. I *hypothesized* that mutant p53 preserves HSC

function in response to genotoxic stress and promotes their clonal expansion. I challenged HSCs expressing mutant p53 with irradiation or the chemotherapeutic agent Fluorouracil (5-FU) and performed competitive transplantation assay to examine whether HSCs expressing mutant p53 are resistant to genotoxic stress. (Chapter 4)

Specific Aim 3: Determine the role of GOF mutant p53 in the pathogenesis of MDS. I *hypothesized* that mutant p53 contributes to the pathogenesis of myelodysplastic syndrome in mice during ageing. I monitored the disease development of $p53^{+/+}$ and $p53^{R248W/+}$ mice to determine whether mutant p53 mice developed features recapitulating human myelodysplastic syndrome. (Chapter 5)

Specific Aim 4: Elucidate the mechanisms by which GOF mutant p53 drives the development of pre-leukemic HSCs. I *hypothesized* that mutant p53 promotes the development of pre-leukemic HSCs through regulating genes or/and pathways critical for HSC function. I utilized RNA-seq, gene expression profiling, and ATAC-seq assays to decipher the molecular mechanisms by which mutant p53 contributes to pre-leukemic HSC development. (Chapter 6)

Specific Aim 5: Determine the interactions between GOF mutant p53 and epigenetic regulators in HSCs. I *hypothesized* that mutant p53 interacts with epigenetic regulators to modulate genes or/and pathways essential for HSC maintenance. I performed biochemical experiments and ChIP-seq assay to explore how mutant p53 collaborates with epigenetic factors in regulating HSC self-renewal. (Chapter 7)

Significance

Pre-leukemic HSCs may transform to leukemic stem cells, especially under stress conditions (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Pre-leukemic HSCs are resistant to chemotherapy and serve as reservoirs for disease, generating new cancerous clones and leading to relapse (Corces-Zimmerman et al., 2014; Shlush et al., 2014). A major barrier to development of effective therapeutics for hematological malignancies is lack of in-depth knowledge of the origins and the development of pre-leukemic HSCs. Therefore, there is a critical need to identify the aberrations and elucidate the underlying mechanisms that lead to pre-leukemic HSCs formation. While *TP53* mutations have been identified in aged healthy individuals as well as patients with MDS, the role of mutant p53 in clonal hematopoiesis and pathogenesis of MDS is largely unknown. I discovered that GOF mutant p53 drives pre-leukemic HSC and MDS development through epigenetic modulation.

Thus, our studies provide novel insights into the mechanisms of by which mutant p53 drives pre-leukemic HSC development. Understanding of the molecular basis of pre-leukemic HSC with p53 mutations has implication for cancer prevention at pre-malignant stage. Moreover, it will facilitate the development of therapeutic strategies that would be effectively in eliminating chemotherapy resistant pre-leukemic HSCs and MDS stem cells.

Given that MDS patients with *TP53* mutations are associated with a poor prognosis and bone marrow transplantation fails to improve their clinical outcomes (Chang, et al. 2017; Volkert, et al. 2014; Welch, et al 2016; Christopeit,

et al. 2016; Lindsley, et al. 2017), these patients are desperately in need of more effective therapeutic options. Therefore, our humanized mutant p53 knock-in $p53^{R248W/+}$ mouse model provides a valuable research tool to study the pathogenesis of MDS with *TP53* mutations and test novel therapeutic agents.

CHAPTER TWO

Materials and methods

Materials

1. Mice

All mice were maintained at the Indiana University Laboratory Animal Research Center (Indianapolis, IN, USA) according to the Institutional Animal Care and Use Committee (IACUC) of the Indiana University School of Medicine in the Indiana University Animal Facility.

1.1 HUPKI (*p53*$^{+/+}$) and *p53*$^{R248W/+}$ mice

The HUPKI (*p53*$^{+/+}$) and *p53*$^{R248W/+}$ mice used in our studies have been backcrossed to the C57BL6 background for 8 generations (Song et al., 2007). All young *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice used in these studies are 8 to 12 weeks old and are tumor free. We maintained both *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice for more than 12 months for aging studies.

1.2 C57BL/6 mice

The C57BL/6 mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

1.3 BoyJ mice

The BoyJ mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

1.4 F1 C57BL/6/ BoyJ mice

The F1 mice were purchased from the In Vivo Therapeutics Core at Indiana University School of Medicine.

2. Primers

Table 2.1 Primers

	Gene	Forward (5'-3')	Reverse (5'-3')
qPCR primer	Actin	GCCCTGAGGCTCTTTTCCAG	TGCCACAGGATTCCATACCC
	p21	GACAAGAGGCCCAGTACTTC	GCTTGGAGTGATAGAAATCTGTC
	Necdin	GAGTTTGCCCTGGTCAAAGC	CATAGATGAGGCTCAGGATCATGA
	Mll1	CGGGCTCATCAACGATAAGC	CAGGCCCAGATGTCAGGTG
	Mll2	GGATCTATGACAGGGCTTTCCC	ACCATGTGACATCATTCCTTGC
	Moz	GTGCTGCTACACCGATGGTG	CTCTCGCTGAAGCCCCTAAA
	Ezh2	CAATGATTTTGTGGTGGATG	CAAAGATGCCTATCCTGTGGT
	Cxcl9	GCCATGAAGTCCGCTGTTCT	TAGGGTTCCTCGAACTCCACA
	Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
	Mef/Elf4	GCAGGACTGGAAAGGTTGCT	CTGAGCCTCTGGGGAGGATA
	Gadd45g	GTCCTGAATGTGGACCCTGA	GCAGAACGCCTGAATCAACG
ChIP-qPCR primer	ELF4-TSS	CTGCACTTTAAGCGGCCTTC	TCTCTTGAGTCAGCGGATCG
	ELF4-UP 100	TTATTTCTTGCTCCCGCCCC	GGACTCACTTTCCTCCCAGC
	Gadd45g-TSS	CGCGCCGAGCCTGTCTATAA	AAGATTCCCAGAGTCCGATGC
	Gadd45g-UPS	GCATCAGAAAGCGGGCG	GCCGGGAGAGCCCTTTTAT
	Gadd45g-Down	CATTGATATCGTGCGCGTGG	GGGGGACACTCACCGAAATG

3. Antibodies

Table 2.2 Antibodies

Antibody	Company	Catalog Number
CD3-PE	Biolegend	145-2C11
B220-PECY7	Biolegend	RA3-6B2
Gr-1-APC	Biolegend	RB6-8C5
Mac1-APC	Biolegend	M1/70
Ter119-FITC	Biolegend	TER-119
CD45.2-FITC	Biolegend	104
CD45.1-PE	Biolegend	2B8
CD48-PE	eBioscience	HM48-1
CD150-Percpcy5.5	eBioscience	TC15-12F12.2
cKit-APC	eBioscience	2B8
CD16/CD32-PE-Cy7	eBioscience	93
Sca-1-PE-Cy7	eBioscience	D7
CD34-FITC	eBioscience	RAM34
Strepdividin-APCcy7	eBioscience	RA3-6B2
Sca-1-Percpcy5.5	eBioscience	D7

4. Commercial Kits

Table 2.3 Commercial Kits

Kit	Company	Catalog Number
EZ ChIP Chromatin Immunoprecipitation Kit	Millipore	17-371
Lin- Cell Depletion Kit, mouse	Miltenyi Biotec	130-090-858
PE Mouse Anti-Human Ki-67	BD Pharmingen	556027
FastStart Universal SYBR Green Master (Rox)	Roche	04913850001
MicroRNA Reverse Transcription Kit	Qiagen	4366596
GeneJET PCR purification Kit	Thermo Scientific	K0702
RNeasy Plus Micro Kit	Qiagen	74034

5. Reagents

Table 2.4 Reagents

Reagent	Company	Catalog Number
RIPA buffer	Sigma	R0278
NuPAGE 10% Bis-Tris Gel	Life technologies	NP0316BOX
Immobilon Western HRP Substrate Peroxide Solution	Millipore	16144B2
SuperSignal West Femto	Thermo Scientific	1856190
Protease Inhibitor	Roche	
Streptavidin MicroBeads	Miltenyi Biotec	130-048-101
APC Annexin V	Biolegend	640920
FITC Annexin V	Biolegend	640906
10x Annexin V Binding Buffer	BD Pharmingen	51-66121E
Red Blood Cell Lysis Solution (10x)	Miltenyi Biotec	130-094-183
SuperScript IV First-Strand Synthesis System	Invitrogen	18091050
MethoCult GF M3434	StemCell	03434
GoTag G2 Hot Start Green Master Mix	Promega	M7423

Methods

1. Stem and progenitor cell assays

Progenitors as assayed by colony formation were determined in methylcellulose medium (MethoCult GF M3234, StemCell Technologies) using 2 x 10^4 BM cells per well (6-well plate). Colonies were scored after 7 days of the initial culture, and all cells were collected and washed twice in PBS. Subsequently cells were cultured at 2 x10^4 per well in the same medium. Colony scoring and replating were repeated every 7 days at least two times, or until no colonies were observed in the cultures.

2. Flow cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells was performed as described previously (Liu et al., 2009b). Murine hematopoietic stem and progenitor cells were identified and evaluated by flow cytometry using a single cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers listed in the Table 1.1 (See page 5). Bone marrow (BM) cells were obtained from tibia, femur and iliac crest (6 from each mice) by flushing cells out of the bone using a syringe and phosphate-buffered saline (PBS) with 2mM EDTA. Red blood cells (RBCs) were lysed by RBC lysis buffer (eBioscience) listed in Table 2.4 prior to staining by antibodies listed in Table 2.2. Experiments were performed on FACS LSR IV cytometers (BD Biosciences) and analyzed by using the FlowJo Version 9.3.3 software (TreeStar).

3. Ki-67 staining

Bone marrows were stained for cell surface markers as described above. After staining, cells were washed with 0.2% BSA in PBS, fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) listed in Table 2.3 and then incubated with PE-conjugated-antibody against Ki-67 (BD Biosciences) for more than 30 minutes on ice. Cells were washed, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) and acquired using LSR IV flow cytometer machine. Data analysis was performed using FlowJo software.

4. AnnexinV staining

Bone marrows were stained for cell surface markers as described above. After staining, cells were washed with 0.2% BSA in PBS, and then suspended in 1X AnnexinV-binding buffer listed in Table 2.4. Suspended cells were stained with FITC-conjugated-AnnexinV (BD Biosciences) for 30 minutes at room temperature. DAPI (Sigma) was then added to cell suspension and LSR IV flow cytometer machine was used for acquisition. Data analysis was performed using FlowJo software

5. Transplantation

5.1 Competitive bone marrow transplantation

We injected 5 x 10^5 total BM cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice (CD45.2$^+$) plus 5 x 10^5 competitor total BM cells (CD45.1$^+$) into 9.5Gy lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$). Peripheral blood was obtained by tail vein bleeding every 4-week after transplantation, RBC lysed, and the PB mononuclear cells stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. 16 weeks following transplantation, bone marrow cells from recipient mice were analyzed to evaluate donor chimerism in bone marrows. For secondary transplantation, 3X10^6 BM cells from mice reconstituted with *p53*$^{+/+}$ or *p53*$^{R248W/+}$ BM cells were injected into 9.5Gy lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$).

5.2 Competitive HSC transplantation

We injected 200 CD48$^-$CD150$^+$LSK cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice (CD45.2$^+$) plus 3 x 10^5 competitor bone marrow cells (CD45.1$^+$) into lethally

irradiated F1 mice ($CD45.1^+CD45.2^+$). The percentage of donor-derived ($CD45.2^+$) cells in peripheral blood was analyzed every 4 weeks after transplantation as described above. 16 weeks following transplantation, we harvested bone marrow cells from recipient mice and performed flow cytometry analysis to evaluate HSC repopulating capability.

5.3 Whole bone marrow transplantation

Recipient mice were lethally irradiated (9.5Gy) followed by injection of 1 x 10^6 to 3 x 10^6 BM cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice.

6. Limiting dilution assay

Different doses (10,000, 20,000, 40,000, 80,000) of BM cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice ($CD45.2^+$) with 200,000 competitor cells ($CD45.1^+$) were transplanted into lethally irradiated (9.5Gy) F1 recipient mice ($CD45.2^+CD45.1^+$). The percentage of donor-derived (CD45.2+) cells were analyzed 16-week following transplantation as described above. HSC frequency was calculated using L-Calc software (StemCell Technologies Inc.) and ELDA software (bioinf.wehi.edu.au/software/elda/). Poisson statistics was used to calculate the p value.

7. Homing assays

1 x 10^7 *p53*$^{+/+}$ and *p53*$^{R248W/+}$ BM cells ($CD45.2^+$) were injected into lethally irradiated recipient mice ($CD45.1^+$). BM cells were harvested 18 hours following injection and the frequency of donor-derived cells ($CD45.2^+$) was evaluated by flow cytometry.

8. Histology

All tissue samples were collected following a detailed LARC approved lab animal protocol. Tissues were fixed overnight at room temperature in 10% NBF after which they were transferred through graded concentrations of alcohol to xylene inside a Leica Automated Vacuum Tissue Processor. They were embedded in paraffin before being sliced into five micron thick sections, mounted onto positively charged slides, and baked at 60 °C. The slides were stained with H&E for pathologic evaluation.

9. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using RNeasy Plus Micro Kit (Qiagen) in Table 2.3 and cDNA was prepared from total RNA using SuperScript IV First-Strand cDNA Synthesis Kit (Invitrogen Life Technologies) and oligo (dT) primers, following manufacturer's instructions. qRT- PCR assay was performed by using the 7500 Real Time PCR machine (Applied Biosystems) with FastStart Universal SYBR Green Master (ROX) (Roche). Primers for PCR are listed in Table 2.1.

10. Quantification of γ-H2Ax by ImageStream Flow Cytometry

Lineage-depleted BM cells were first stained with antibodies against appropriate HSPC surface markers, then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), as described by the manufacturer, and finally stained with an Alexa-488-conjugated anti- γ-H2Ax antibody (Cell Signaling Technology).

11. Quantification of H3K27me3 by ImageStream Flow Cytometry

Lineage-depleted BM cells were first stained with antibodies against HSPC surface markers, then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences), as described by the manufacturer, and finally stained with an Alexa-488-conjugated anti- H3K27me3.

12. Co-IP

Co-Immunoprecipitation (Co-IP) experiments were performed as described previously (Gao et al., Mol Cell 2012) with certain modifications. H1299 cells were co-transfected with FLAG-HA-EZH2 and wild-type or mutant p53, respectively, or transfected with FLAG-HA-EZH2 alone. Nuclear extract was prepared from these cells and incubated with a polyclonal p53 antibody (FL393) (Santa Cruz) prior to addition of protein G beads. After overnight incubation, beads were then washed 5 times and eluted with glycine (0.1 M, pH 2.0), and then neutralized by adding Tris solution (1.5 M, pH 8.8). The eluates were mixed with SDS sample buffer and analyzed by SDS-PAGE, followed by immunoblotting.

13. RNA sequencing

Total RNA is extracted from cells using RNeasy MicroPlus Kit (Qiagen). Then the mRNA is enriched with the oligo (dT) magnetic beads (for eukaryotes), and is fragmented into short fragments (about 100 bp). With random hexamer-primer, the first strand of cDNA is synthesized, and then the second strand is synthesized. The double strand cDNA is purified with magnetic beads. The ends of the double strand cDNA are repaired, and a single nucleotide A (adenine) is

added to the 3′-ends. Finally, sequencing adaptors are ligated to the fragments. The ligation products are amplified with PCR. For quality control, RNA and library preparation integrity are verified using Agilent 2100 BioAnalyzer system and ABI StepOnePlus Real-Time PCR System. RNA sequencing library was then constructed and then sequenced with Hiseq 4000. Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed on gene sets from the Molecular Signatures Database (MSigDB, https://www.broadinstitute.org/msigdb) and additional gene sets curated from publications. Gene sets with FDR q-value < 0.05 were considered significantly enriched.

14. Expression microarray analysis

Transcript profiling of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs (CD48-CD150+LSKs) were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Raw data will be available for download from Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo/, accession number x). For data analysis, CEL files were analyzed using GenePattern (Broad Institute) according to current protocols. Pearson correlation values were determined from all genes that passed normalization and filters. Gene Set Enrichment Analysis (GSEA) was conducted according to current protocols.

15. Chromatin immunoprecipitation sequencing (ChIP-seq)

Cells were fixed with 1% formaldehyde for 15 min and quenched with 0.125 M glycine. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated and the DNA

sheared to an average length of 300-500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K and heat for de-crosslinking, followed by ethanol precipitation. Pellets were resuspended and the resulting DNA was quantified on a NanoDrop spectrophotometer. An aliquot of chromatin (30 ug) was precleared with protein A agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 ug of antibody against Histone H3K27me3 (clone: 39155, Active Motif) Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Crosslinks were reversed by incubation overnight at 65 C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation. Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end-polishing, dA-addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina's NextSeq 500 (75 nt reads, single end). Reads were aligned to the mouse genome (mm10) using the BWA algorithm (default settings). Duplicate reads were removed and only uniquely mapped reads (mapping quality >= 25) were used for further analysis.

Alignments were extended in silico at their 3'-ends to a length of 200 bp, which is the average genomic fragment length in the size-selected library, and assigned to 32-nt bins along the genome. The resulting histograms (genomic "signal maps") were stored in bigWig files. H3K27me3 enriched regions were identified using the SICER algorithm with a MaxGap parameter setting of 600 bp. Signal maps and peak locations were used as input data to Active Motifs

proprietary analysis program, which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations.

16. ATAC sequencing

100,000 *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSK cells were harvested and frozen in culture media containing 5% DMSO. Frozen cells were sent to Active Motif to perform the Open Chromatin Sequencing Assay. The cells were then thawed in a 37^{o}C water bath, pelleted, washed with cold PBS, and tagmented as previously described (Buenrostro et al. 2013), with some modifications. Briefly, cell pellets were resuspended in lysis buffer, pelleted, and tagmented using the enzyme and buffer provided in the Nextera Library Prep Kit (Illumina). Tagmented DNA was then purified using the MinElute PCR purification kit (Qiagen), amplified with 10 cycles of PCR, and purified. Resulting material was quantified using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems), and sequenced with PE42 sequencing on the NextSeq 500 sequencer (Illumina).

17. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad software, Inc). All data are presented as mean ± standard error of the mean (SEM). The sample size for each experiment and the replicate number of experiments are included in the figure legends. Statistical analyses were performed using unpaired, two-tailed Student's t test where applicable for comparison between two groups, and a One-way ANOVA test or Two-way ANOVA was used for experiments involving more than two groups. Statistical

significance was defined as *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

CHAPTER THREE

GOF mutant p53 enhances the repopulating potential of hematopoietic stem cells

Introduction

We have been investigating the role of tumor suppressor p53 in normal hematopoiesis. We have discovered that p53 regulates HSC quiescence by analyzing the p53 knock out mice at the age of two to three months (Liu et al., 2009b). In addition, studies from other groups suggest that p53 regulates proliferation, differentiation and ageing in HSCs (Kastan et al., 1991; Shounan et al., 1996; Park et al., 2003; Liu et al., 2009a). Given that p53 is a critical regulator of HSCs, alteration of p53 may lead to overt blood malignancies. Indeed, *TP53* mutations are found in approximately 10% AML or MDS patients (Rücker, et al. 2012; Sperling, et al. 2017). Moreover, among AML patients with complex karyotypes, the frequency of *TP53* mutations and/or deletions is almost 70% (Chang, et al. 2017; Volkert, et al. 2014; Welch, et al 2016). Previously, *TP53* mutations and/or deletions were regarded as secondary events in the evolution of leukemia (Harutyunyan et al., 2011). However, *TP53* mutations have recently been found in the blood of aged healthy individuals by next generation sequencing studies, and *TP53* mutations have been identified as one of the most common alterations in clonal hematopoiesis (Genovese et al., 2014). These findings implicate a role for*TP53* mutations in the early steps of leukemia development. Therefore, it was tempting for us to study whether *TP53* mutations

are pre-leukemic mutations that drive the development of pre-leukemic hematopoietic stem cells.

p53 knock-out mice have been widely used to determine the role of the p53 pathway in hematopoietic malignancy (Liu et al., 2009b). However, *TP53* deletions are mainly found in advanced phase of hematological malignancies (Harutyunyan et al., 2011). Further, most *TP53* mutations in AML or MDS are mono-allele missense mutations which can have dominant negative effects and/or gain-of-function (GOF) features (Freed-Pastor et al., 2012). These aspects of *TP53* mutations in regulating HSC behavior cannot be delineated by using $p53^{-/-}$ mice. Thus, the role of *TP53* mutations in the pathogenesis of MDS and leukemia remains elusive.

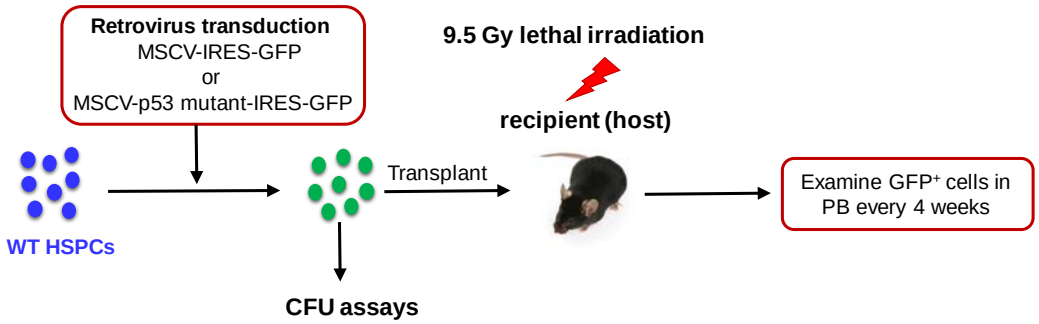
In order to study the role of *TP53* mutations in HSCs, we performed a functional screening of 8 hot-spot *TP53* mutations identified in AML or MDS (Barlow et al., 2010). The experimental designs were illustrated in Figure 3.1 A. Briefly, hotspot p53 mutants were introduced into WT fetal live cells and transduced cells (GFP^{+}) cells were then utilized to perform serial replating assays. Some of the mutants, including $p53^{R248W}$, $p53^{R273H}$, $p53^{Y220C}$, $p53^{R175H}$, $p53^{R248Q}$, and $p53^{C238Y}$, enhanced the serial replating potential of HSPCs *in vitro* compared to control viruses (MIGR1) transduced cells (Figure 3.1.B). Competitive transplantation assays were then performed to determine the role of some GOF p53 mutants, such as $p53^{R248W}$, $p53^{R273H}$ and $p53^{Y220C}$, in HSCs *in vivo*. We found that ectopic expression of GOF mutant p53 proteins enhance the repopulating potential of murine hematopoietic stem and progenitor cells (Figure

3.1.C). Our data indicate that GOF mutant p53 may enhance the repopulating potential of HSCs *in vivo*.

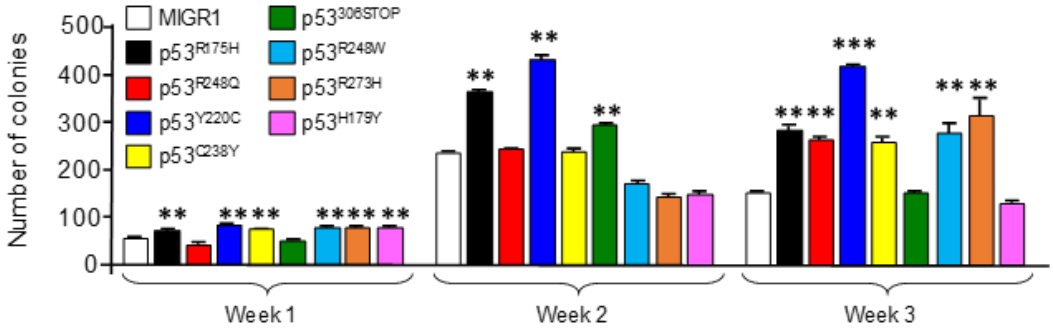
Based upon our preliminary studies and clinical findings, I hypothesized that **GOF mutant p53 increases the competitive advantage of HSCs without impairing normal differentiation, leading to the development of pre-leukemic hematopoietic stem cells**.

To test this hypothesis, I investigated the function of mutant p53 in HSCs utilizing mouse models. I focused on studying $p53^{R248W}$ mutant as codon R248 of p53 is most frequently mutated in myeloid malignancies (Rücker et al., 2012; Welch et al., 2016). Moreover, mutations on R248 codon have been found in the blood of aged healthy people (Genovese et al., 2014). I, therefore, utilized the humanized $p53^{R248W}$mice, where the humanp53 protein is expressed from the endogenous murine *Trp53* promoter. I examined HSC behavior in young adult (2-3 month old) $p53^{+/+}$ and $p53^{R248W/+}$ mice. SLAM cell surface markers were used to determine the frequency of immunophenotypic LT-HSCs in mice. I analyzed the frequency of quiescent and apoptotic HSCs utilizing Ki67 and Annexin V staining.

A


Retrovirus transduction
MSCV-IRES-GFP
or
MSCV-p53 mutant-IRES-GFP
9.5 Gy lethal irradiation
recipient (host)
WT HSPCs
Transplant
Examine GFP+ cells in PB every 4 weeks
CFU assays

B


Number of colonies
MIGR1
p53R175H
p53R248Q
p53Y220C
p53C238Y
p53306STOP
p53R248W
p53R273H
p53H179Y
Week 1
Week 2
Week 3

C

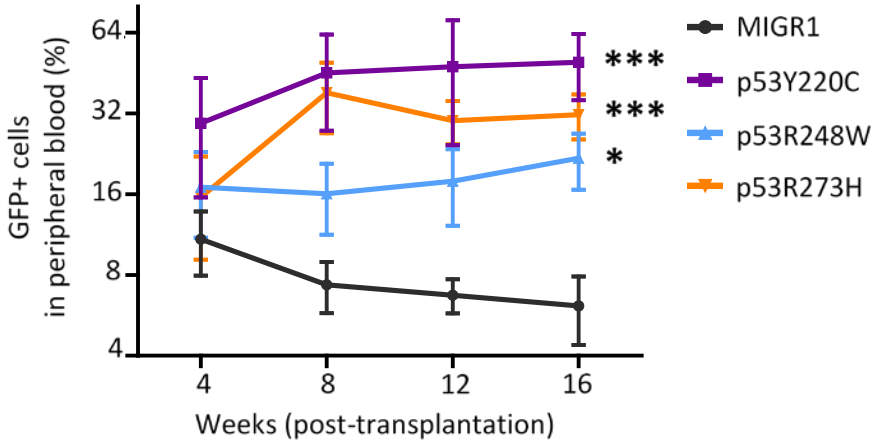

GFP+ cells in peripheral blood (%)
Weeks (post-transplantation)
MIGR1
p53Y220C
p53R248W
p53R273H

Figure 3.1 Functional screening of mutant p53 in hematopoietic stem cells.

(A) Hot-spot p53 mutants were introduced into wild type fetal liver cells using retrovirus-mediated transduction. *In vitro* and *in vivo* stem and progenitor cell assays were then performed using sorted GFP^+ cells.

(B) Serial replating assays of fetal liver cells transduced with p53 mutants. **p<0.01, ***p<0.001, n=3.

(C) Percentage of GFP^+ cells in the peripheral blood of recipient mice following competitive transplantation. n=5 mice per group, *p<0.05, ***p<0.001, ****p<0.0001.

HSCs have the capacity to provide long-term reconstitution of all hematopoietic lineages upon transplantation into lethally irradiated mice (Orkin et al., 2008). Thus, serial competitive transplantation assay is the gold standard to measure HSC self-renewal capability *in vivo* (Orkin et al., 2008; Ng et al., 2017). I first performed serial competitive bone marrow transplantation and competitive HSC transplantation assays in order to investigate whether heterozygote $p53^{R248W}$ mutant has an impact on HSC self-renewal and differentiation *in vivo*. I also performed limiting dilution assay to quantify the number of functional HSCs in the BM of $p53^{+/+}$ and $p53^{R248W/+}$ mice. Homing assay was conducted in order to evaluate whether $p53^{R248W}$ mutant affects HSC homing ability in transplantation settings. My ultimate ***goal*** is to determine the role of mutant p53 in the development of pre-leukemic HSCs.

Results

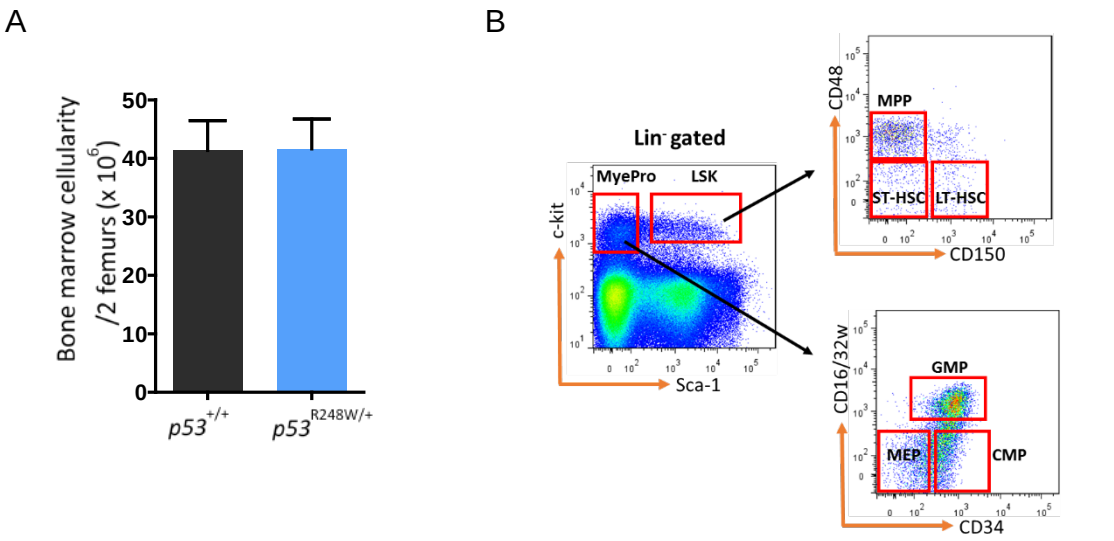
1. GOF mutant p53 does not affect HSC frequency

In our preliminary studies, we showed that some hot-spot GOF mutant p53 proteins, including $p53^{R248W}$, $p53^{R273H}$, and $p53^{Y220C}$, enhances the repopulating potential of fetal liver cells *in vivo* (Figure 3.1.C). However, the role of GOF mutant p53 in HSC self-renewal and differentiation *in vivo* is not known. Codon 248 of the p53 protein ($p53^{R248}$) is most frequently mutated in MDS and AML (Rücker et al., 2012; Welch et al., 2016). In addition, mutations in $p53^{R248}$ ($p53^{R248Q}$ and $p53^{R248W}$) were identified in aged healthy individuals and were found further proceed the development of AML (Genovese et al., 2014; Wong et al., 2015). Therefore, I focused on studying the role of $p53^{R248W}$ in HSC regulation. Considering that over-expression of mutant p53 from a MSCV promoter may not reflect accurate function at physiological levels, I used *p53*^{R248W} knock-in mice where $p53^{R248W}$ was introduced into the humanized *p53* knock-in (*HUPKI*) allele in mice, expressing human p53 mutant protein from the endogenous murine *Trp53* promoter (Song et al., 2007). The *HUPKI* allele encodes a human/mouse chimeric protein consisting primarily of human p53 sequence (amino acids 33–332) flanked by the conserved extreme amino and carboxyl-termini of mouse p53 (Luo et al., 2001). *HUPKI* mice were described as *p53*$^{+/+}$ mice in the text below.

Most *TP53* mutations in hematological malignancies are mono-allele missense mutations. To mimic clinical situations, I utilized heterozygous *p53*$^{R248W/+}$ mice to study how mutant p53 affects HSC behavior. Analysis of bone

marrow (BM) of young (8 to 12 week-old) *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice revealed that *p53*$^{R248W/+}$ and *p53*$^{+/+}$ mice have comparable bone marrow cellularity. (Figure 3.2.A). To examine the frequency of primitive HSPCs in BM, I then performed flow cytometry based on SLAM surface markers to distinguish LT-HSCs (Lin$^-$ Sca1$^+$c-kit$^+$CD150$^+$CD48$^-$), ST-HSCs (Lin$^-$Sca1$^+$c-kit$^+$CD150$^-$CD48$^-$), MPPs (Lin$^-$ Sca1$^+$c-kit$^+$CD150$^-$CD48$^+$) and LSKs (Lin$^-$Sca1$^+$c-kit$^+$) population in the BM (Figure 3.2.B). Interestingly, expression of p53^{R248W} does not affect the frequency of LT-HSCs, ST-HSCs, MPPs and LSK cells (Figure 3.2.C). I also found that the frequency of hematopoietic progenitor cells, including MEPs, GMPs and CMPs, are comparable between *p53*$^{R248W/+}$ and *p53*$^{+/+}$ mice (Figure 3.2.B and Figure 3.2.D).

Collectively, *p53*$^{R248W/+}$ does not affect immunophenotypic hematopoietic stem and progenitor cells during steady state hematopoiesis.


A
Bone marrow cellularity /2 femurs (x 10^6)
50
40
30
20
10
0
p53+/+
p53R248W/+
B
Lin- gated
MyePro
LSK
c-kit
Sca-1
CD48
MPP
ST-HSC
LT-HSC
CD150
CD16/32w
GMP
MEP
CMP
CD34

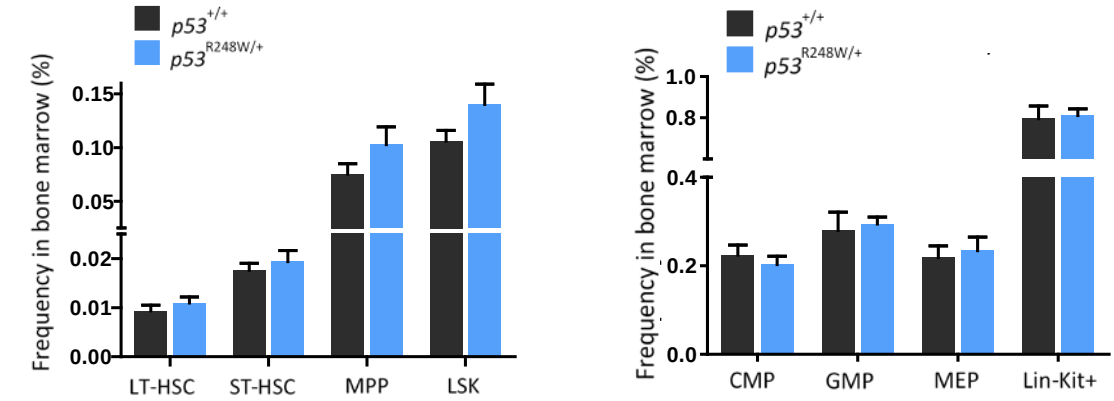

C
p53+/+
p53R248W/+
Frequency in bone marrow (%)
0.15
0.10
0.05
0.02
0.01
0.00
LT-HSC
ST-HSC
MPP
LSK
D
p53+/+
p53R248W/+
Frequency in bone marrow (%)
1.0
0.8
0.4
0.2
0.0
CMP
GMP
MEP
Lin-Kit+

Figure 3.2 Mutant p53 does not affect the frequency of hematopoietic stem and progenitor cells.

(A) Bone marrow cell numbers from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice (8 to 12 weeks old). n=10 mice per group.

(B) Representative flow cytometry plots of hematopoietic stem and progenitor cells gating were shown.

(C) The frequency of LT-HSCs, ST-HSCs, MPPs, and LSKs in the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. n=10 mice per genotype.

(D) The frequency of CMPs, MEPs, and GMPs in the bone marrow of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. n=10 mice per genotype.

2. GOF mutant p53 does not affect HSC survival in steady state hematopoiesis

Wild-type p53 plays a critical role in programmed cells death (Bálint et al., 2001). Given that wild-type p53 activates Bax, a pro-apoptotic protein of the BCL-12 family, loss of p53 decreases cell apoptosis (Mihara et al. 2003). While we reported that wild-type p53 is important for HSC survival (Liu et al., 2009b), the impact of GOF mutant p53 on HSC survival in steady state remains elusive. Therefore, I examined whether $p53^{R248W}$ mutant affect apoptosis of HSCs in steady state. AnnexinV and DAPI staining were then performed in freshly isolated BM cells following cell surface markers staining to determine the apoptotic state of HSCs. The early apoptotic cells are positively stained for Annexin V but not for DAPI, shown as the left lower panel on the profile. (Figure 3.3.A). Flow cytometry analysis showed no change in the apoptosis of $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$ HSCs (Figure 3.3.B), suggesting that mutant p53 does not affect the survival of HSC in steady state.

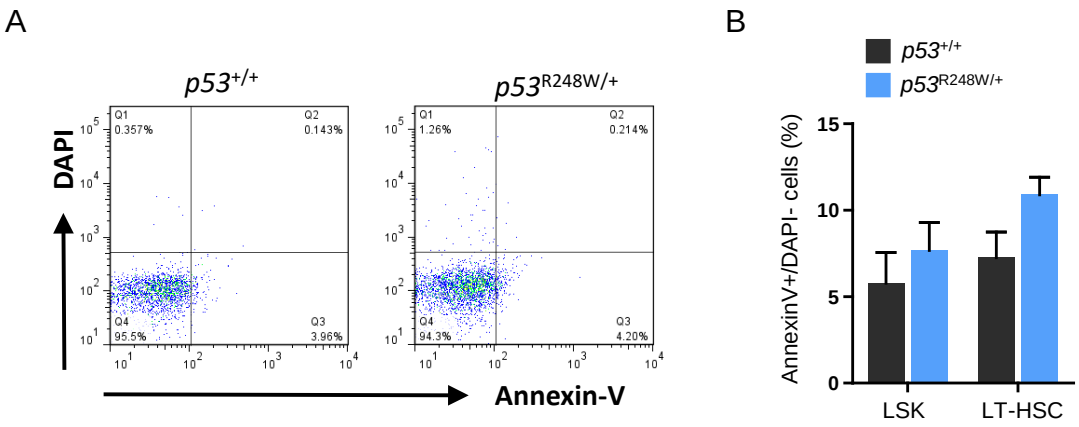


Figure 3.3 Mutant p53 does not affect survival of hematopoietic stem cells.

(A) Representative flow cytometry plots of Annexin V and DAPI staining of hematopoietic stem cells were shown.

(B) The percentage of early apoptotic *p53*$^{+/+}$ and *p53*$^{R248W/+}$ hematopoietic stem and progenitor cells was measured by Annexin V and DAPI staining. n=4 mice per genotype.

3. GOF mutant p53 does not affect HSC quiescence in steady state

Quiescence (G0) is a physiological state where cells exit the cell cycle and remain dormant (Orkin et al., 2008). It has been well established that most HSCs in an organism are quiescent in order to maintain long-term self-renewal potential and to prevent the stem cell pool from exhaustion (Orkin et al., 2008; Ng et al., 2017). In response to stress signals or cytokine stimulations, quiescent HSCs enter the cell cycle to replenish hematopoietic system and self-renew to maintain the stem cell pool (Zon et al., 2008). In addition, quiescence protects HSCs from acquiring mutations, decreasing the risk of leukemic transformation (Orkin et al., 2000; Orkin et al., 2008; Ng et al., 2017).

Ki67 is a cell proliferation marker that is commonly used for examining cell proliferation. The size of quiescent (G0) stem cell pool can be determined by the absence of the proliferation marker Ki67 (Figure 3.4.A). Wild-type p53 maintains HSC quiescence as loss of p53 enhances HSC proliferation (Liu et al., 2009b). However, I observed no significant change on the quiescent state of $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$ HSCs as assessed by Ki67 and DAPI staining (Figure 3.4.B), suggesting that $p53^{R248W}$ mutant does not affect HSC quiescence in steady state.

A

B

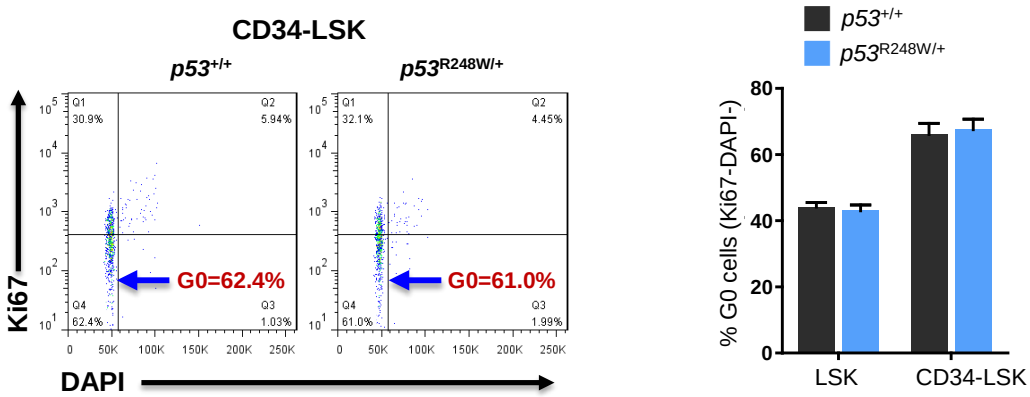


Figure 3.4 Mutant p53 does not affect quiescence of hematopoietic stem cells.

(A) Representative flow cytometry plots of Ki67 and DAPI staining of hematopoietic stem and progenitor cells were shown.

(B) The percentage of quiescent (G0) hematopoietic stem and progenitor cells was measured by Ki67 and DAPI staining. n=5 mice per genotype.

4. GOF mutant p53 enhances the repopulating potential of HSCs without affecting terminal differentiation

The serial replating assay assesses the preservation of “stemness” in progenitor compartment (Liu et al., 2009b). We performed CFU assays using 20,000 BM cells from young *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice and found that *p53*$^{R248W/+}$ bone marrow cells showed increased replating potential compared to *p53*$^{+/+}$ bone marrows. (Figure 3.5), implicating a positive role of p53^{R248W} in regulating HSC self-renewal *in vivo*.

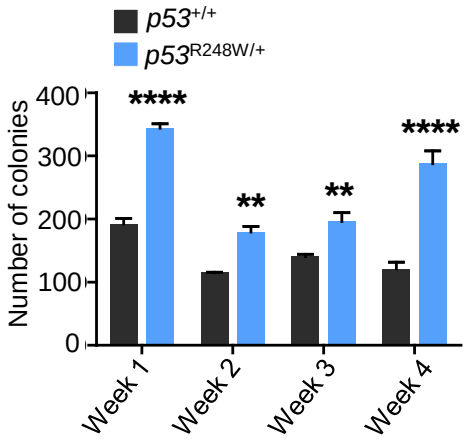
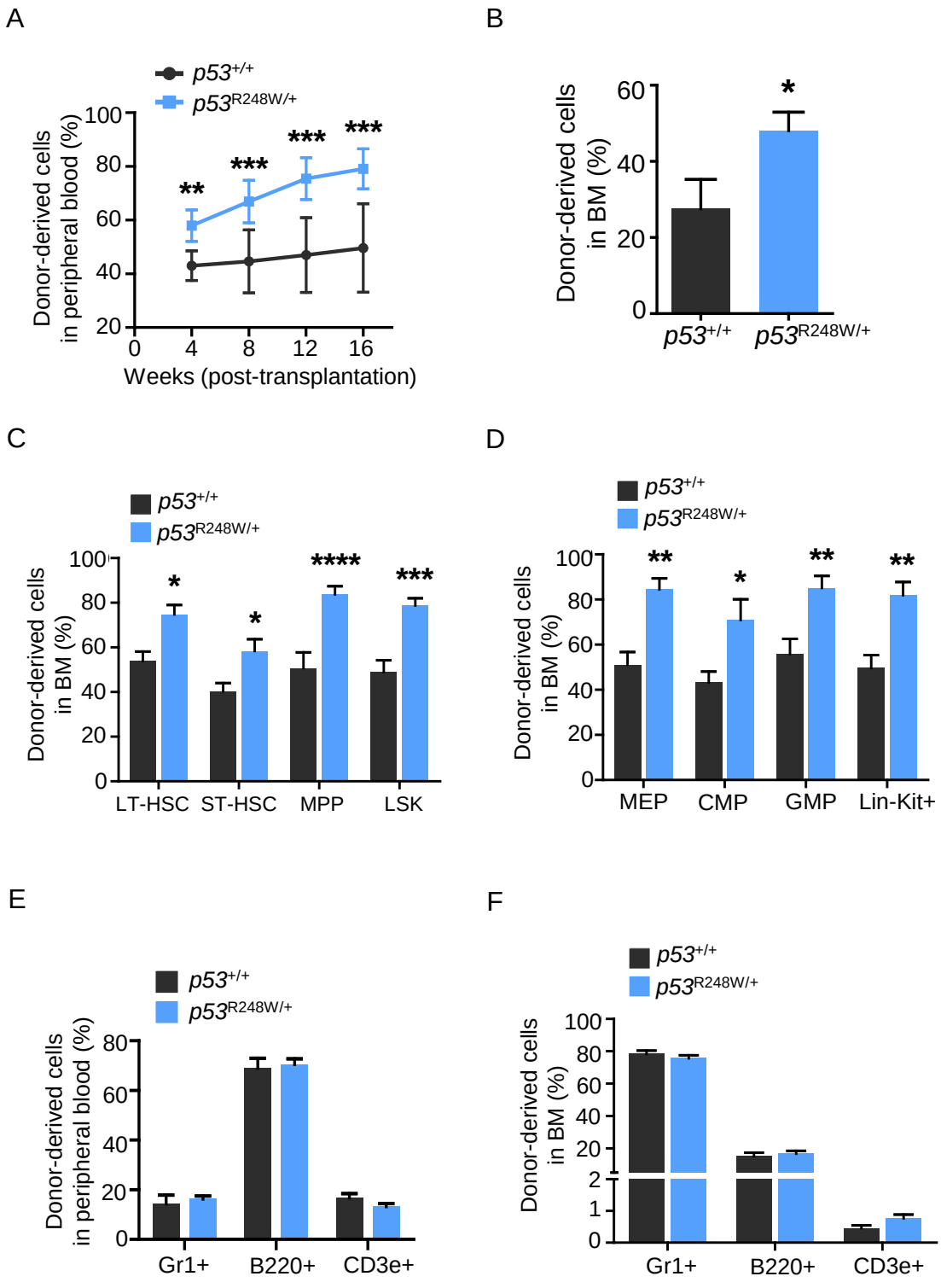


Figure 3.5 Mutant p53 enhances the replating potential of hematopoietic progenitor cells.

Serial replating studies. Myeloid progenitors were quantified by methylcellulose culture using BM cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. The methylcellulose cultures were serially replated, weekly, for 3 weeks. n = 3 per genotype, **p<0.01, ****p<0.0001.

To determine the impact of mutant p53 on HSC functions *in vivo*, I first performed serial competitive bone marrow transplantation assays, where 5 x 10^5 donor BM cells (*p53*$^{+/+}$ or *p53*$^{R248W/+}$, CD45.2$^+$) were transplanted into lethally irradiated (9.5 Gy) F1 recipient mice (CD45.1$^+$CD45.2$^+$) along with 5 x 10^5 competitor BM cells (CD45.1$^+$). 16 weeks after transplantation, the repopulating potential of *p53*$^{R248W/+}$ BM cells was significantly higher than that of the wild type cells, reflecting by increased percentage of donor-derived (CD45.2+) in the peripheral blood (PB) and BM of recipient mice (Figure 3.6.A and 3.6.B). Moreover, the percentage of CD45.2$^+$ HSCs and progenitors in the BM of recipient mice repopulated with *p53*$^{R248W/+}$ BM cells were significantly higher than those from *p53*$^{+/+}$ group (Figures 3.6.C and 3.6.D). However, p53^{R248W} did not affect myeloid and lymphoid differentiation in both PB and BM of primary recipients (Figure 3.6.E and 3.6.F). I then transplanted 3 x 10^6 BM cells isolated from the primary recipient mice repopulated with *p53*$^{+/+}$ or *p53*$^{R248W/+}$ cells into lethally irradiated secondary F1 recipients. *p53*$^{R248W/+}$ cells continued to show increased repopulating ability compared to *p53*$^{+/+}$ cells (Figure 3.6.G). No changes in myeloid and lymphoid differentiation were observed in peripheral blood of secondary transplantation recipients at 16-week post transplantation (Figure 3.6.H).

Thus, I demonstrated that *p53*$^{R248W/+}$ enhances repopulating potential of HSCs without affecting myeloid and lymphoid differentiation in serial competitive bone marrow transplantation experiments.


A
p53+/+
p53R248W/+
Donor-derived cells in peripheral blood (%)
100
80
60
40
20
0
4
8
12
16
Weeks (post-transplantation)
**
***
***
***
B
Donor-derived cells in BM (%)
60
40
20
0
*
p53+/+
p53R248W/+
C
p53+/+
p53R248W/+
Donor-derived cells in BM (%)
100
80
60
40
20
0
*
*
****
***
LT-HSC
ST-HSC
MPP
LSK
D
p53+/+
p53R248W/+
Donor-derived cells in BM (%)
100
80
60
40
20
0
**
*
**
**
MEP
CMP
GMP
Lin-Kit+
E
p53+/+
p53R248W/+
Donor-derived cells in peripheral blood (%)
80
60
40
20
0
Gr1+
B220+
CD3e+
F
p53+/+
p53R248W/+
Donor-derived cells in BM (%)
100
80
60
40
20
2
1
0
Gr1+
B220+
CD3e+

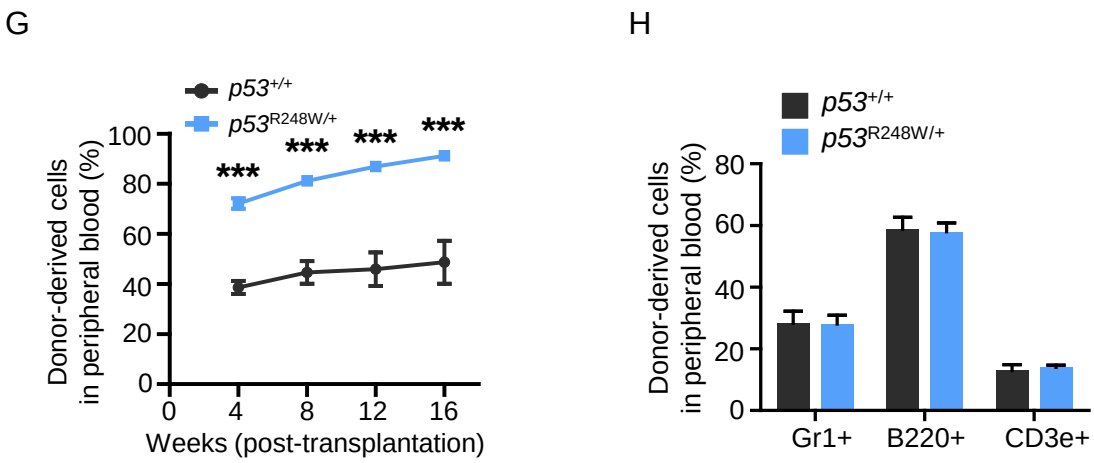


Figure 3.6 Mutant p53 enhances repopulating potential of hematopoietic stem cells in serial competitive bone marrow transplantations.

(A) Percentage of donor-derived ($CD45.2^+$) cells in the peripheral blood of primary recipient mice post-transplantation, measured at 4-week intervals. n=7 mice per group, **p<0.01, ***p<0.001.

(B) The percentage of donor-derived cells ($CD45.2^+$) in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group, *p<0.05.

(C) The frequency of donor-derived LT-HSCs, ST-HSCs, MPPs, and LSK cells in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group, *p<0.05, ***p<0.001, ***p<0.0001.

(D) The frequency of donor-derived MEPs, CMPs, GMPs, and Lin^-Kit^+ cells in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group, *p<0.05, **p<0.01.

(E) Percentage of donor-derived myeloid cells ($Gr1^+$), B cells ($B220^+$) and T cells ($CD3^+$) in the peripheral blood of primary recipient mice 16 weeks following transplantation. n=7 mice per group.

(F) The frequency of myeloid cells ($Gr1^+$), B cells ($B220^+$) and T cells ($CD3^+$) in donor-derived bone marrow of the primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group.

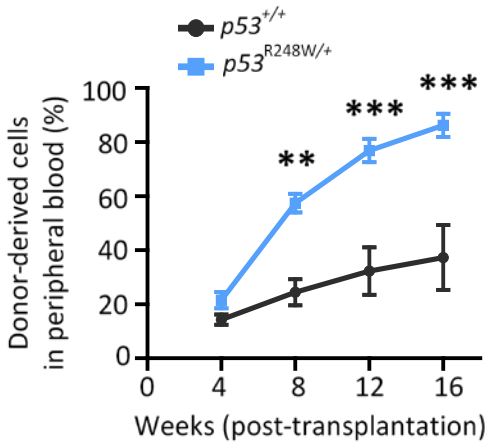
(G) Percentage of donor-derived cells in the peripheral blood of secondary recipient mice. n=7 mice per group, ***$p<0.001$

(H) The frequency of myeloid cells ($Gr1^+$), B cells ($B220^+$) and T cells ($CD3^+$) in donor-derived peripheral blood of the secondary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group.

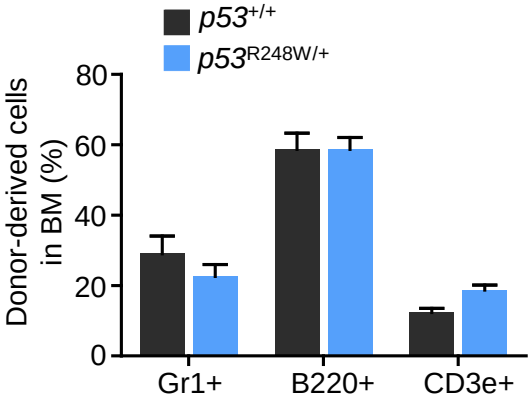
To further determine whether mutant p53 affects HSC self-renewal and differentiation, I transplanted 200 purified CD45.2^{+} *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs (CD48^{-}CD150^{+}LSKs) into 9.5Gy lethally irradiated F1 recipient mice along with 300,000 wild-type competitor CD45.1^{+} BM cells. I found that *p53*$^{R248W/+}$ HSCs exhibited a substantially higher reconstitution to peripheral blood production compared to *p53*$^{+/+}$ HSCs (Figure 3.7.A). Consistent with serial competitive BM transplantation assays, p53^{R248W} did not affect myeloid and lymphoid differentiation in bone marrows and peripheral blood of recipient mice 16-week following transplantation (Figure 3.7.B). Interestingly, the absolute numbers of CD45.2^{+} HSCs in the BM of recipient mice repopulated with *p53*$^{R248W/+}$ HSCs was significantly higher than that of the *p53*$^{+/+}$ HSCs (Figure 3.7.C), suggesting mutant p53 enhances the repopulating potential of HSCs in competitive transplantation assays.

To quantify the numbers of functional HSCs in the BM of *p53*$^{R248W/+}$ mice, I then performed competitive BM transplantation experiments with different dose of donor cells. Calculating the number of non-engrafted recipient mice revealed that the frequency of competitive repopulation units (CRU) in the BM of *p53*$^{R248W/+}$ mice is 3 to 4 fold higher than that of the *p53*$^{+/+}$ mice (Figures 3.8.A and 3.8.B), indicating that there are more functional HSCs in *p53*$^{R248W/+}$ mice compared to that of *p53*$^{+/+}$ mice.

A


p53+/+
p53R248W/+
Donor-derived cells in peripheral blood (%)
100
80
60
40
20
0
**
***
***
0
4
8
12
16
Weeks (post-transplantation)

B


p53+/+
p53R248W/+
Donor-derived cells in BM (%)
80
60
40
20
0
Gr1+
B220+
CD3e+

C

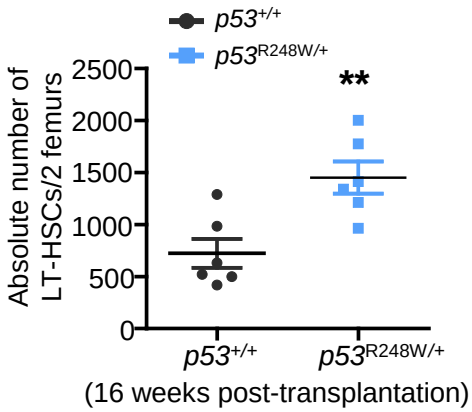

p53+/+
p53R248W/+
Absolute number of LT-HSCs/2 femurs
2500
2000
1500
1000
500
0
**
p53+/+
p53R248W/+
(16 weeks post-transplantation)

Figure 3.7 Mutant p53 enhances the repopulating potential of hematopoietic stem cells in competitive HSC transplantation.

(A) Percentage of donor-derived cells in the peripheral blood of recipient mice flowing HSC transplantation. n=7 mice per group, **p<0.01, ***p<0.001.

(B) Absolute number of donor-derived LT-HSCs in the BM of recipient mice 16 weeks following HSC transplantation. n=6 mice per group, **p<0.01.

(C) The frequency of myeloid cells ($Gr1^+$), B cells ($B220^+$) and T cells ($CD3^+$) in donor-derived bone marrow of the secondary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group.

A

Competitor (CD45.1+ MNCs)	**Donor** (CD45.2+ MNCs)	Non-respondent / Tested recipients	
		***p53*$^{+/+}$**	***p53*$^{R248W/+}$**
200K	10K	6/10	3/10
	20K	4/10	0/10
	40K	2/8	0/7
	80K	0/7	0/8
CRU frequency (±95% confidence interval)		1/22,469 (1/13,927-1/36,250)	1/6,554 (1/3,694-1/11,629)

B

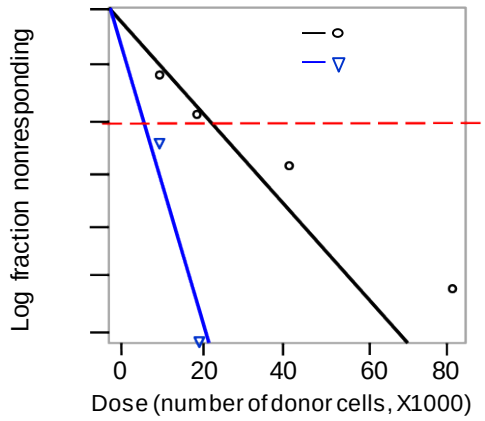


Figure 3.8 Limiting dilution assays show that mutant p53 increases the number of functional HSCs *in vivo*.

(A) Measuring HSC frequency in the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice utilizing limiting dilution assays. Recipients with less than 2% donor-derived cells in the peripheral blood were defined as non-respondent. n=7-10 mice per group, **p<0.01.

(B) Poisson statistical analysis of data from Figure 2H using L-Calc software. Shapes represent the percentage of negative mice for each dose of cells. Solid lines indicate the best-fit linear model for each data set.

Collectively, I demonstrated that heterozygote $p53^{R248W}$ mutant increases the competitive advantage of HSCs but does not affect terminal differentiation in *vivo*.

5. GOF mutant p53 does not affect HSC Homing

Increased repopulating potential of *p53*$^{R248W/+}$ HSCs in transplantation assays may due to enhanced homing capability. Therefore, I performed homing assays to evaluate the homing capability of *p53*$^{R248W/+}$ HSCs. Briefly, bone marrow cells ($CD45.2^+$) from either *p53*$^{+/+}$ or *p53*$^{R248W/+}$ mice were *ex vivo*-labeled with CFSE and injected into lethally irradiated recipient mice. 18hrs after transplantation, bone marrow cells of recipient mice were analyzed by flow cytometry. I found that there was no difference on the percentage of $CD45.2^+$ BM cells and $CD45.2^+$ HSCs ($Lin^-Sca\text{-}1^+CD48^-CD150^+$) in the recipient mice repopulated with *p53*$^{R248W/+}$ BM cells compared to those of *p53*$^{+/+}$ BM cells (Figures 3.9.A and 3.9.B). Thus, mutant p53 does not affect HSC homing.

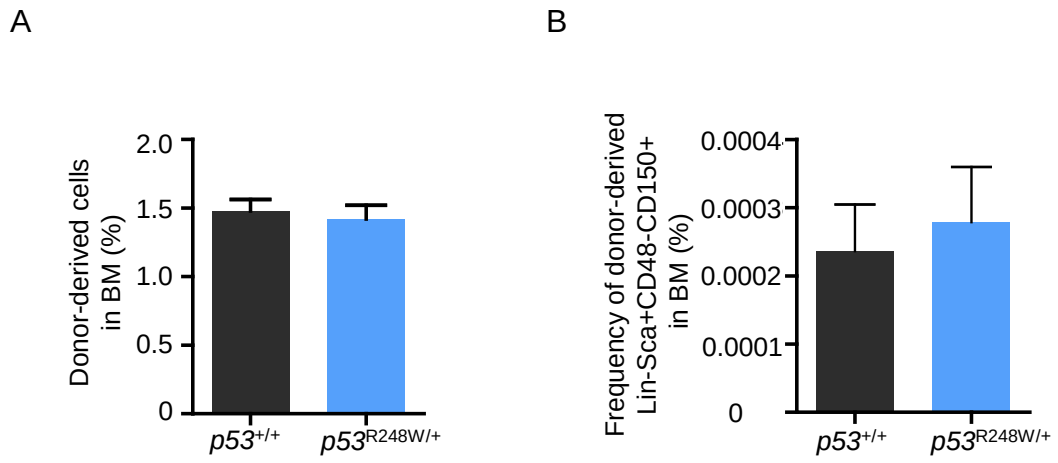


Figure 3.9 Mutant p53 does not affect HSC homing.

(A) 1 x 10^7 bone marrow cells ($CD45.2^+$) from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice were transplanted into lethally irradiated recipient mice ($CD45.1^+$). At 18 hours after transplantation, the percentage of donor-derived cells in the bone marrow of recipient mice was determined by flow cytometry. n=7 mice per group.

(B) The frequency of donor-derived LSKs in the bone marrow of recipient mice 18 hours after transplantation was determined by flow cytometry. n=7 mice per group.

Conclusion and Discussion (Chapter Three)

GOF *TP53* mutations have been found in the blood of aged healthy individuals as well as in patients with MDS, implying their important role in clonal hematopoiesis and pre-leukemic HSC development (Genovese et al., 2014). In this study, I analyzed HSC behavior in young adult $p53^{+/+}$ and $p53^{R248W/+}$ mice and discovered that heterozygote mutant p53 enhances the repopulating potential of HSCs without affecting terminal differentiation. Further, I found that GOF mutant p53 does not affect the quiescence, survival, and homing of HSCs.

Previously, we have reported that *p53* knockout (KO) mice have an increase number of proliferating HSCs (Liu et al., 2009b), demonstrating the importance of p53 in regulating p53 quiescence. Given that the majority of missense *TP53* mutations are located at the DNA-binding domain (DBD) of p53 protein, its sequence-specific DNA-binding activity may be altered (Brosh et al., 2009), which may acquire “gain-of-function” (GOF) properties in human cancer (Song et al., 2007; Brosh et al., 2009). Here, I speculated that GOF mutant p53 proteins have distinct biological impact on HSCs in comparison to the effect of p53 loss. Indeed, I found that $p53^{+/+}$ and $p53^{R248W/+}$ mice had similar numbers of LT-HSCs (Figure 3.2.C) and heterozygote mutant p53 does not affect HSC quiescence (Figure 3.4.B). These findings suggest that $p53^{R248W}$ mutant is not a loss-of-function mutant.

In an effort to examine the role of mutant p53 on HSC self-renewal, I performed *in vivo* transplantation in completive settings and discovered that $p53^{R248W}$ mutant enhanced repopulating potential of HSCs without affect terminal

differentiation. Furthermore, limiting dilution transplantation assays revealed that there are more functional HSC in *p53*$^{R248W/+}$ mice compared to *p53*$^{+/+}$ mice. In addition, I demonstrated that the enhanced repopulating potential of mutant HSCs in transplantation assay is not due to increased homing capability. Taken together, these results demonstrate that HSCs harboring *TP53* mutations outcompete wild type HSCs to drive clonal expansion.

Pre-leukemic HSCs have increased competitive advantage allowing clonal expansion (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Meanwhile, Pre-leukemic HSCs maintain normal hematopoietic differentiation (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Given that HSCs expressing mutant p53 recapitulate these two key features of pre-leukemic HSCs in steady state, I conclude that p53^{R248W} mutant drives the development of pre-leukemic HSCs.

CHAPTER FOUR

GOF mutant p53 protects HSCs from genotoxic stress and promotes their clonal expansion

Introduction

p53 plays a pivotal role in regulating cell cycle arrest, DNA damage repair, apoptosis, and senescence in response to genotoxic stress (Bálint et al., 2001; Brown et al., 2009). Loss of p53 decreases cell apoptosis induced by genotoxic stress (Mihara et al. 2003; Brown et al., 2009). Previously, we have demonstrated that p53 promotes DNA damage repair in HSCs under genotoxic stress (Liu et al., 2009b). We found that p53 and MEF double knock-out HSCs have more r-H2AX foci, a DNA damage indicator, than MEF-deficient HSCs following irradiation (Liu et al., 2009b). p53-null HSCs are resistant to chemotherapeutic agents treatment and show enhanced repopulation potential following chemotherapy compared to WT HSCs (Shounan et al., 1996; Park et al., 2003; Liu et al., 2009a). These studies suggest that p53 sensitizes HSCs to genotoxic stress.

Pre-leukemic HSCs are resistant to chemotherapy and frequently progress to leukemia stem cells (LSCs) under stress conditions (Corces-Zimmerman et al., 2014; Shlush et al., 2014), thereby serving as a reservoir for disease relapse. I found that mutant p53 enhances repopulation potential of HSCs and drives the development of pre-leukemic HSCs. However, whether HSCs expressing mutant p53 are resistance to irradiation and chemotherapy treatment is largely unknown. I hypothesized that **mutant p53 preserves HSC**

function in response to genotoxic stress and promotes clonal expansion of HSCs under stress conditions.

To test my hypothesis, I first challenged *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice with 9Gy total body irradiation (TBI) and monitored their survival. I then examined the survival of HSCs after exposing to low dose irradiation. r-H2Ax staining was performed to assess the sensitivity of mutant HSCs to DNA damages. To evaluate the effect of irradiation on HSC function, I performed serial competitive transplantation of BM cells isolated from irradiated *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. I *predicted* that HSCs expressing mutant p53 are not sensitive to irradiation and mutant p53 preserves HSC functions.

I next investigated whether HSCs expressing mutant p53 are resistant to chemotherapy. I employed weekly 5-FU treatment to *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice and recorded their survival. I also monitored hematopoietic recovery of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice after one-dose 5-FU injection by examining white blood cell counts. In addition, I assessed the apoptosis of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs following 5-FU treatment. Further, I calculated the absolute number of total bone marrow cells and HSCs from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice 7 days after a single-dose 5-FU administration. In order to assess the function of mutant HSCs after chemotherapy, I performed competitive transplantation assays using BM cells from 5-FU treated *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. I *predicted* that HSCs expressing mutant p53 are resistant to chemotherapy and maintain their repopulating potential *in vivo*.

Finally, I performed clonal expansion experiments by transplanting BM cells consisting CD45.1+ *p53*$^{+/+}$ and CD45.2+ *p53*$^{R248W/+}$ cells at a ratio of 10:1 into lethally irradiated recipient mice. Irradiation or chemotherapeutic drug ENU were applied to recipient mice 8-week post transplantations. The engraftment of *p53*$^{R248W/+}$ BM cells were examined 16-week after treatment. I ***predicted*** that mutant p53 promotes clonal expansion of hematopoietic cells in recipient mice after irradiation or chemotherapy. The ***objective*** of this study was to investigate the biological impact of genotoxic stress on mutant p53 HSC function.

Results

1. Mutant p53 HSCs are not sensitive to irradiation

p53 plays important role in response to irradiation (Bálint et al., 2001; Brown et al., 2009). While p53 null HSCs are resistant to irradiation (Liu et al., 2009), the response of HSCs expressing mutant p53 to irradiation is largely unknown. Thus, I investigated the impact of GOF mutant p53 on HSC function flowing irradiation. I challenged $p53^{+/+}$ and $p53^{R248W/+}$ mice with 9Gy total body irradiation (TBI) and found that all $p53^{+/+}$ mice died 5 weeks following 9Gy TBI, whereas 90% of $p53^{R248W/+}$ mice were still alive (Figure 4.1.A). I then examined the survival of mutant p53 HSCs following irradiation. I first irradiated $p53^{+/+}$ and $p53^{R248W/+}$ mice at dose of 2Gy and then isolated HSCs from the mice 2hrs after irradiation and assessed for apoptosis. As shown in Figure 4.2.B, $p53^{R248W/+}$ HSCs and HSPCs show decreased apoptosis compared to $p53^{+/+}$ cells. When I irradiated freshly isolated HSCs, I found that $p53^{R248W/+}$ HSCs showed decreased apoptosis 2hrs after irradiation (Figures 4.1.C). Using phosphorylation of histone H2AX (γH2AX) as an indicator of DNA damage, I found that $p53^{R248W/+}$ HSCs were largely devoid of γH2AX foci, whereas $p53^{+/+}$ HSCs stained positive for γH2AX following irradiation. (Figures 4.1.D and 4.1.E). These findings demonstrate that HSCs expressing mutant p53 are less sensitive to irradiation.

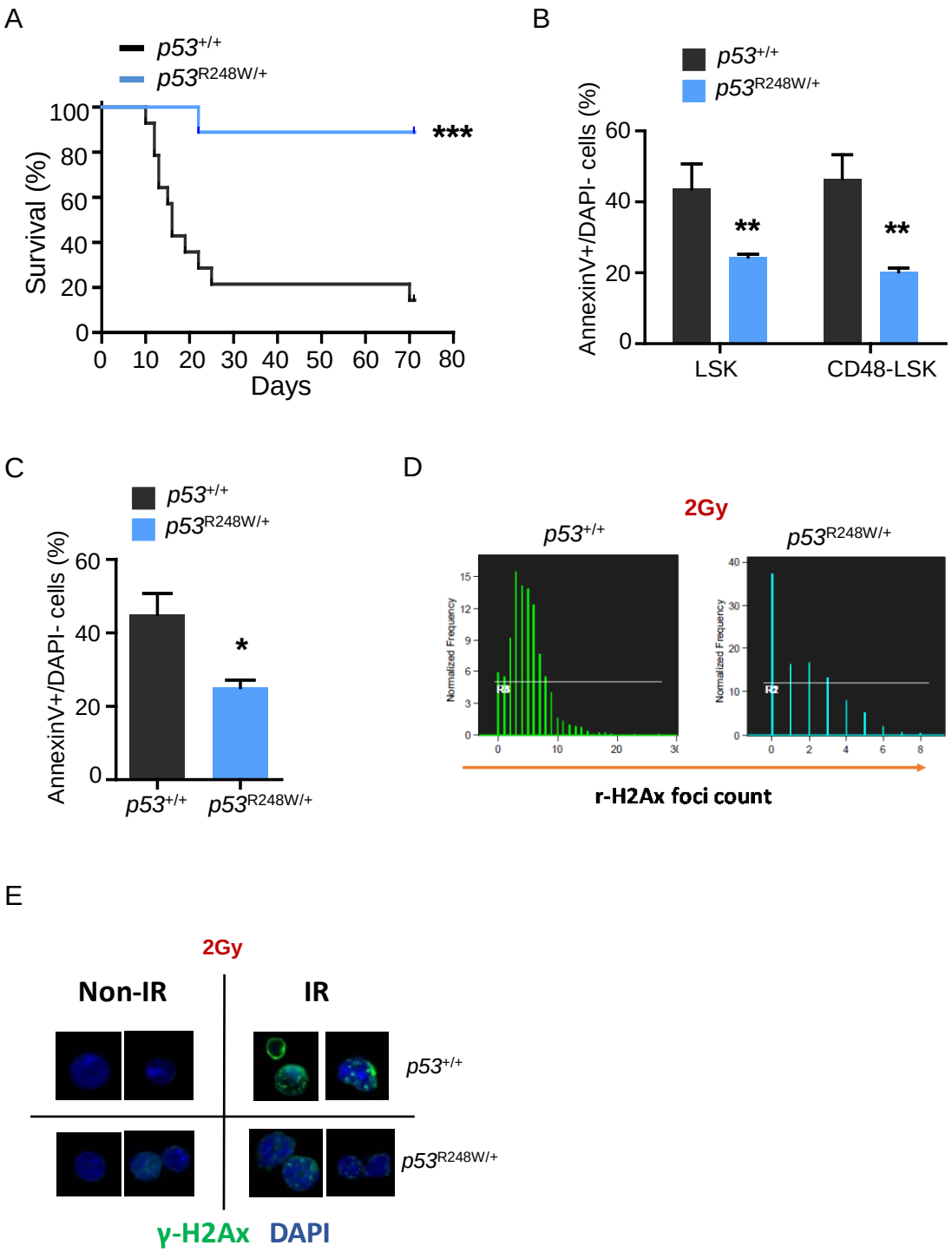

A
p53+/+
p53R248W/+
Survival (%)
Days
***
B
AnnexinV+/DAPI- cells (%)
LSK
CD48-LSK
**
C
AnnexinV+/DAPI- cells (%)
*
D
2Gy
Normalized Frequency
r-H2Ax foci count
E
2Gy
Non-IR
IR
γ-H2Ax
DAPI

Figure 4.1 HSCs expressing mutant p53 are less sensitive to irradiation.

(A) Kaplan-Meier survival curve of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice after 9 Gy total body irradiation (TBI). ***p<0.001, n = 10 mice per group.

(B) Hematopoietic stem and progenitor cells from the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice were assessed for apoptosis 2 hours after 2Gy irradiation. n = 3 mice per group, **p<0.01.

(C) HSCs from the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice treated with 2 Gy TBI were assessed for apoptosis. n = 3 mice per group, *p<0.05.

(D) Fluorescence intensity of γ-H2AX in LT-HSCs at 4 hours after 2 Gy irradiation was determined by ImageStream flow cytometry.

(E) γ-H2AX foci generation in HSCs following irradiation. LT-HSCs from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice were immunostained for γ-H2AX at 4 hours after 2 Gy irradiation. LT-HSCs were stained with DAPI to identify the nuclei.

2. Mutant p53 maintains HSC function following irradiation

To determine the impact of irradiation on *p53*$^{R248W/+}$ HSC function *in vivo*, I first treated *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice with 2 Gy TBI and then isolated BM cells from irradiated mice for competitive transplantation assays. Irradiated *p53*$^{R248W/+}$ BM cells displayed enhanced repopulating potential in both primary and secondary transplantation assays compared to irradiated *p53*$^{+/+}$ BM cells (Figures 4.2.A and 4.2.E). I observed increased frequency of hematopoietic stem and progenitor cells in the bone marrow of primary recipient mice repopulated with irradiated *p53*$^{R248W/+}$ BM cells (Figures 4.2.C and 4.2.D). While *p53*$^{R248W/+}$ had no effect on multi-lineage differentiation of HSCs and progenitors in peripheral blood of primary recipient mice (Figure 4.2.B), I found decreased myeloid differentiation and increased B cell differentiation in the secondary recipient mice repopulated with *p53*$^{R248W/+}$ BM cells compared to *p53*$^{+/+}$ BM cells (Figure 4.2.F).

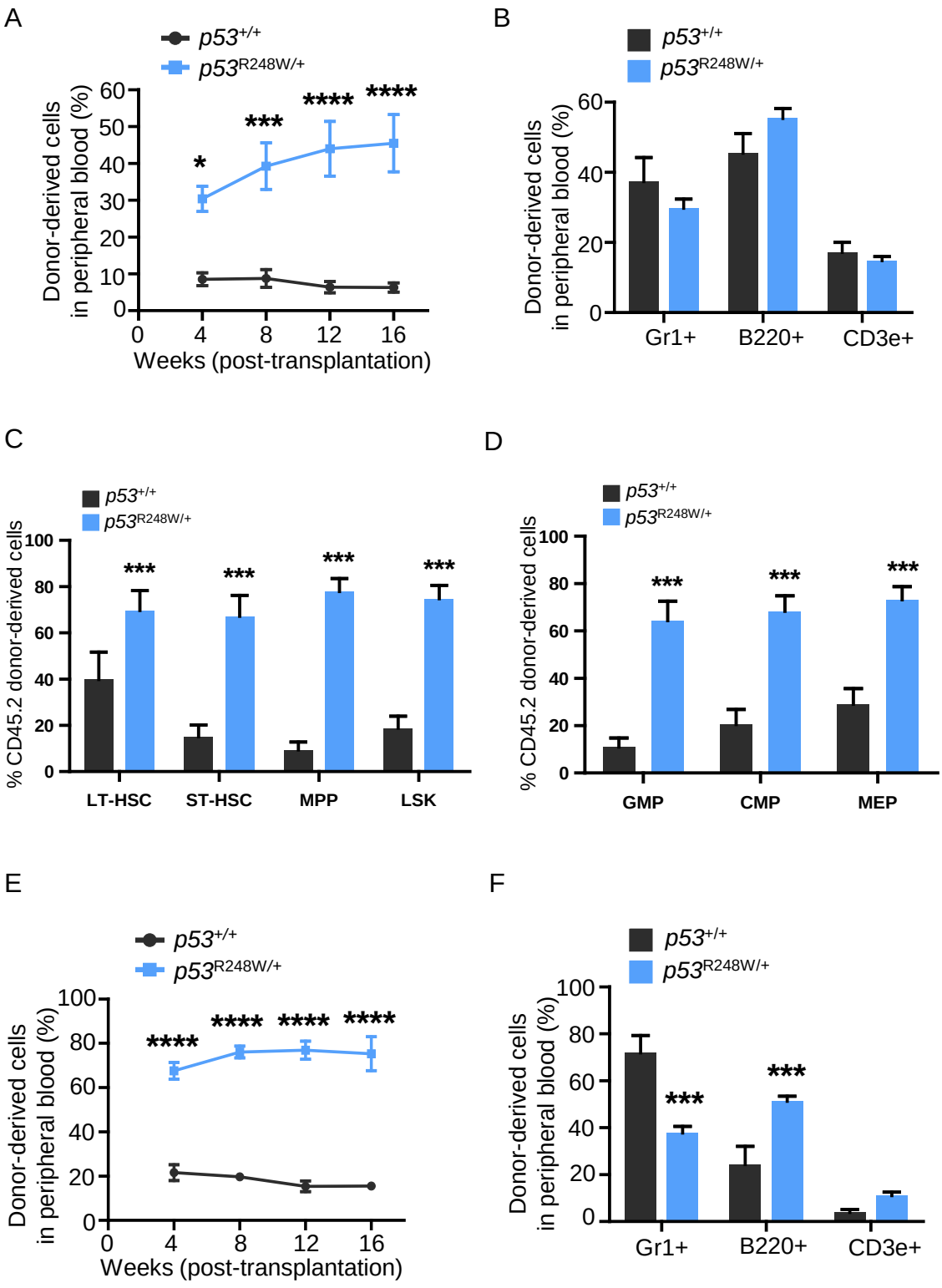

A
p53+/+
p53R248W/+
Donor-derived cells in peripheral blood (%)
Weeks (post-transplantation)
B
Gr1+
B220+
CD3e+
C
% CD45.2 donor-derived cells
LT-HSC
ST-HSC
MPP
LSK
D
GMP
CMP
MEP
E
F

Figure 4.2 Mutant p53 maintains the repopulating potential of irradiated hematopoietic stem cells in serial competitive bone marrow transplantation assays.

(A) The percentage of donor-derived cells in PB of primary recipient mice. n=7 mice per group. ****p<0.001 n = 7 mice per group, *p<0.05, ***p<0.001, ****p<0.0001.

(B) The percentage of donor-derived myeloid cells, B cells, and T cells in peripheral blood of the primary recipient mice 16 weeks following transplantation. n = 7 mice per group.

(C) The percentage of donor-derived LT-HSCs, ST-HSCs, MPPs, and LSK cells in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group, ***p<0.001.

(D) The percentage of donor-derived MEPs, CMPs, and GMPs in the bone marrow of primary recipient mice 16 weeks following transplantation was determined by flow cytometry analysis. n = 7 mice per group, ***p<0.001.

(E) The percentage of donor-derived cells in PB of secondary recipient mice. n=7 mice per group. ****p<0.001

(F) The percentage of donor-derived myeloid cells, B cells and T cells in peripheral blood of the secondary recipient mice 16 weeks after transplantation. n = 7 mice per group, ***p<0.001, ****p<0.0001.

3. Mutant p53 HSCs are not sensitive to chemotherapy

To investigate whether HSCs expressing mutant p53 are chemo-resistant, I challenged *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice with weekly 5-FU treatment and found that more than 80% of the *p53*$^{+/+}$ mice died within 7 weeks, whereas all of the *p53*$^{R248W/+}$ mice survived, suggesting that *p53*$^{R248W/+}$ mice were resistance to 5-FU treatment (Figure 4.3.A). I next monitored peripheral blood counts of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice after a single-dose of 5-FU treatment. *p53*$^{+/+}$ mice underwent myelosuppression following 5-FU treatment and recovered back to normal 28 days after treatment (Figure 4.3.B). However, *p53*$^{R248W/+}$ mice exhibited a rapid recovery with their white blood cell count went above normal level in 10 days after 5-FU injection (Figure 4.3.B). I hypothesized that the rapid recovery rate was due to decreased apoptosis of mutant hematopoietic cells. Indeed, Annexin V/DAPI staining of LT-HSCs after a single-dose of 5-FU treatment revealed that *p53*$^{R248W/+}$ HSCs were less apoptotic following 5-FU injection (Figure 4.3.A). I also observed increased bone marrow cellularity and increased number of LT-HSCs in *p53*$^{R248W/+}$ mice 7 days after 5-FU administration compared to *p53*$^{+/+}$ mice (Figure 4.3.D, 4.3.E and 4.3.F).

To examine the functional impact of chemotherapy on mutant p53 HSCs, I isolated bone marrow cells from 5-FU treated *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice and then performed competitive transplantation assay. I found that *p53*$^{R248W/+}$ BM cells showed enhanced reconstitution capability compared to *p53*$^{+/+}$ BM cells 16-week post transplantation, manifested by increased percentage of donor-derived

mutant cells in PB of recipient mice (Figure 4.3.G). However, mutant p53 did not affect terminal differentiation in the PB of recipient mice (Figure 4.3.H).

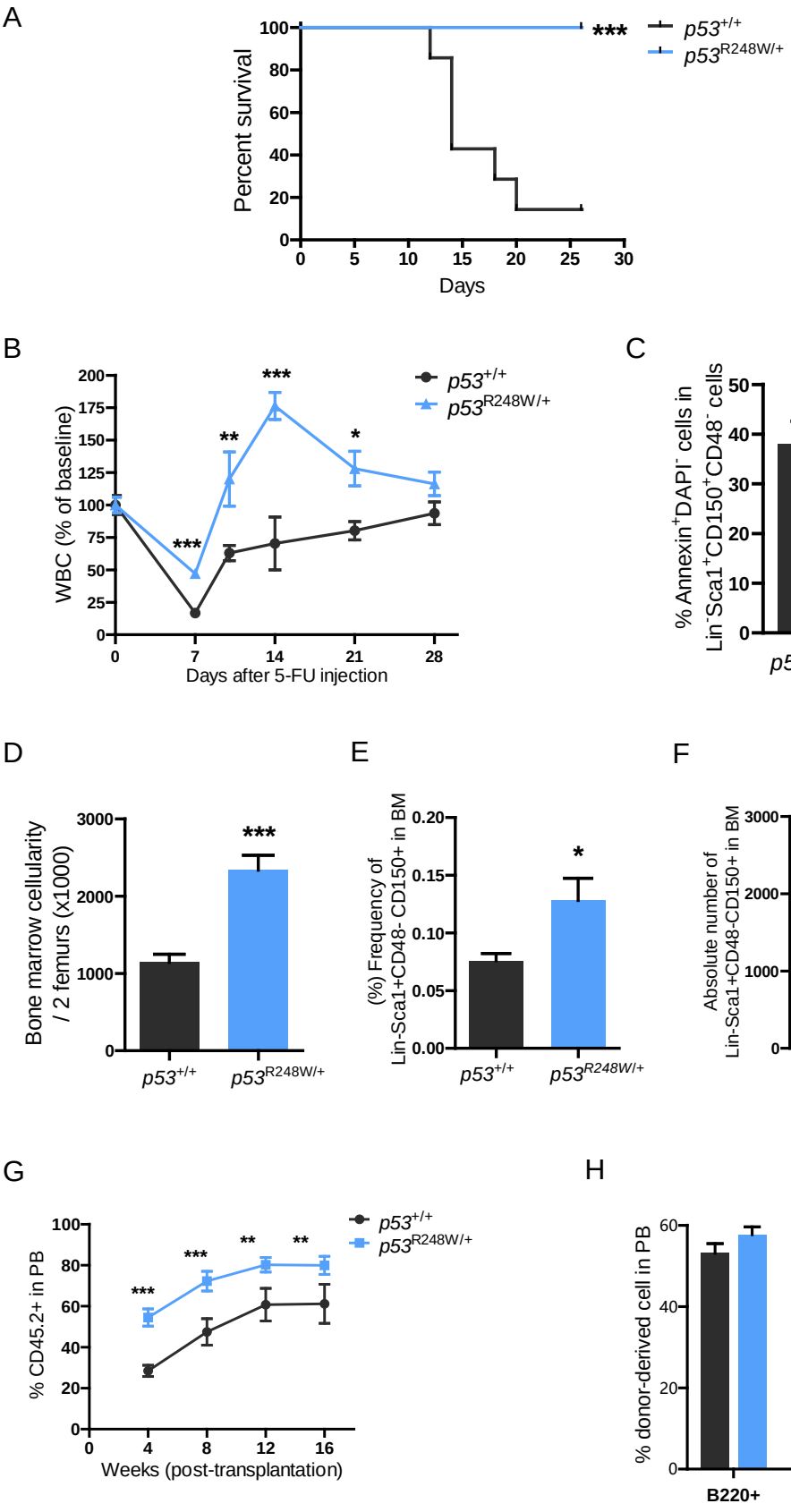

A
Percent survival
Days
***
p53+/+
p53R248W/+
B
WBC (% of baseline)
Days after 5-FU injection
C
% Annexin+DAPI- cells in Lin-Sca1+CD150+CD48- cells
D
Bone marrow cellularity / 2 femurs (x1000)
E
(%) Frequency of Lin-Sca1+CD48- CD150+ in BM
F
Absolute number of Lin-Sca1+CD48-CD150+ in BM
G
% CD45.2+ in PB
Weeks (post-transplantation)
H
% donor-derived cell in PB
B220+
CD3+
Gr1+

Figure 4.3 HSCs expressing mutant p53 are less sensitive to 5-FU administration.

(A) Kaplan-Meier survival curve of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice after weekly 5-FU administration. n = 10 mice per group. ***p<0.001.

(B) White blood cell counts of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice after single-dose 5-FU treatment. n = 7 mice per group, *p<0.05, **p<0.01, ***p<0.001.

(C) Percentage of early apoptotic cells in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs after single-dose 5-FU treatment. n = 3 mice per group, **p<0.01.

(D) Bone marrow cellularity of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice 7 days after single-dose 5-FU treatment. n = 5 mice per group, ***p<0.001.

(E) Frequency of HSCs in the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice 7 days after single-dose 5-FU treatment. n = 5 mice per group, *p<0.05.

(F) Absolute number of HSCs in the BM of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice 7 days after single-dose 5-FU treatment. n = 5 mice per group, **p<0.01.

(G)The frequency of donor-derived cells in PB of primary recipient mice. n=7 mice per group. n = 7 mice per group, **p<0.01, ***p<0.001.

(H) The frequency of donor-derived myeloid cells, B cells, and T cells in peripheral blood of the primary recipient mice 16 weeks following transplantation. n = 7 mice per group.

4. Mutant p53 promotes clonal expansion of hematopoietic cells following genotoxic stress

To determine whether functional GOF *TP53* mutation promotes HSC expansion following genotoxic stress, I generated mixed BM chimaeras containing both $p53^{+/+}$ and $p53^{R248W/+}$ cells with a 10:1 ratio of $p53^{+/+}$ cells (CD45.1^{+}) to $p53^{R248W/+}$ cells (CD45.2^{+}). Eight weeks following transplantation, recipient mice were treated with DMSO, chemotherapeutic drug ENU, or 5 Gy TBI (Figure 4.5.A). $p53^{R248W/+}$ HSCs outcompeted $p53^{+/+}$ cells, and underwent clonal expansion following total body irradiation or ENU treatment (Figure 4.5.B). In addition, total body irradiation treatment significantly increased frequency of $p53^{R248W/+}$ HSCs in the BM of recipient mice (Figure 4.5.C). Thus, mutant p53 protects HSCs from genotoxic stress and promotes their clonal expansion.

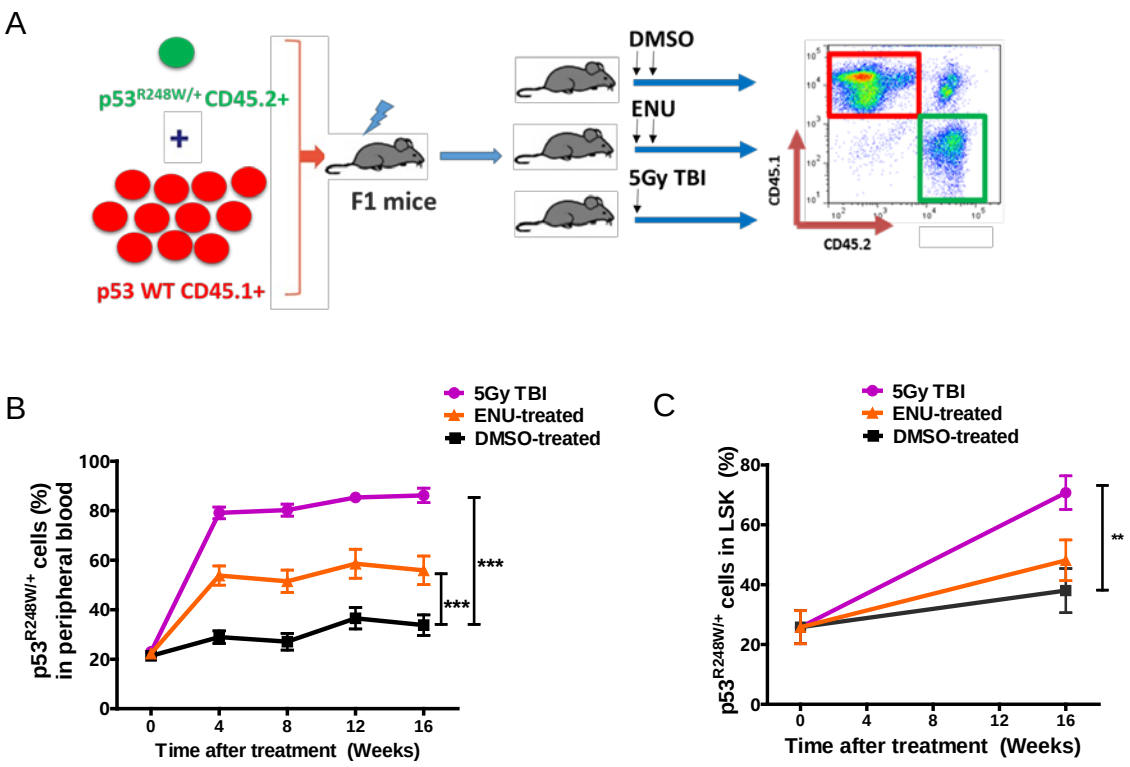


Figure 4.4 Mutant p53 promotes clonal evolution of HSCs under genotoxic stress *in vivo*.

(A) Bone marrow chimaeras were generated by transplanting a 10:1 ratio of *p53*$^{+/+}$ cells (CD45. 1^{+}) to *p53*$^{R248W/+}$ cells (CD45.2^{+}) into irradiated recipient mice (CD45.1^{+}CD45.2^{+}). After hematopoietic reconstitution (8 weeks), mice were treated with DMSO, ENU or 5Gy TBI.

(B) Frequency of *p53*$^{R248W/+}$ (CD45.2^{+}) cells in the peripheral blood of recipient mice following DMSO, ENU, or TBI treatment. n = 7 or 8 mice per group, ***p<0.001.

(C) Frequency of *p53*$^{R248W/+}$ LSK cells (CD45.2^{+}) in the peripheral blood of recipient mice following DMSO, ENU, or TBI treatment. n = 7 or 8 mice per group, **p<0.01.

Conclusion and Discussion (Chapter Four)

In this study, I demonstrated that mutant p53 protects HSCs from genotoxic stress and promotes their clonal expansion. These results recapitulate one of the key features of pre-leukemic HSCs, which is chemo-resistance (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Therefore, this study provides additional evidence to support my conclusion that mutant p53 drives the development of pre-leukemic HSCs.

Tumor suppressor p53 is the guardian of the genome (Bálint et al., 2001; Brown et al., 2009). In response to stress signals, such as oncogene activation, DNA damage or hypoxia, p53 is activated, triggering cell cycle arrest, DNA damage repair, senescence or apoptosis (Bálint et al., 2001; Mihara et al. 2003; Brown et al., 2009). We have reported that p53 promotes DNA damage repair in HSCs following irradiation and p53-null HSCs are resistant to genotoxic stress manifested by decreased apoptosis (Liu et al., 2009). These studies identified a critical role for p53 in HSCs under stress conditions. Here, I found that mutant HSCs were less apoptotic than WT HSCs in response to low dose irradiation. Moreover, there were less γH2AX foci formation in $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$ HSCs. These findings demonstrate that HSCs expressing mutant p53 are less sensitive to irradiation. Furthermore, serial competitive transplantation assays using BM cells isolated from irradiated $p53^{+/+}$ and $p53^{R248W/+}$ mice revealed that $p53^{R248W/+}$ bone marrows show enhanced repopulating potential compared to that of $p53^{+/+}$bone marrow cells, suggesting that mutant p53 preserves HSC function following irradiation.

p53 affects the proliferation and exhaustion of hematopoietic stem and progenitor cells in the bone marrow after 5-FU treatment (Shounan et al., 1996; Park et al., 2003; Liu et al., 2009a). Here, I observed that mutant p53 facilitated the regeneration of hematopoietic systems after myelosuppression caused by 5-FU treatment. This effect was due to decreased apoptosis of HSCs bearing p53 mutation. I also found that the pool of HSCs in *p53*$^{R248W/+}$ bone marrow was exhausted more slowly than that of the *p53*$^{+/+}$ bone marrow following 5-FU treatment. These results suggest that HSCs bearing p53 mutation are less sensitive to chemotherapy and mutant p53 promotes hematopoietic regeneration after chemotherapy treatment. Further, competitive transplantation of BM from 5-FU treated *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice showed that *p53*$^{R248W/+}$ bone marrow cells show enhanced engraftment in lethally irradiated recipients compared to *p53*$^{+/+}$bone marrow cells, suggesting that mutant p53 preserves HSC function in response to chemotherapeutic agents.

Finally, clonal expansion experiments under stress conditions indicate that mutant p53 promotes clonal expansion of HSCs in response to genotoxic stress. These results suggest that AML and MDS patients with *TP53* mutations should not be treated with conventional chemotherapy and radiotherapy. Collectively, my findings demonstrate that mutant p53 protects HSCs from genotoxic stress, thereby promoting the clonal expansion of pre-leukemic HSCs. These studies underscore the importance of developing novel therapeutic approaches to target chemotherapy and irradiation resistant pre-leukemic HSCs bearing *TP53* mutations.

CHAPTER FIVE

Aged heterozygous mutant p53 mice develop MDS

Introduction

While the roles of mutant p53 in oncogenesis of solid tumors have been well delineated, its functional impact on myeloid malignancies remains elusive. Myelodysplastic syndrome (MDS) is a heterogeneous group of disease characterized by dysplastic hematopoietic cells, peripheral blood cytopenia and high risk of progression to AML (Nimer, et al. 2008; Sperling, et al. 2017; Woll, et al. 2014). Although progress has been made on MDS treatment, the clinical outcome of MDS patients is still very poor (Sperling, et al. 2017). *TP53* mutations present in 10% of primary MDS, and characterized by severe thrombocytopenia, elevated blast count and complex karyotypes (Rücker, et al. 2012; Sperling, et al. 2017). Moreover, *TP53* mutations are associated with drug resistance and shortest survival (Chang, et al. 2017; Volkert, et al. 2014; Welch, et al 2016). Allogenic bone marrow transplantation does not improve the outcome of MDS patients with *TP53* mutations due to increased relapse post-transplantation (Christopeit, et al. 2016; Lindsley, et al. 2017). Notably, the frequency of *TP53* mutations increases to a large extent after patients are exposed to irradiation or alkylating agents (Metzgeroth, et al. 2016). Despite the clinical importance of *TP53* mutations in MDS, little is known regarding how these mutations contribute to the development of MDS. Therefore, it is of great importance to advance our understanding of the role of *TP53* mutations in the pathogenesis of MDS.

MDS is not easy to study in the laboratory due to the difficulty of engrafting human MDS in immunodeficient mice (Rhyasen, et al. 2014). Meanwhile, lack of proper MDS murine models increases the challenge of studying the disease development (Zhou, et al, 2015). It has been evaluated that most *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice develop spontaneous tumors, including lymphoma, thymoma, and sarcoma, and die within 3 to 6 months after birth (Song et al., 2007); whereas disease spectrums of *p53*$^{R248W/+}$ mice is not clear. Actually, *TP53* deletions are mainly found in advanced phases of hematological malignancies (Harutyunyan, et al. 2011, Rücker, et al. 2012). Importantly, most *TP53* mutations identified in AML or MDS are mono-allele missense mutations having gain-of-function properties that promote tumorigenesis (Peller, et al. 2003). Therefore, *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice are not good models for studying the pathogenesis of MDS. Thus, I ***speculated*** that *p53*$^{R248W/+}$ mice may develop different disease spectrums compared to *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice. I hypothesized that ***p53*$^{R248W/+}$ mice develop features recapitulating human MDS during aging**.

To investigate the role of mutant p53 in the pathogenesis of MDS, I first maintained the secondary recipient mice repopulated with *p53*$^{+/+}$ and *p53*$^{R248W/+}$ BM cells for 48 weeks and examined their multi-lineage differentiation ability in the PB of recipient mice. Given that mutant p53 causes genome instability, I expected to observe impaired lineage differentiation over time in recipient mice repopulated with *p53*$^{R248W/+}$ BM cells. Next, I monitored the survival and disease development of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice during ageing. Peripheral blood,

tumor or organs relevant to hematopoiesis, such as bone marrow, spleen, thymus and liver, were isolated for pathological analysis. I ***predicted*** that *p53*$^{R248W/+}$ mice develop MDS-like disease that mimics human conditions. The ***objective*** of the study is to determine the role of mutant p53 in the development of MDS.

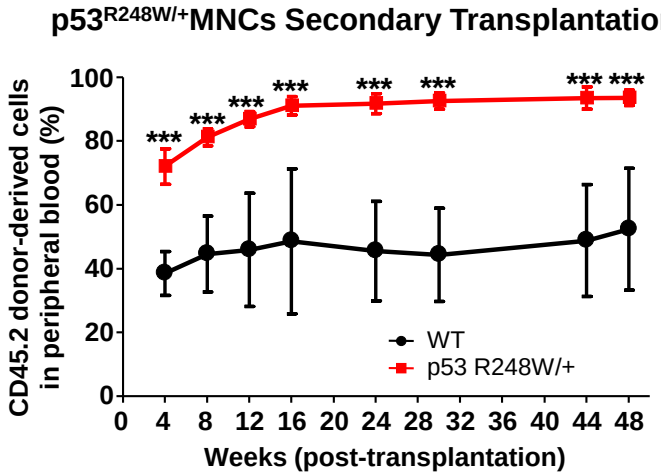
Results

1. Mutant p53 alters terminal differentiation during aging

In Chapter Three, I found that mutant p53 does not affect multi-lineage differentiation in the PB of both primary and secondary recipient mice after competitive BM transplantation, demonstrating that HSCs expressing mutant p53 retain normal differentiation capabilities. However, the impact of mutant p53 on HSC differentiation during aging is largely unknown.

To determine the impact of mutant p53 on terminal differentiation during aging, I maintained the recipient mice from the secondary competitive bone marrow transplantation experiments (describe in Chapter Three) for 48-weeks. *p53*$^{R248W/+}$ BM cells still retained enhanced engraftmentability in the peripheral blood compared to *p53*$^{+/+}$ BM cells. Strikingly, I observed an decrease of myeloid cells (Gr1^{+}) and increase of B lymphoid cells (B220^{+}) in the PB of recipient mice repopulated with *p53*$^{R248W/+}$ BM cells compared to those of *p53*$^{+/+}$ BM cells, suggesting that mutant p53 impaired myeloid differentiation over time. These results indicate that GOF mutant p53 may predispose mice to MDS development during aging.

A



B

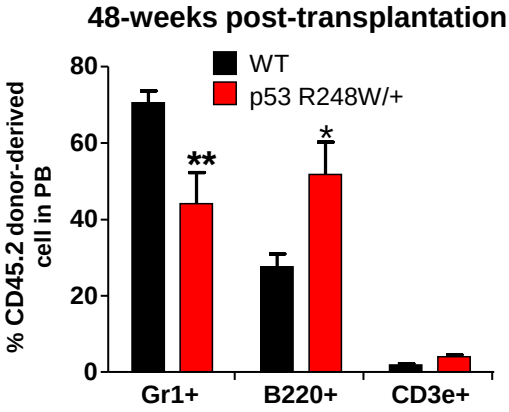


Figure 5.1 Mutant p53 impairs multi-lineage differentiation during ageing.

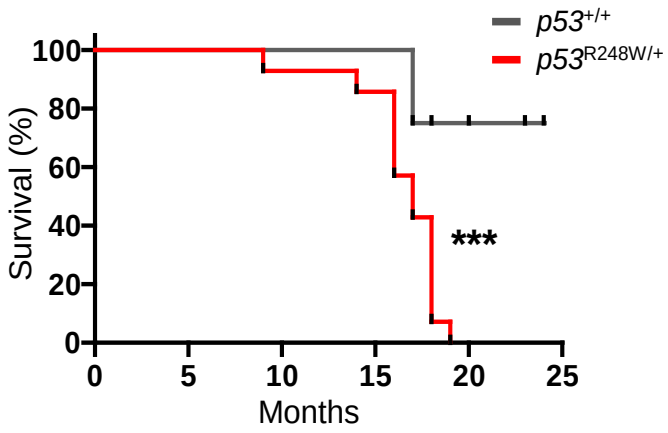
(A) Percentage of donor-derived cells in PB of secondary recipient mice repopulated the *p53*$^{+/+}$ and *p53*$^{R248W/+}$ BM. n=7 mice per group, ***p<0.001.

(B) The frequency of donor-derived myeloid cells, B cells, and T cells in peripheral blood of the secondary recipient mice 16 weeks following transplantation. n = 7 mice per group. *p<0.05, **p<0.01.

2. Characterize aged heterozygous mutant p53 mice

To evaluate the role of GOF mutant p53 in the pathogenesis of myeloid malignancies, I maintained *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice till their death to monitor their survival rate and tumor development. I observed that *p53*$^{R248W/+}$ mice have a median age of 18-month-old (Figure 5.2.A), which is much longer than *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice. I measured peripheral blood cell counts and performed peripheral blood smear before their death. I also harvested, organs, including bone, spleen, liver, kidney, lung and tumors for H&E staining. Pathology analysis revealed that approximately 30 % of *p53*$^{R248W/+}$ mice developed MDS (Figure 5.2.B). Other aged *p53*$^{R248W/+}$ mice developed MDS/lymphoma, MDS/sarcoma, lymphoma, and sarcoma (Figure 5.2.B); whereas most of the aged-matched *p53*$^{+/+}$ mice were normal. Thus, *p53*$^{R248W/+}$ mice developed different diseases compared to *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice.

A



B

Disease Type	Number of Mice	Frequency
MDS	5	33.3%
MDS/Lymphoma	3	20%
MDS/Carcinoma	1	6.7%
MDS/Fibrosarcoma	1	6.7%
Lymphoma	3	20%
Osterosarcoma	2	13.3%

Figure 5.2 Survival rate and disease spectrum of aged *p53*$^{R248W/+}$ mice.

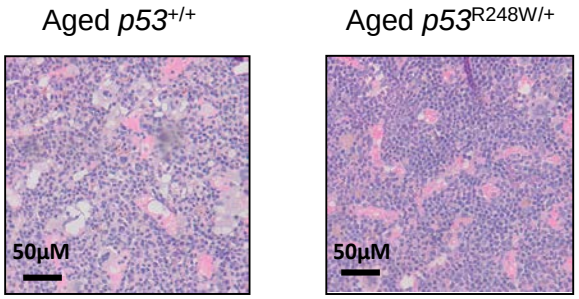
(A) Kaplan-Meier survival curve of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. n=12-15 mice per group, ***p<0.001.

(B) *p53*$^{R248W/+}$ mice developed various diseases during aging. Disease types and frequencies were shown.

3. Some aged heterozygous mutant p53 mice developed MDS

Histological assessment revealed hypercellular BM in some aged *p53*$^{R248W/+}$ mice (Figure 5.3.A). *p53*$^{R248W/+}$ mice also show splenomegaly with enlarged spleen and increased spleen weight (Figures 5.3.B and 5.3.C). Indeed, hypercellular spleens were found in *p53*$^{R248W/+}$ mice compared to *p53*$^{+/+}$ mice (Figure 5.3.D). Moreover, the spleens of aged *p53*$^{R248W/+}$ mice have increased numbers of hematopoietic stem and progenitor cells, indicating extramedullary hematopoiesis (EMH) in the spleen of mutant mice (Figure 5.3.E).

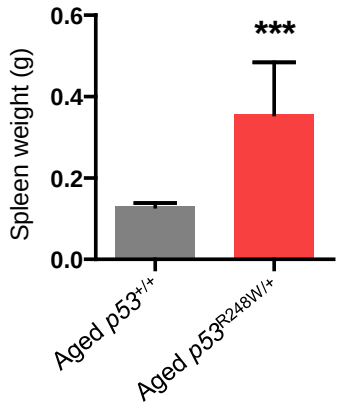
A


Aged p53+/+
Aged p53R248W/+
50μM
50μM

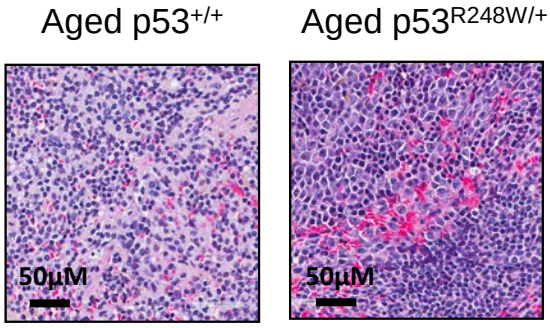
B


Aged p53+/+
Aged p53R248W/+

C


Spleen weight (g)
0.6
0.4
0.2
0.0
***
Aged p53+/+
Aged p53R248W/+

D


Aged p53+/+
Aged p53R248W/+
50μM
50μM

E

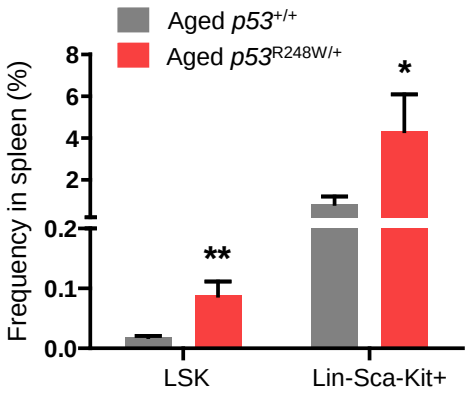

Aged p53+/+
Aged p53R248W/+
Frequency in spleen (%)
8
6
4
2
0.2
0.1
0.0
**
*
LSK
Lin-Sca-Kit+

Figure 5.3 Aged *p53*$^{R248W/+}$ mice show hypercellular bone marrow and extramedullary hematopoiesis in spleen.

(A) H&E staining of bone marrow sections from aged *p53*$^{+/+}$ mice and aged *p53*$^{R248W/+}$ mice.

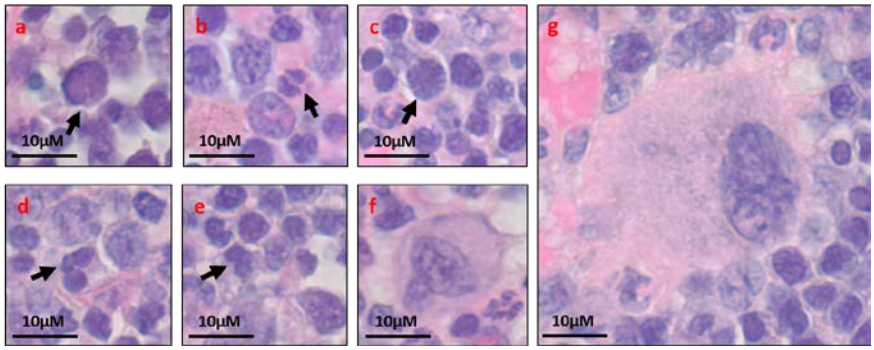
(B) Representative images of spleens from aged *p53*$^{+/+}$ mice and aged *p53*$^{R248W/+}$ mice.

(C) Spleen weight of aged *p53*$^{+/+}$ mice and aged *p53*$^{R248W/+}$ mice. n = 6 mice per group, ***p<0.001.

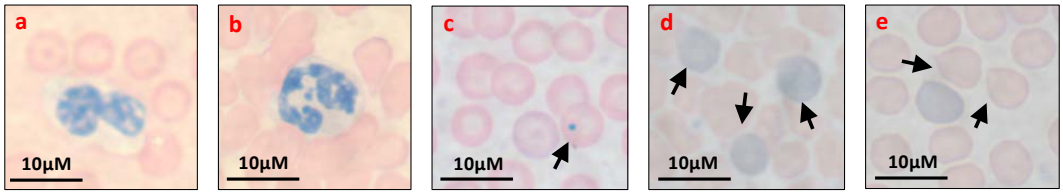
(D) H&E staining of spleen sections from aged *p53*$^{+/+}$ mice and aged *p53*$^{R248W/+}$ mice.

Furthermore, BM of some aged *p53*$^{R248W/+}$ mice show neutrophil, erythroid precursor and megakarocyte dysplasia (Figure 5.4.A). *p53*$^{R248W/+}$ peripheral blood smears revealed hypersegmented neutrophils, Pseudo-Pelger-Huet, nucleated red cells, and tear-drop red cells (Figure 5.4.B). Significant leukopenia and thrombocytopenia were seen in aged *p53*$^{R248W/+}$ mice with MDS compared to age-matched *p53*$^{+/+}$ mice (Figure 5.4.C and 5.4.D). In addition, mild but significant anemia was detected in aged *p53*$^{R248W/+}$ mice with MDS, manifested by low red blood cell counts and decreased levels of hemoglobin (Figure 5.4.E and 5.4.F). However, the mean corpuscular volume (MCV) of red blood cells was normal (Figure 5.4.G).

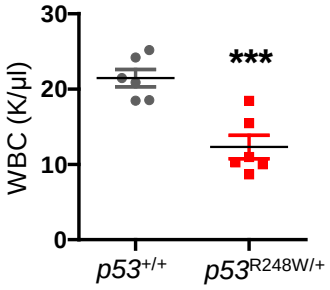
A


a
b
c
g
d
e
f
10μM

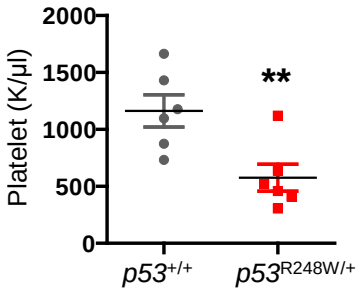
B


a
b
c
d
e
10μM

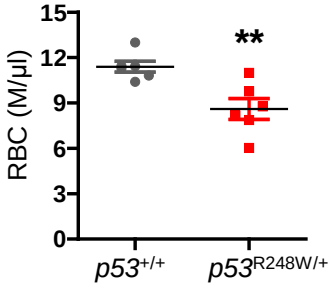
C


WBC (K/μl)
30
20
10
0
***
p53+/+
p53R248W/+

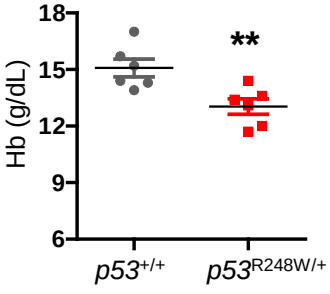
D


Platelet (K/μl)
2000
1500
1000
500
0
**
p53+/+
p53R248W/+

E


RBC (M/μl)
15
12
9
6
3
0
**
p53+/+
p53R248W/+

F


Hb (g/dL)
18
15
12
9
6
**
p53+/+
p53R248W/+

G

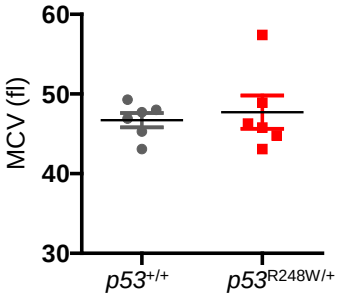

MCV (fl)
60
50
40
30
p53+/+
p53R248W/+

Figure 5.4 Aged heterozygous mutant p53 mice developed MDS.

(A) H&E-stained BM sections from *p53*$^{R248W/+}$ mice developed MDS. Representative images show binucleated myeloid precursor (a, b), karyorrhexis in erythroid precursors (c), erythroid precursors with nuclear budding (d), binucleated erythroid precursor (e), a giant megakaryocyte (g) in comparison to a megakaryocyte from age-matched *p53*$^{+/+}$ mice (f).

(B) Giemsa-stained peripheral blood smears from *p53*$^{R248W/+}$ mice developed MDS. Representative images show Pseudo-Pelger-Huet (a), hypersegmented neutrophil (b), Howell-Jolly body in red blood cells (RBCs) (c), polychromatophilic RBCs (d, e), tear-drop RBCs (e).

(C) White blood cell (WBC) counts in the peripheral blood of *p53*$^{R248W/+}$ mice developed MDS and age-matched *p53*$^{+/+}$ mice. n=6 mice per group, ***p<0.001.

(D) Platelet counts in the peripheral blood of *p53*$^{R248W/+}$ mice developed MDS and age-matched *p53*$^{+/+}$ mice. n=6 mice per group, **p<0.01.

(E) Red blood cell (RBC) counts in the peripheral blood cells of *p53*$^{R248W/+}$ mice developed MDS and age-matched *p53*$^{+/+}$ mice. n=6 mice per group, **p<0.01.

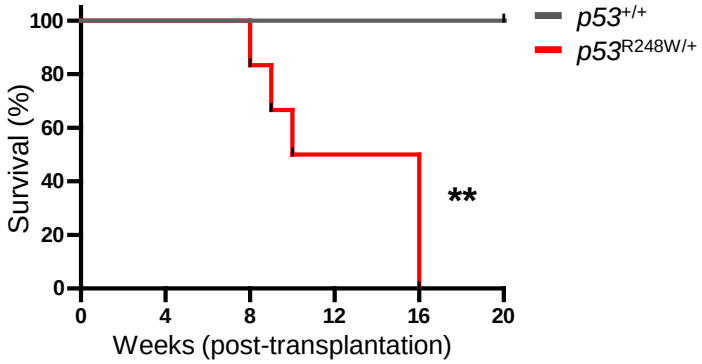
(F) Hemoglobin (Hb) levels in the peripheral blood cells of *p53*$^{R248W/+}$ mice developed MDS and age-matched *p53*$^{+/+}$ mice. n=6 mice per group, **p<0.01.

(G) MCV of red blood cells of aged *p53*$^{+/+}$ mice and *p53*$^{R248W/+}$ mice developed MDS. n=6 mice per group, p>0.05, n.s.

4. Mutant p53-driven MDS is transplantable

MDS is a clonal disease originated from mutated MDS stem cells (Nimer, et al. 2008; Sperling, et al. 2017). Thus, recipient mice repopulated with bone marrow cells from MDS mice might develop MDS over time. To determine whether mutant p53-driven MDS is transplantable, I injected whole BM cells from aged wild-type mice and *p53*$^{R248W/+}$ mice with MDS into lethally–irradiated recipient mice. I found that recipient mice repopulated with MDS BM cells died within 24 weeks, whereas no mice transplanted with aged *p53*$^{+/+}$ BM cells died during observation period (Figure 5.5.A). Furthermore, recipient mice repopulated with MDS cells show decreased WBC and RBC counts compared to that of the aged *p53*$^{+/+}$ cells (Figure 5.5.B and 5.5.C). Importantly, pathology analysis of the dead mice revealed MDS and bone marrow failure (Figure 5.5.D), suggesting that the MDS phenotype-induced by GOF mutant p53 was transplantable. Therefore, I demonstrated that some aged *p53*$^{R248W/+}$ mice developed MDS, recapitulating several key features of human MDS.

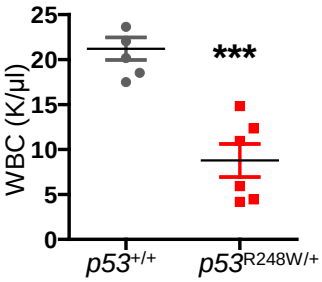
A



B

Disease Type	Number of Mice
MDS	3
Bone marrow failure	1
Not determined due to death	2

C



D

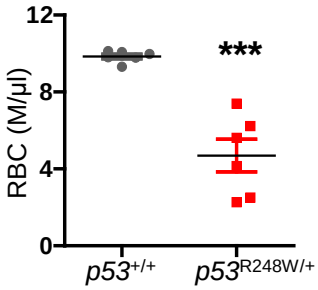


Figure 5.5 Mutant p53 driven MDS is transplantable.

(A) Kaplan-Meier survival curve of recipient mice repopulated with BM cells from aged *p53*$^{+/+}$ mice or *p53*$^{R248W/+}$ mice with MDS. n=6 mice per group, **p<0.01.

(B) Recipient mice developed MDS and bone marrow failure following transplantation of BM cells isolated from *p53*$^{R248W/+}$ mice with MDS. n = 6 mice.

(C) WBC counts in the peripheral blood of recipient mice following BM transplantation. n=5 mice per group, ***p<0.001.

(D) RBC counts in the peripheral blood of recipient mice following BM transplantation. n=5 mice per group, ***p<0.001.

Conclusion and Discussion (Chapter Five)

TP53 mutations are present in 10% of primary MDS patients, who have severe thrombocytopenia, elevated blast count and complex karyotypes (Rücker, et al. 2012; Sperling, et al. 2017). Genetic studies identified *TP53* mutations as one of the common recurrent lesions in clonal hematopoiesis (Genovese, et al. 2014; Jaiswal, et al. 2014), implicating a role for *TP53* mutations in early steps of MDS development. However, how these mutations contribute to MDS and whether they are sufficient to drive MDS development is poorly understood. In this study, I observed that aged *p53*$^{R248W/+}$ mice developed features recapitulating human MDS, demonstrating that GOF mutant p53 drives MDS development.

I have demonstrated that *p53*^{R248W} is a pre-leukemic mutation driving the development of pre-leukemic HSCs without affecting normal differentiation (date shown in Chapter Three and Chapter Four). Given that p53 is the guardian of the genome (Brown, et al. 2009; Liu, et al. 2009a), mutations on p53 may lead to genome instability, resulting in ineffective hematopoiesis over time. I then monitored secondary recipient mice repopulated with *p53*$^{+/+}$ and *p53*$^{R248W/+}$ BM cells for 48 weeks and examined their multi-lineage differentiation in the PB of recipient mice. Interestingly, I observed that p53^{R248W} mutant altered terminal differentiation over time, manifested by decreased myeloid differentiation and increased lymphoid differentiation compared to WT group. Thus, I demonstrated that GOF mutant p53 inhibits myeloid differentiation during ageing.

Next, I monitored the survival and disease development of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. *p53*$^{R248W/+}$ mice had a median survival rate of 16 months, which

is much longer than what has been reported for *p53*$^{-/-}$ and *p53*$^{R248W/R248W}$ mice (3-6 months old). Strikingly, 30% of *p53*$^{R248W/+}$ mice developed features of human MDS, including cytopenia, thrombocytopenia, anemia and multi-lineage dysplasia in bone marrow cells and peripheral blood (Harutyunyan, et al. 2011, Rücker, et al. 2012). Furthermore, transplantation of bone marrow cells from MDS mice resulted in lethal MDS or bone marrow failure in lethally irradiated recipient mice, suggesting that mutant p53 induced MDS is transplantable. Collectively, these results demonstrated that GOF mutant p53 drives the pathogenesis of MDS in mice.

In addition, I observed that some *p53*$^{R248W/+}$ mice display lymphoma, sarcoma, and mixed disease with lymphoma and MDS. These phenomenon were consistent with what have been reported in human cases with heterozygote *TP53* mutations. Current studies suggest that pre-leukemic state may exist in both myeloid and lymphoid malignancies, and diverse diseases may carry the same recurrent mutation (Corces-Zimmerman, et al. 2014). My findings that *p53*$^{R248W/+}$ mice developed different hematological malignancies highlighted the clinical importance of eliminating pre-leukemic HSCs harboring *TP53* mutations.

TP53 mutations are associated with drug resistance and dismal prognosis in MDS (Chang, et al. 2017; Volkert, et al. 2014; Welch, et al 2016). Allogenic bone marrow transplantation does not improve the outcome of MDS patients with *TP53* mutations due to increased relapse post-transplantation (Christopeit, et al. 2016; Lindsley, et al. 2017). Our humanized *p53*$^{R248W/+}$ mice developing MDS will

be a valuable tool to test therapeutic agents for the prevention and treatment of MDS patients with *TP53* mutations.

CHAPTER SIX

GOF mutant p53 drives pre-leukemic stem cell development through regulating gene expression

Introduction

p53 regulates gene transcription through its DNA-binding domain (DBD) (Patricia et al., 2013). Majority of mono-allele missense mutations on the DNA-binding domain of p53 alter its sequence-specific binding activity, leading to distinct gene expression patterns compared to WT p53 (Brosh et al., 2009). As a result, these changes are key mediators of the biological impacts of mutant p53 on tumorigenesis.

We have previously identified *Gfi-1* and *Necdin* as p53 target genes in HSCs (Liu et al., 2009b). p53 transcriptionally activates both *Gfi-1* and *Necdin* genes to maintain HSC quiescence (Liu et al., 2009b). Downregulation of *Gfi-1* and *Necdin* promote HSC proliferation as seen in p53-null mice (Liu et al., 2009b). In Chapter Three, I have shown that mutant p53 did not affect HSC quiescence, indicating $p53^{R248W}$ is not a loss-of-function mutant. Moreover, I demonstrated that $p53^{R248W}$ mutant enhances the competitive advantage of HSCs, leading to clonal expansion and the development of pre-leukemic HSCs. However, the mechanisms by which mutant p53 drives pre-leukemic HSC development remain elusive. I hypothesized that **$p53^{R248W}$ enhances HSC self-renewal through regulating genes or/and pathways important for HSC function**.

To uncover the molecular mechanisms by which mutant p53 drives pre-leukemic HSCs, I assessed genome-wide changes in the transcriptome by performing RNA-seq assay of LSK cells isolated from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. Gene Set enrichment analysis (GSEA) and DAVID analysis were performed to examine whether gene signatures or pathways related to HSC regulations were altered in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs.

Changes in chromatin accessibility play a critical role in transcriptional regulation, reprogramming, and epigenetic controls (Tsompana et al., 2014). Chromosome accessibility, reflected by the positioning and distribution of nucleosomes throughout a genome, determines the availability of binding regions to transcription factors and epigenetic machinery, and affects DNA-dependent process such as transcription, thus correlates with gene expression levels (Lamparter et al., 2017; Su et al., 2017). Mutant p53 has been shown to execute its GOF properties through interacting with transcriptional machineries, activating or inhibiting their activity (Brosh et al., 2009; Patricia et al., 2013). Hence, mutant p53 may regulate transcription through modulating chromatin accessibility. However, this aspect of mutant p53 has not been explored. Therefore, it is necessary to determine the state of chromatin accessibility in pre-leukemic HSC harboring p53 mutations. I hypothesized that **GOF mutant p53 drives the development of pre-leukemic HSCs through altering chromatin accessibility to genes important for HSC function**.

The Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) is one of the approaches for mapping genome-wide

chromatin accessibility (Buenrostro et al., 2013). It utilizes hyperactive Tn5 transposase to cut and ligate adapters (Goryshin and Reznikoff, 1998; Adey et al., 2010). The insertion ends of DNA is then applied to high-throughput sequencing allowing for identifying the DNA regions of opened accessibility (Buenrostro et al., 2013; Tsompana et al., 2014). I employed ATAC-seq assay to compare chromatin accessibility landscape of *p53*$^{+/+}$ LSKs and *p53*$^{R248W/+}$ LSKs. In addition, I searched genome-wide transcription factor binding motifs in mutant p53 LSK cells, in an effort to identify transcription factors interacting with mutant p53.

The ***aim*** of this study was to elucidate the mechanisms underlying the contribution of mutant p53 to pre-leukemic HSC development. This study will facilitate the identification of therapeutic targets in pre-leukemic HSCs expressing *TP53* mutations.

Results

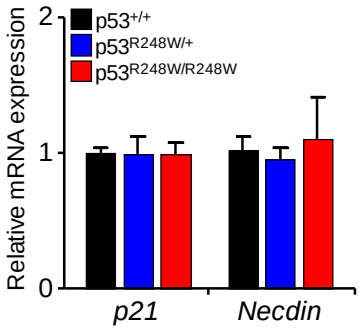
1. GOF mutant p53 HSCs show gene expression signatures important for stem cell maintenance and leukemia development

Mutations on the DNA binding domain of p53 disrupt specific DNA binding activity, which may result in loss of wild-type p53 functions (Brosh et al., 2009; Patricia et al., 2013). Therefore, I first examined the expression of bona fide wild type p53 target genes in HSCs, including *p21* and *Necdin*. As shown in Figure 6.1.A, HSCs expressing $p53^{R248W}$ does not affect the expression of *p21* and *Necdin,* suggesting that $p53^{R248W}$ is not a loss-of-function mutant.

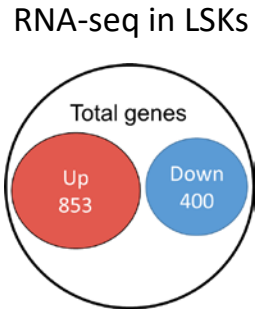
To identify $p53^{R248W}$ specific target genes in HSC, I utilized RNA-seq technique to investigate genome-wide gene expression changes in purified LSKs from $p53^{+/+}$ and $p53^{R248W/+}$ mice. Computational analysis revealed that 853 genes were significantly upregulated and 400 genes were significantly down-regulated in $p53^{R248W/+}$ LSKs compared to $p53^{+/+}$ LSKs (Figure 6.1.B). I then performed Gene Set Enrichment Assays (GESA) analysis to group potential $p53^{R248W}$ target genes into specific pathways important for HSC function. I found that HSC signatures were significantly enriched in $p53^{R248W/+}$ LSKs compared to $p53^{+/+}$ LSKs (Figure 6.1.C). Interestingly, several hematological malignancy signatures, including AML, chronic myeloid leukemia (CML), and leukemia stem cell signatures, were enriched in $p53^{R248W/+}$ LSKs compared to $p53^{+/+}$ LSKs (Figure 6.1.D). I also performed DAVID pathway analysis and found that several pathways important for HSC maintenance, including Regulation of Hematopoiesis, Hematopoietic organ development, Immune response, and

Positive regulation of cytokine response, were significantly enriched in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs (Figure 6.1.E). These findings support my hypothesis that mutant p53 drives the development of pre-leukemic HSCs.

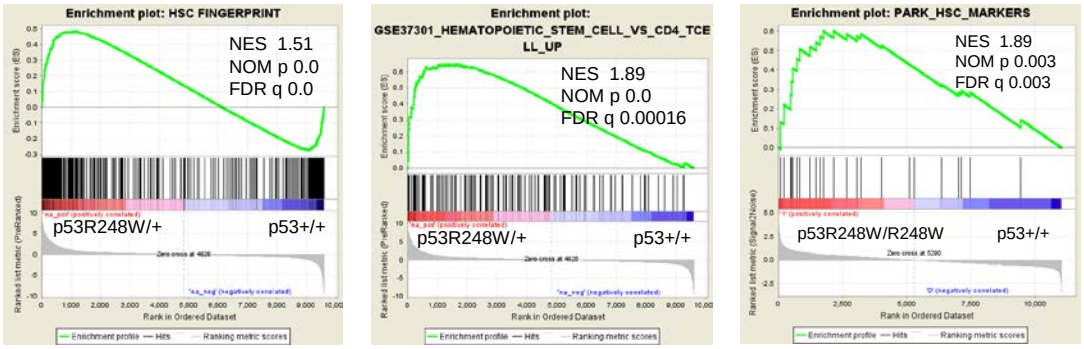
A


Relative mRNA expression
2
1
0
p53+/+
p53R248W/+
p53R248W/R248W
p21
Necdin

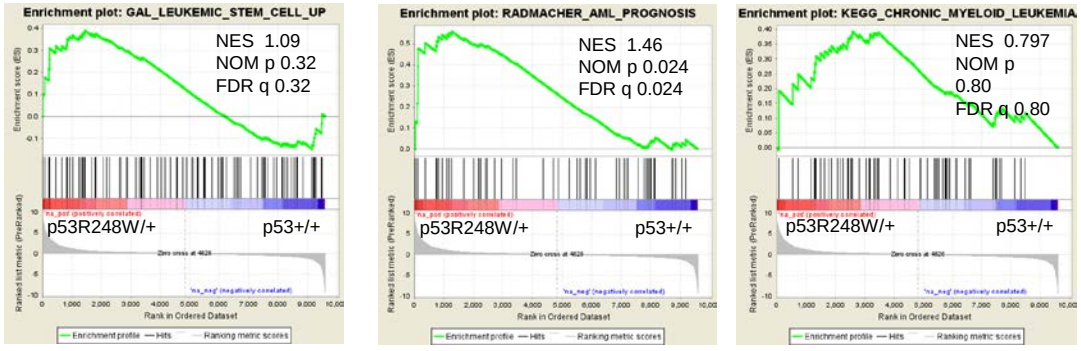
B


RNA-seq in LSKs
Total genes
Up
853
Down
400

C


Enrichment plot: HSC FINGERPRINT
NES 1.51
NOM p 0.0
FDR q 0.0
p53R248W/+
p53+/+
Enrichment plot: GSE37301_HEMATOPOIETIC_STEM_CELL_VS_CD4_TCELL_UP
NES 1.89
NOM p 0.0
FDR q 0.00016
p53R248W/+
p53+/+
Enrichment plot: PARK_HSC_MARKERS
NES 1.89
NOM p 0.003
FDR q 0.003
p53R248W/R248W
p53+/+

D


Enrichment plot: GAL_LEUKEMIC_STEM_CELL_UP
NES 1.09
NOM p 0.32
FDR q 0.32
p53R248W/+
p53+/+
Enrichment plot: RADMACHER_AML_PROGNOSIS
NES 1.46
NOM p 0.024
FDR q 0.024
p53R248W/+
p53+/+
Enrichment plot: KEGG_CHRONIC_MYELOID_LEUKEMIA
NES 0.797
NOM p
0.80
FDR q 0.80
p53R248W/+
p53+/+

E

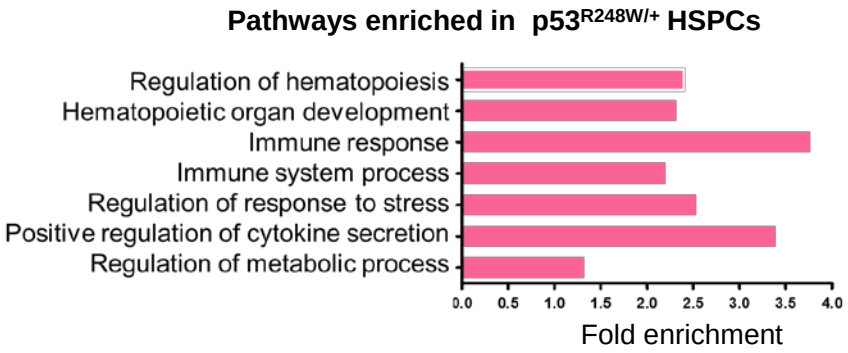


Figure 6.1 *p53*$^{R248W/+}$ HSCs show gene expression signatures important for stem cell maintenance and leukemia development

(A) Quantitative RT-PCR analysis of mRNA levels of p53 target genes, *p21* and *Necdin*, in HSCs. n=3 biological replicates, p>0.05.

(B) Number of genes significantly up-regulated or down-regulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs in RNA-seq assays. n=2 biological repeats, **p<0.01.

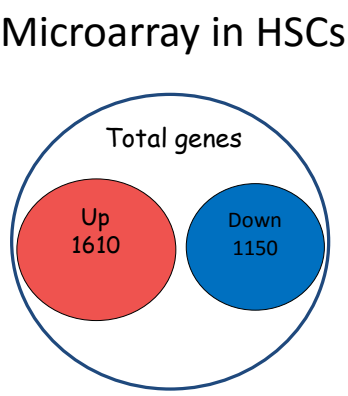
(C) RNA-seq data was applied to Gene Set Enrichment Analysis (GSEA) to identify stem cell related gene sets enriched in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs in molecular signature database (MsigDB).

(D) GESA analysis showed enrichment of CML, AML and leukemia stem cell signatures in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs.

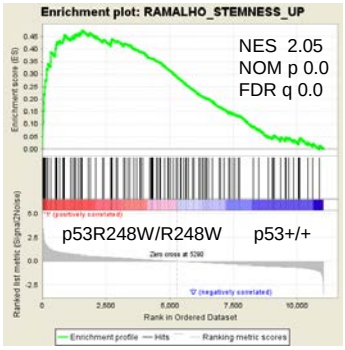
(E) DAVID pathway analysis of genes upregulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs.

To rule out the potential dominant-negative (DN) effect of mutant p53 on gene expression in HSCs, I performed microarray analysis to compare gene expression in HSCs ($CD48^{-}CD150^{+}$LSKs) isolated from $p53^{+/+}$ and $p53^{R248W/R248W}$ mice. I found that 1, 610 genes were significantly upregulated, and 1,150 genes were down-regulated in $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$, respectively (Figure 6.2.A). GESA analysis indicate that HSC signatures and several hematological malignancy signatures, including CML, AML, and poor-prognosis AML genes, were enriched in $p53^{R248W/R248W}$ HSCs compared to $p53^{+/+}$ HSCs (Figures 6.2.B and 6.2.C). Taken together, the gene expression profiling data provided additional evidence that GOF mutant p53 modulates specific pathways associated with hematopoiesis and leukemogenesis, thereby predisposing normal HSCs to leukemic transformation.

A



B



C

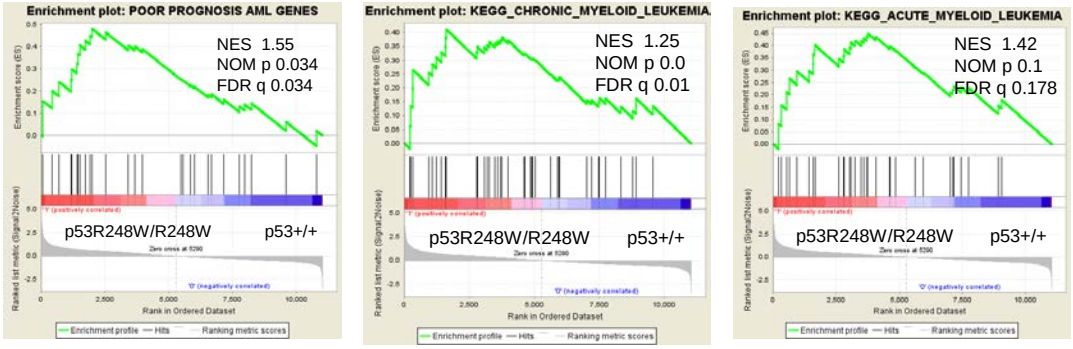


Figure 6.2 *p53*$^{R248W/R248W}$ HSCs show gene expression signatures important for stem cell maintenance and leukemia development

(A) Number of genes significantly up-regulated or down-regulated in *p53*$^{R248W/R248W}$ HSCs compared to *p53*$^{+/+}$ HSCs in microarray assays. n=3 biological repeats, **p<0.01.

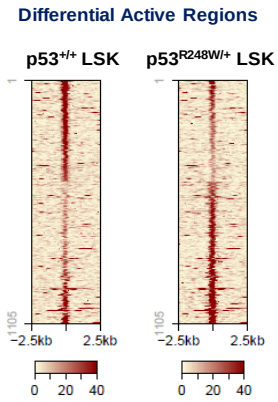
(B) GESA analysis showed enrichment of HSC signature in *p53*$^{R248W/R248W}$ HSCs compared to *p53*$^{+/+}$ HSCs.

(C) GESA analysis showed enrichment of CML and AML signatures in *p53*$^{R248W/R248W}$ HSCs compared to *p53*$^{+/+}$ HSCs.

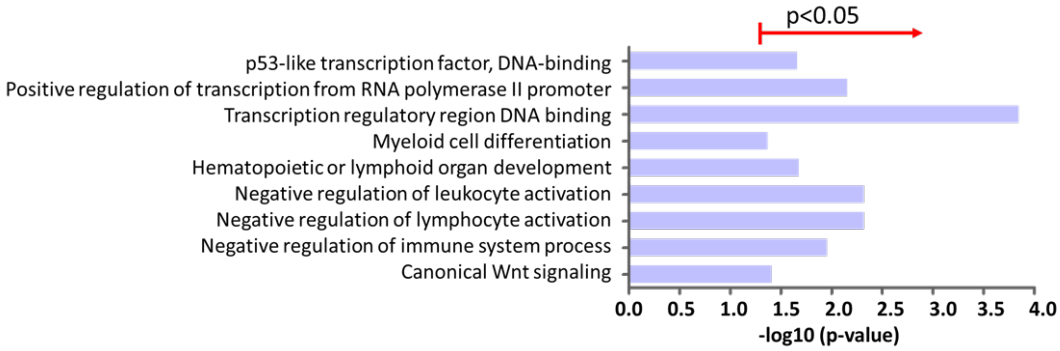
2. GOF mutant p53 alters chromatin accessibility in HSPCs

Given that p53 functions as a transcription factor binding to chromatin to regulate the transcription of its target genes (Brosh et al., 2009; Patricia et al., 2013), it is possible that GOF mutant p53 may change gene expression through altering chromatin accessibility. Therefore, I utilized ATAC-seq assay to identify differential regions of chromatin accessibility (Buenrostro et al., 2013) of *p53*$^{R248W/+}$ LSKs in comparison to *p53*$^{+/+}$ LSKs. Approximately 600 peaks were significantly upregulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs (Figure 6.3.A), and around 600 peaks were significantly downregulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs (Figure 6.3.A). DAVID pathway analysis of the ATAC peaks that were significantly upregulated in *p53*$^{+/+}$ LSKs revealed enrichment in p53 pathway, canonical Wnt signaling, and negative regulation of immune system processes (Figure 6.3.B), which is consistent with known p53 functions. Interestingly, HSC maintenance and HSC self-renewal pathways were enriched in *p53*$^{R248W/+}$ LSKs (Figure 6.3.C). Additionally, I found that pathways related to mRNA splicing and SNF-2 interaction, which have been reported to regulate HSC functions, are enriched in in *p53*$^{R248W/+}$ LSKs (Figure 6.3.C). Furthermore, pathway analysis result revealed that p53^{R248W} may function as both transcriptional co-activator and co-repressor in HSPCs.

A


Differential Active Regions
p53+/+ LSK
p53R248W/+ LSK
1
105
-2.5kb
2.5kb
0
20
40

B


p<0.05
p53-like transcription factor, DNA-binding
Positive regulation of transcription from RNA polymerase II promoter
Transcription regulatory region DNA binding
Myeloid cell differentiation
Hematopoietic or lymphoid organ development
Negative regulation of leukocyte activation
Negative regulation of lymphocyte activation
Negative regulation of immune system process
Canonical Wnt signaling
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
-log10 (p-value)

C

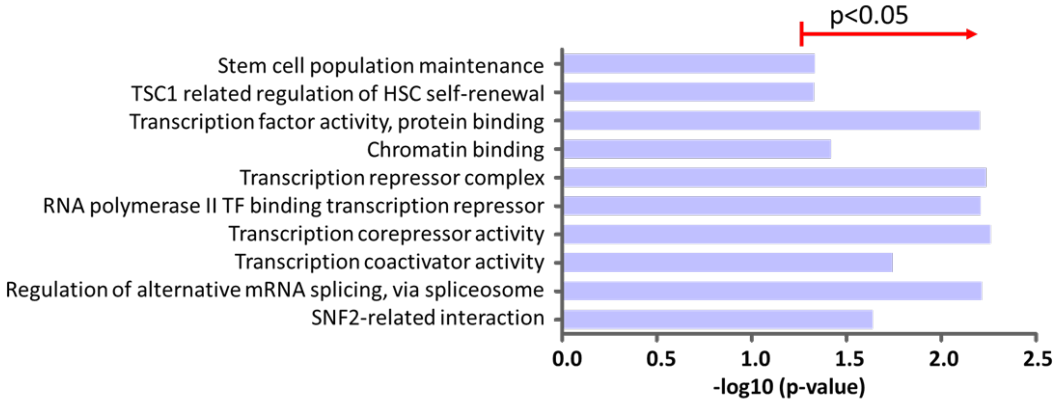

p<0.05
Stem cell population maintenance
TSC1 related regulation of HSC self-renewal
Transcription factor activity, protein binding
Chromatin binding
Transcription repressor complex
RNA polymerase II TF binding transcription repressor
Transcription corepressor activity
Transcription coactivator activity
Regulation of alternative mRNA splicing, via spliceosome
SNF2-related interaction
0.0
0.5
1.0
1.5
2.0
2.5
-log10 (p-value)

Figure 6.3 Enriched biological processes of genes associated with upregulated ATAC-seq peaks in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSKs.

(A) Heat map of differential active regions in *p53*$^{R248W/+}$ and *p53*$^{+/+}$ LSKs.

(B) Enriched biological processes of genes associated with significantly upregulated ATAC-seq peaks in *p53*$^{+/+}$ LSKs analyzed by DAVID Functional Annotation Tool.

(C) Enriched biological processes of genes associated with significantly upregulated ATAC-seq peaks in *p53*$^{R248W/+}$ LSKs analyzed by DAVID Functional Annotation Tool.

3. GOF mutant p53 interacts with transcription factors to regulate chromatin accessibility

Mutant p53 may function as a transcription cofactor in regulating gene expression as implicated in Figure 6.3.C. I decided to identify potential transcription factors that are associated with mutant p53. I performed motif search to analyze the differential accessible peaks in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSK cells. I identified strong enrichment for transcription factor (TF) binding sites, including CTCF, ETS1, MEIS1, and FOXO1(Figure 6.4), which play important roles in hematopoiesis (Degner et al., 2011; Barton et al., 1998; Kocabas et al., 2012; Tothova et al., 2007). For example, both MESI1 and FOXO1 are critical mediators of HSCs via limiting oxidative stress and this preserving HSC functions. The motif searching results may provide additional mechanisms by which mutant p53 maintains HSC functions.

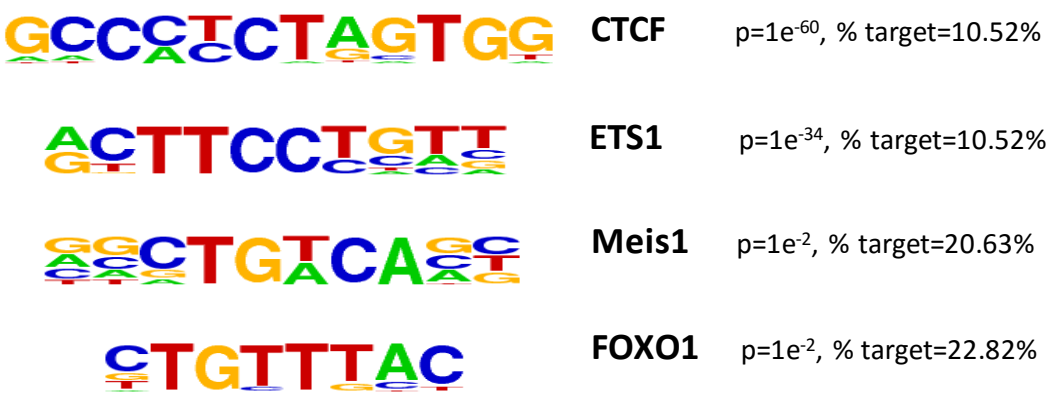


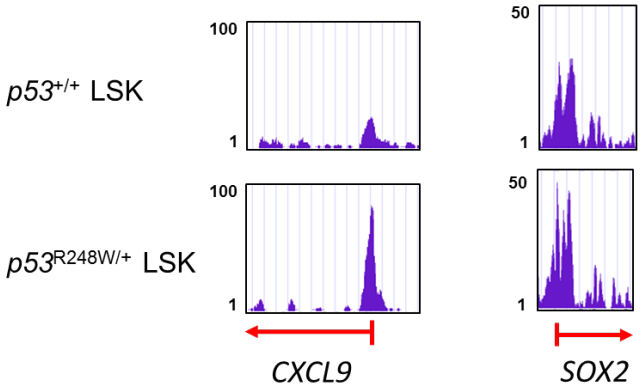
Figure 6.4 Enrichment of transcription factor motifs in peaks that gain open accessibility in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs determined by HOMER *de novo* or Known Motif Analysis.

4. GOF mutant p53 increases the chromatin accessibility to genes important for HSC maintenance

To identify specific genes with changes in chromatin accessibility upon expression of mutant p53, I next wanted to investigate chromatin accessibility changes on specific genes regulated by mutant p53. I found that mutant p53 increased chromatin accessibility to *Cxcl9* and *Sox2* genes as visualized on UCSC Genome browser (Figure 6.5.A). Chemokine gene *Cxcl9* has been shown to regulate HSPC expansion and protect HSPC from chemotherapy (Broxmeyer et al., 2006; Lu, et al., 2012). *Sox2* is a pluripotency gene important for stem cell maintenance in embryonic stem cells (Takahashi et al., 2006). Upregulation of *Sox2* has been suggested to be responsible, at least in part, for *ex vivo* expansion of cord-blood CD34+ induced by HADC inhibitor (Chaurasia et al., 2014), implicating a potential role of *Sox2* in HSC clonal expansion.

By performing qPCR analysis, I validated that both *Cxcl9* and *Sox2* were upregulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs (Figure 6.5.B). Thus, I demonstrate that GOF mutant p53 alters chromatin accessibility to genes important for HSC maintenance.

A



B

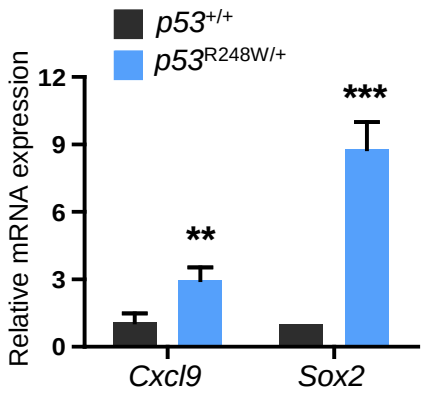


Figure 6.5 Expression of *Cxcl9* and *Sox2* are increased in *p53*$^{R248W/+}$ HSCs.

(A) UCSC genome browser tacks of normalized ATAC-seq peaks at the TSS region of *Cxcl9* and Sox2 genes in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSKs.

(B) Quantitative RT-PCR analysis of *Cxcl9 and Sox2* expression in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSKs. n=3 biological replicates, **p<0.01, ***p<0.01.

Conclusion and Discussion (Chapter Six)

In previous chapters, I have demonstrated that mutant p53 enhances the repopulating potential of HSCs and promotes their clonal expansion, driving the development of pre-leukemic HSCs. I next tried to uncover the molecular mechanisms by which mutant p53 promotes pre-leukemic HSC development. In this study, I demonstrated that mutant p53 regulates genes or/and pathways important for HSC function.

Mutant p53 may exhibit loss-of-function, dominant-negative effect or gain-of-function properties in driving tumorigenesis in human cancer (Brosh et al., 2009; Patricia et al., 2013). p21 is a classic target of p53 in regulating cell cycle (Wang et al., 2009). Additionally, we have identified *Necdin* and *Gfi-1* as p53 target genes in regulating HSC quiescence (Liu et al., 2009b). Loss of p53 leads to downregulation of *Necdin* and *Gfi-1* in HSCs (Liu et al., 2009b), resulting in decreased number of quiescent HSCs. However, I observed no changes on quiescent state between $p53^{+/+}$ and $p53^{R248W/+}$ HSCs, ruling out loss-of-function effect of mutant p53 on HSCs. To validate the result from the molecular perspective, I conducted qPCR assays to examine the expression of bona fide p53 targets in HSCs expressing $p53^{R248W}$. Interestingly, mRNA levels of p21 and Necdin were changed in neither $p53^{R248W/+}$ HSCs nor $p53^{R248W/R248W}$ HSCs, in comparison to those of $p53^{+/+}$ HSCs. These data further demonstrate that mutant p53 does not affect HSC quiescence and that $p53^{R248W}$ is not a loss-of-function mutant.

To further understand how mutant p53 affects transcriptome, I performed RNA-seq assays of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSK cells. Comprehensive bioinformatic analysis revealed that HSCs harboring p53^{R258W} mutation showed enriched HSC signatures as well as leukemic stem cell signatures, consistent with my findings that mutant p53 enhances the repopulating potential of HSCs and aged *p53*$^{R248W/+}$ mice developed MDS. Therefore, results from RNA-seq analysis support my conclusion that *TP53* mutation is a pre-leukemic mutation that predisposes pre-leukemic HSC to leukemic transformation.

I also performed gene expression profiling assays using *p53*$^{+/+}$ and *p53*$^{R248W/R248W}$ LT-HSCs and found that HSC and leukemia stem cell signatures were enriched in mutant HSCs. Given that *p53*$^{R248W/+}$ HSCs and *p53*$^{R248W/R28W}$ HSCs share similar gene expression profiles compared to WT HSCs, I demonstrate that p53^{R258W} does not have a dominant-negative effect on gene expression in HSCs.

To decipher the gain-of-function mechanisms underlying GOF mutant p53 on HSC function, I employed ATAC-seq assays to map open chromatin regions of pre-leukemic HSCs expressing mutant p53. Comparison of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSK cells revealed pathways regulated by wild-type p53 in WT cells consistent with published results. For example, pathway related to p53-like transcription factor and DNA binding activity were significantly enriched in in *p53*$^{+/+}$ LSKs. On the contrary, pathways relevant to stem cell maintenance and HSC self-renewal were enriched in *p53*$^{R248W/+}$ LSK cells, further supporting the findings from RNA-seq analysis that mutant p53 contributes to pre-leukemic HSC

development through regulating pathways involved in HSC maintenance. Interestingly, I also observed pathways related to transcription coactivator activity, transcription corepressor activity, and RNA Pol II relevant chromatin binding activity were enriched in mutant HSCs, implying a potential role for mutant p53 in transcriptional regulation. Moreover, enrichment of spliceosome and SWI/SNF related interaction pathways were also found in mutant HSCs. Mounting evidence suggest a critical role of spliceosome in normal and malignant hematopoiesis (Abdel-Wahab et al., 2011; Lee et al., 2016). Importantly, high frequency of spliceosome gene mutations were identified in aged individuals with clonal hematopoiesis and in patients with MDS (Yoshida et al., 2011; Inoue et al., 2016), suggesting that alteration of spliceosome causes hematopoietic disorders. SWI/SNF is a chromatin remodeling complex mediating transcriptional machinery (Kadoch et al., 2015). GOF mutant p53 associated with SWI/SNF complex in breast cancer cells, promoting their tumorigenesis (Pfister et al., 2015). My findings that mutant p53 may interfere spliceosome activity and/or associate with SWI/SNF complex are intriguing, as that may be novel mechanisms underlying the gain-of-function activity of mutant p53 in HSCs.

Furthermore, ATAC-assay facilitates the discovery of open chromatin regions that are occupied by transcription factors (Lamparter et al., 2017; Su et al., 2017). By performing comprehensive motif analysis, I was able to identify transcription factors that may cooperate with mutant p53 in regulating gene expression in HSCs. These transcription factors are CTCF, ETS1, MEIS1 and FOXO1, which have been implicated as important regulators in hematopoiesis

(Degner et al., 2011; Barton et al., 1998; Kocabas et al., 2012; Tothova et al., 2007). Particularly, MEIS1 and FOXO1 are critical mediators of HSC by limiting oxidative stress (Kocabas et al., 2012; Tothova et al., 2007). MEIS1 is required for expansion of adult HSC (Kocabas et al., 2012). Deletion of MEIS1 resulted in dramatic decrease of repopulating potential of HSCs due to accumulation of reactive oxygen species (ROS), demonstrating that MEIS1 preserves HSC functions through restricting oxidative stress (Kocabas et al., 2012). FOXO family proteins, including FOXO1, FOXO3, FOXO4, are required for HSC long-term regenerative potential, as they decrease the accumulation of ROS in HSCs and protect HSCs from physiological oxidative stress (Tothova et al., 2007). Therefore, my findings that mutant p53 interacts with MEIS1 and FOXO1 may help explain how mutant p53 enhances repopulating potential of HSCs *in vivo*.

Finally, I found that mutant p53 increases the chromatin accessibility to several genes, including *Sox2* and *Cxcl9*. Consistently, the transcription levels of these genes were increased in *p53*$^{R248W/+}$ LSK cells. CXCL9 is a chemokine that regulates HSPC expansion and protect HSPC from chemotherapy treatment (Broxmeyer et al., 2006; Lu, et al., 2012). Upregulation of *Cxcl9* in mutant HSCs may provide the molecular basis by which mutant p53 drives clonal expansion and preserves HSCs from chemotherapeutic agents (Lu, et al., 2012). *Sox2* is a well-known pluripotency gene important for stem cell maintenance in embryonic stem cells (ESC) (Liu et al., 2013). Overexpression of crucial pluripotency genes has been shown to induce both pluripotency and self-renewal of ESCs (Takahashi et al., 2006). In addition, Hoffman and colleagues reported that

HDAC inhibition induces *ex vivo* expansion of cord blood CD34+ cells and this was due to upregulation of pluripotency genes, including *Sox2* (Chaurasia et al., 2014). This evidence suggested a novel role of pluripotency genes in regulating HSC expansion. Therefore, I speculated that mutant p53 driven clonal expansion of HSCs may be, at least in part, due to increased levels of *Sox2* in *p53*$^{R248W/+}$ LSK cells.

Collectively, I demonstrated that mutant p53 plays a gain-of-function role in HSCs by regulating genes or/and pathways important for HSC maintenance, leading to the development of pre-leukemic HSCs. These studies identify a novel GOF property for mutant p53 in changing genome-wide chromatin accessibility landscape. Moreover, understanding molecular mechanism by which mutant p53 contributes to clonal expansion of HSCs may facilitate the development of novel therapeutic approaches for MDS patients with *TP53* mutations.

CHAPTER SEVEN

GOF mutant p53 interacts with EZH2 to drive pre-leukemic stem cell development

Introduction

Epigenetic modifiers are key regulators of HSC behavior and play critical roles in normal and malignant hematopoiesis (Shih, et al. 2012; Sun, et al. 2014; Beerman, et al. 2015). Thus, increased fitness of pre-leukemic HSCs could be due to dysregulation of epigenetic pathways. Indeed, genes involved in epigenetic regulations are most commonly mutated in pre-leukemic HSCs and clonal hematopoiesis, such as *DNMT3A*, *TET2*, *ASXL1* (Genovese, et al 2014; Sperling, et al. 2017), highlighting the importance of epigenetic controls in the development of pre-leukemic HSCs. While *TP53* is one of the top 5 genes that are commonly mutated in pre-leukemic HSCs and clonal hematopoiesis (Genovese, et al 2014), it is not clear whether mutant p53 interacts with epigenetic regulators to promote clonal expansion of pre-leukemic HSCs.

Recently, GOF mutant p53 was found to bind the regulatory regions of several epigenetic regulatory genes, including *MLL1*, *MLL2* and *MOZ*, and upregulate their expression, leading to cancer cell proliferation in breast cancer (Zhu, et al. 2015). MLL1 and MLL2 are SET family members of histone methyltransferases that mediate the tri-methylation of histone 3 at lysine 4 (H3K4me3) (Krivtsov, et al. 2007; Rao, et al. 2015). MOZ is a histone acetyltransferase that mediates acetylation of histone 3 at lysine 9 (H3K9ac) (Sun, et al. 2015). Both H3K4me3 and H3K9ac are histone marks for

transcription activation (Krivtsov, et al. 2007; Sun, et al. 2015). The study linked the GOF mutant p53 with chromatin pathways in tumorigenesis. As the mechanisms of action for mutation p53 is dependent on cell context, the mechanism identified in transformed cancer cells may not be applicable to pre-malignant adult stem cells.

In Chapter 6, I demonstrated that mutant p53 altered the landscape of chromatin accessibility across the genome and changed the expression of genes important for HSC self-renewal. Given that epigenetic factors affect the accessibility of regulatory factors to chromatin during transcription initiation (Buenrostro, et al. 2013), it is likely that mutant p53 cooperates with epigenetic factors in regulating gene transcription. DAVID analysis of upregulated ATAC-seq peaks in *p53*$^{R248W/+}$ LSKs indicated that p53^{R248W} functions as a transcriptional corepressor and involves in transcription repression related to RNA polymerase II (RNA Pol II) regulation (Figure 6.3.C). Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2) repress transcription through inhibiting RNA Pol II (Bernstein, et al. 2006; Bracken et al., 2009). Thus, I hypothesized that **mutant p53 cooperates with epigenetic regulators to modulate genes or/and pathways essential for HSC maintenance.**

I first examined the mRNA levels of several epigenetic regulatory genes, including *MLL1*, *MLL2*, *MOZ*, and *EZH2*, in mutant HSCs. To determine which epigenetic pathways are altered in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs, I

employed GSEA analysis to search epigenetic gene signatures in RNA-seq data I described in Chapter 6.

Next, I performed flow cytometry analysis to evaluate the intensity of histone marks in HSCs. Moreover, I performed biochemical experiments to uncover potential mechanisms underlying epigenetic alterations in mutant HSCs. Finally, I employed ChIP-seq assays in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSPCs in order to profile genome-wide epigenetic landscape in mutant HSPCs and understand how these alterations influence biological processes.

The ***objective*** of this study was to explore how mutant p53 interacts with epigenetic pathways in regulating pre-leukemic HSC development, facilitating the development of therapeutic approaches for pre-leukemic HSCs harboring *p53* mutations.

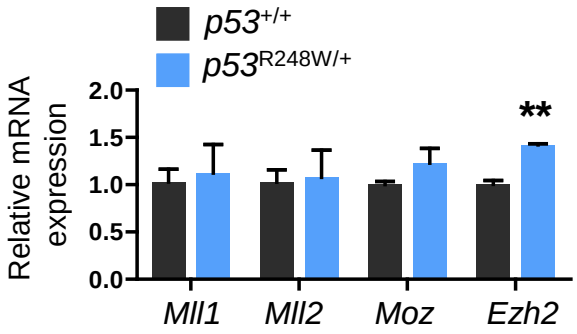
Results

1. GOF mutant p53 proteins enhance EZH2 activity in HSCs

Recent studies from the Berger lab revealed that GOF mutant p53 proteins increase the expression of several epigenetic factors, including *MLL1*, *MLL2*, and *MOZ,* in human breast cancer cells (Zhu et al., 2015). I therefore examined the expression of these epigenetic factors in $p53^{+/+}$ and $p53^{R248W/+}$ HSCs. As shown in Figure 7.1.A, the mRNA levels of *Mll1*, *Mll2*, and *Moz* were comparable in both groups. Interestingly, the expression of histone methyltransferase *Ezh2* were modestly increased in $p53^{R248W/+}$ HSCs compared to $p53^{+/+}$ HSCs (Figure 7.1.A). Consistently, GESA analysis of RNA-seq data from $p53^{+/+}$ and $p53^{R248W/+}$ LSKs showed that the expression of Ezh2 targets were significantly downregulated in $p53^{R248W/+}$ LSKs compared to $p53^{+/+}$ LSKs (Figure 7.1.B), indicating that Ezh2 activity was enhanced in $p53^{R248W/+}$ LSKs.

EZH2 mediates methylation of histone H3K27me3, which is a repressive histone mark (Bracken et al., 2009). I examined the levels of H3K27me3 in $p53^{+/+}$ and $p53^{R248W/+}$ HSCs using ImageStream flow cytometry analysis. As shown in Figure 7.1.C, $p53^{R248W/+}$ HSCs have increased H3K27me3 intensity compared to $p53^{+/+}$ HSCs. Collectively, these findings suggest that GOF mutant p53 may enhance EZH2 activity in HSCs.

A



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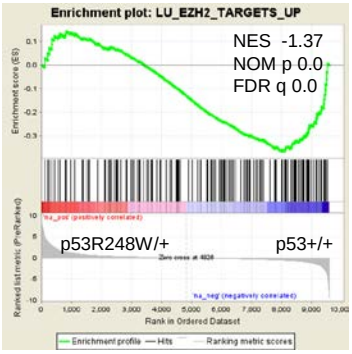


C

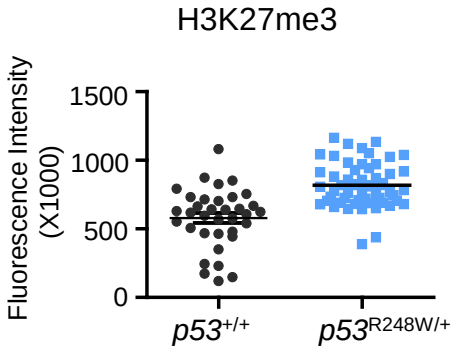


Figure 7.1 GOF mutant p53 enhances H3K27me3 in HSCs.

(A) Quantitative RT-PCR analysis of *Mll1, Mll2, Moz, and Ezh2* expression in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSKs. n=3 biological replicates, **p<0.01.

(B) GESA analysis shows negative enrichment of EZH2 signatures in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs.

(C) Median fluorescence intensity of H3K27me3 in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs detected by ImageStream flow cytometry. n>=50 cells per group, ***p<0.001.

2. Pharmacological inhibition of EZH2 activity decreased the replating potential of mutant HSPCs

EZH2 is a critical regulator of HSCs (Kamminga, et al. 2006; Xie, et al. 2014b; Mochizuki-Kashio, et al. 2017). While overexpression of EZH2 enhances HSC maintenance (Mochizuki-Kashio, et al. 2017), loss of EZH2 decreases the engraftment of HSCs in repopulation assays (Kamminga, et al. 2006). Remarkably, gain-of-function EZH2 mutations have been discovered in many hematological malignancies, including lymphoma, AML and multiple myeloma (Xie, et al. 2014b). Given that EZH2 inhibitors have been tested in clinical trials to treat cancer patients with high EHZ2 activity (Campbell, et al. 2015), I hypothesized that GOF mutant p53 depends on EZH2 activity in enhancing HSC self-renewal.

To test this hypothesis, I plated 20,000 $p53^{+/+}$ and $p53^{R248W/+}$ BM cells in MethoCult GF M3234 culture with DMSO (0.01%) or EZH2 specific inhibitor EPZ011989 (3uM) and performed serial replating assays. EPZ011989 is a potent oral inhibitor which has been shown to inhibit EZH2-mediated H3K27me3 (Campbell et al., 2015). I found that $p53^{R248W/+}$ bone marrow cells showed enhanced colony formation compared to $p53^{+/+}$ bone marrow cells in both and secondary Methocult culture (Figure 7.2). Interestingly, EZH2 inhibitor treatment decreased the colony formation of $p53^{R248W/+}$ bone marrow cells back to $p53^{+/+}$ cell level (Figure 7.2). Thus, the enhanced replating potential of BM cells expressing mutant p53 is dependent on EZH2 activity.

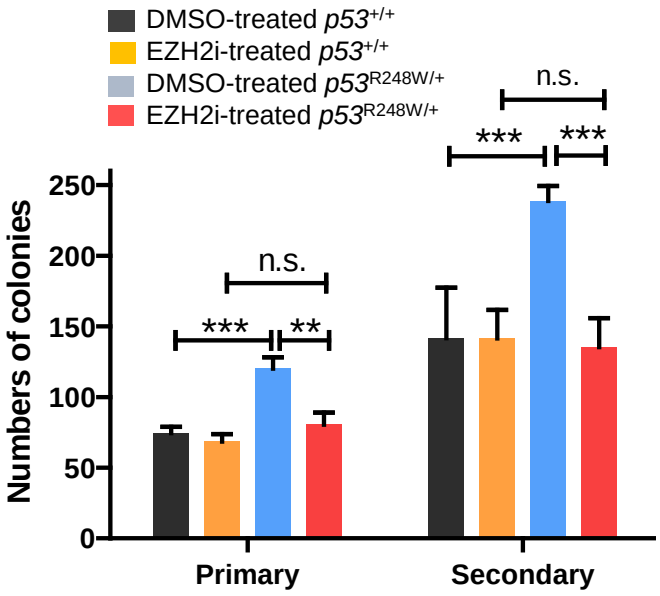


Figure 7.2 Inhibiting EZH2 decreased replating potential of mutant HSPCs *in vitro*. 20x10^3 BM cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ were seeded in methylcult treated with DMSO or EZH2 inhibitor (3μM). Primary colony number was counted at Day 7. Cells were harvested and seeded in new methylcult culture. Secondary colony number was counted at Day 7 after replating.

3. GOF mutant p53 proteins enhances H3K27me3 in genes important for HSC self-renewal

ATAC-seq data from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ LSK cells revealed that mutant p53 may act as a cofactor for EZH2-mediated transcriptional repression (Figure 6.3.C). I hypothesized that mutant p53 enhances H3K27me3 through interacting with EZH2. To test this hypothesis, I expressed wild-type p53 or several mutant p53 together EZH2 in H1299 cells, a human lung cancer cell line with p53 deletion (Wang et al., 1999). I then performed co-immunoprecipitation assays to see whether EZH2 was pulled down by p53 antibody. Interestingly, I found that several mutant p53 proteins, including p53^{R248W}, p53^{R273H} and p53^{R175H}, displayed enhanced association with EZH2 compared to WT p53 (Figure 7.3).

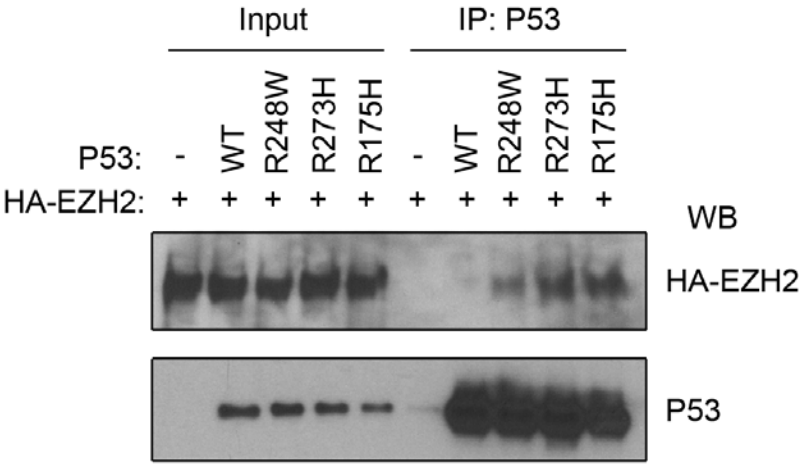


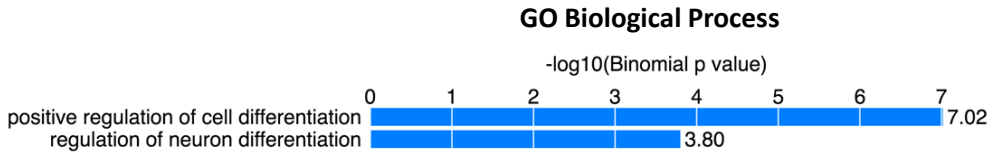
Figure 7.3 Several mutant p53 proteins, but not wild type p53, interact with EZH2. H1299 cells were transfected with EZH2-HA, and wild type p53 or mutant p53 (R248W, R273H or R179H). Immunorecipatation was performed with p53 antibody and blot with HA antibody.

4. GOF mutant p53 proteins interact with EZH2

To test whether GOF mutant p53 affects genome-wide H3K27me3 in HSPCs, we performed H3K27me3 ChIP-seq analysis in purified $Lin^{-}Kit^{+}$ cells from *p53*$^{+/+}$ and *p53*$^{R248W/+}$ mice. GREAT pathway analysis was utilized to group genome-wide H3K27me3 occupancy into biological pathways. I found that *p53*$^{R248W/+}$ cells showed non-overlapping H3K27me3 peaks in genes that positively regulate cell differentiation (Figure 7.4.A), whereas *p53*$^{+/+}$ cells had non-overlapping H3K27me3 peaks in genes important for stem cell proliferation (Figure 7.4.B).

Indeed, increased levels of H3K27me3 were found in genes regulating HSC differentiation and proliferation, including *Gadd45g* and *Elf4*/*Mef* (Thalheimer et al., 2014; Lacorazza et al., 2006; Liu et al., 2009b), in *p53*$^{R248W/+}$ cells compared to *p53*$^{+/+}$ cells (Figure 7.4.C). Consistently, both *Gadd45g* and *Elf4* were significantly downregulated in *p53*$^{R248W/+}$ LSKs compared to *p53*$^{+/+}$ LSKs (Figure 7.4.D). Taken together, these data demonstrate that GOF mutant increases HSC competitive advantage through interacting with EZH2 and enhancing H3K27me3 in HSCs.

A


GO Biological Process
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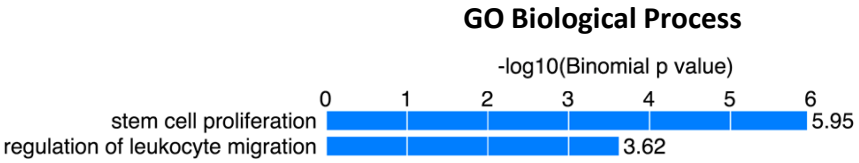
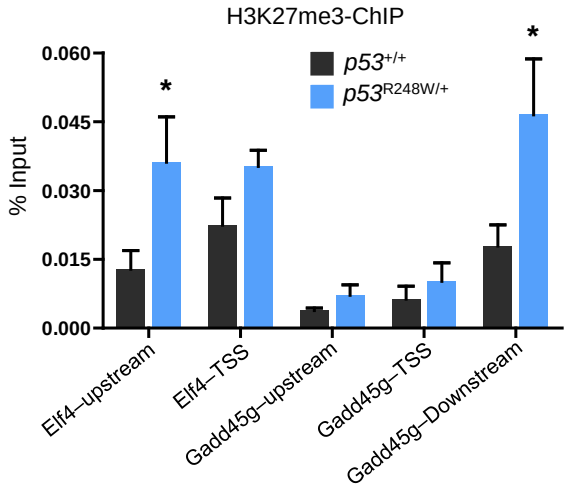
0
1
2
3
4
5
6
7
positive regulation of cell differentiation
7.02
regulation of neuron differentiation
3.80

B


GO Biological Process
-log10(Binomial p value)
0
1
2
3
4
5
6
stem cell proliferation
5.95
regulation of leukocyte migration
3.62

C


H3K27me3-ChIP
p53+/+
p53R248W/+
% Input
0.060
0.045
0.030
0.015
0.000
*
*
Elf4–upstream
Elf4–TSS
Gadd45g–upstream
Gadd45g–TSS
Gadd45g–Downstream

D

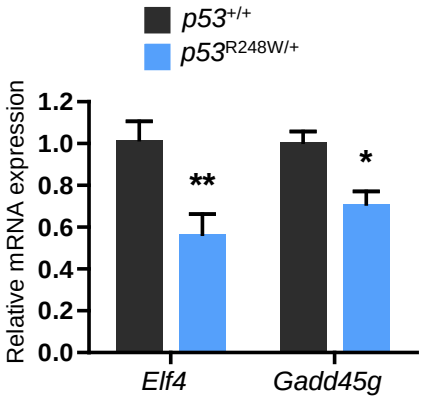

p53+/+
p53R248W/+
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
Elf4
Gadd45g

Figure 7.4 GOF mutant p53 regulates genes important for HSC self-renewal through enhancing H3K27me3

(A) GREAT pathway analysis of non-overlapping H3K27me3 peaks in *p53*$^{R248W/+}$ Lin^{-}Kit^{+} cells.

(B) GREAT pathway analysis of non-overlapping H3K27me3 peaks in *p53*$^{R248W/+}$ Lin^{-}Kit^{+} cells.

(C) H3K27me3 ChIP-seq analysis revealed the abundance and localization of H3K27me3 peaks at *Gadd45g* and *Elf4* genes in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ Lin^{-}Kit^{+} cells.

(D) Quantitative RT-PCR analysis of *Gadd45g* and *Elf4* expression in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSCs. n=3 biological replicates, *p<0.05, **p<0.01.

Conclusion and Discussion (Chapter Seven)

Epigenetic modifications play critical roles in balancing HSC self-renewal with differentiation (Shih, et al. 2012; Sun, et al. 2014; Beerman, et al. 2015). Epigenetic regulatory genes are frequently mutated in aged healthy individuals with clonal hematopoiesis as well as in patients with hematological malignancies, including MDS and AML (Genovese, et al 2014; Sperling, et al. 2017), highlighting their importance in the evolution of hematological malignancies. In this study, I demonstrated that GOF mutant p53 proteins, but not wild-type p53, interact with histone methyltransferase EZH2 and enhance H3K27me3, leading to repression of negative regulators of HSC repopulating potential.

Recent studies reported that GOF mutant p53 directly upregulated the expression of epigenetic regulatory genes, *MLL1*, *MLL2*, and *MOZ*, and increased level of H3K4me3 and H3K9ac, thereby promoting breast cancer cell proliferation (Zhu, et al. 2015). However, I proved that this was not the case in pre-leukemic HSCs with *TP53* mutation. Instead, *Ezh2*, gene encoding a histone methyltransferase, was modestly upregulated in $p53^{R248W/+}$ LSKs compared to $p53^{+/+}$ LSKs. GSEA and flow cytometry analysis further confirmed that mutant p53 proteins enhanced EZH2 activity and increased H3K27me3 in HSCs. These results demonstrate that GOF mutant p53 function is context dependent. Mechanisms seen in cancer cells might not be applicable to pre-malignant stem cells. Further, mechanisms discovered in solid tumors may not function in hematological malignancies.

EZH2 is an important regulator of HSC self-renewal and B cell differentiation (Bracken et al., 2009). Overexpression of EZH2 enhances HSC maintenance (Mochizuki-Kashio, et al. 2017), whereas loss of EZH2 decreases the engraftment of HSCs in repopulation assays (Kamminga, et al. 2006). Notably, gain-of-function mutations on EZH2, have been identified in several hematological malignancies, including AML and MDS (Xie, et al. 2014b). Pharmacological inhibition of EZH2 reduces self-renewal of HSCs, therefore providing a novel therapeutic strategy to treat leukemia patients with high activity of EZH2 (Campbell, et al. 2015). To determine the functional impact of enhanced EZH2 activity on mutant HSCs, I performed serial replating assays of *p53*$^{+/+}$ and *p53*$^{R248W/+}$ bone marrows treated with EZH2 inhibitor. Remarkably, pharmacologic inhibition of EZH2 decreased the colony formation of *p53*$^{R248W/+}$ bone marrow cells back to WT level, suggesting that mutant p53 promotes HSC repopulating potential through enhanced EZH2 activity. These results provide functional links between mutant p53 and epigenetic regulators in pre-leukemic HSC development. Furthermore, to elucidate how mutant p53 enhances EZH2 activity, I performed biochemistry studies and discovered that several mutant p53 proteins, but not WT p53, interact with EZH2. These findings uncovered a novel mechanism of gain-of-function property of mutant p53 in regulating HSC function.

Recently, genome-wide histone modification patterns have been extensively delineated by ChIP-seq assays in hematopoietic cells to understand the effect of epigenetic landscape changes on HSC function (Pepke, et al. 2009). We conducted ChIP-seq analysis of H3K27me3 in *p53*$^{+/+}$ and *p53*$^{R248W/+}$ HSPCs

and found that pathways involved in HSC self-renewal and differentiation were altered in mutant HSPCs. I also noticed that H3K27me3 levels in *Gadd45g and MEF/ELF4 genes were increased in p53*$^{R248W/+}$ HSPCs compared to those of *p53*$^{+/+}$ HSPCs. qPCR experiments and H3K27me3-ChIP assays further confirmed these findings.

Growth arrest and DNA-damage-induced 45 gamma (*Gadd45g*) is one of the member of the *Gadd45* family along with *Gadd45a* and *Gadd45b.* Gadd45 family proteins are known regulators in response to stress conditions and have been implicated in DNA damage repair, growth arrest, senescence and apoptosis. (Chen et al., 2014; Moskalev et al., 2012). Moreover, GADD45G is a tumor suppressor that is frequently silenced in many cancers (Liebermann et al., 2011). Most importantly, GADD45G has been shown to accelerate terminal differentiation of HSCs, and deficiency of *Gadd45g* enhances the repopulation potential of LT-HSCs (Thalheimer et al., 2014). Interestingly, the expression of *Gadd45g* is increased in aged LT-HSCs, which have reduced reconstitution ability (Rossi et al., 2005), and knockdown of *Gadd45g* in aged HSCs enhances HSC self-renewal *in vivo* (Wang et al., 2012). All of these studies provided evidence proved that GADD45G is a negative regulator for HSC repopulating potential. In this study, I found that *Gadd45g* was downregulated in *p53*$^{R248W/+}$ HSPCs through mutant p53 mediated H3K27me3. Therefore, I concluded that enhanced repopulating potential of mutant HSCs, was in part due to epigenetic silencing of *Gadd45g* in HSCs expressing mutant p53*. I also predicted that*

downregulation of Gadd45g contributed to the clonal expansion of mutant HSCs under genotoxic stresses.

MEF, also known as ELF4, is a member of the ETS family of transcription factors. MEF/ELF4 is a key regulator in HSC quiescence and self-renewal (Lacorazza et al., 2006). Mef null LSK cells are more quiescent and thus resistant to chemotherapy and irradiation induced myelosuppression (Lacorazza et al., 2006). I found that mutant p53 mediated epigenetic downregulation of *Mef/Elf4* expression through enhancing H3K27me3. I predicted that pre-leukemic HSC harboring mutant p53 downregulated *Mef/Elf4* expression to escape from the cytotoxic effects of irradiation or chemotherapy.

Collectively, I demonstrated that mutant p53 interacted with EZH2 and enhanced H3K27m3 in genes important for HSC functions. My findings that mutant p53 interacts with EZH2 to promote the repopulating potential of HSCs uncovered a novel gain-of-function mechanism of mutant p53 that contribute to pre-leukemic HSC development. Finally, my studies suggest that EZH2 might be an attractive therapeutic target for prevention and treatment of MDS patients with *TP53* mutations.

CHAPTER EIGHT

Summary and Future Directions

Myelodysplastic syndrome (MDS) is a heterogeneous group of disease characterized by ineffective hematopoiesis, multi-lineage dysplasia and frequent progression to acute myeloid leukemia (AML) (Nimer, et al. 2008; Bejar et al., 2011; Sperling, et al. 2017; Woll, et al. 2014). Cytopenia and transformation to AML attribute to the death of MDS patients (Nimer, et al. 2008; Bejar et al., 2011). Mutations in tumor suppressor gene *TP53* are present approximately in 10% of MDS cases and are associated with dismal prognosis (Rücker, et al. 2012; Volkert, et al. 2014; Welch, et al 2016; Sperling, et al. 2017； Chang, et al. 2017;). Although progress has been made on MDS treatment, the clinical outcome of MDS patients with *TP53* mutations is still very poor (Bejar et al., 2011; Metzgeroth, et al. 2016; Sperling, et al. 2017). Bone marrow transplantation does not improve the outcomes of MDS patients with *TP53* mutations (Christopeit, et al. 2016; Lindsley, et al. 2017). Nevertheless, little is known regarding how these mutations contribute to the development of MDS.

MDS is thought to arise from mutated HSCs (Nimer, et al. 2008; Cazzola et al., 2013; Sperling, et al. 2017). Accumulating studies indicate that the transformation event is initiated from the acquisition of pre-leukemic mutations driving the development of pre-leukemic HSCs, preceding the formation of fully transformed MDS stem cells (Corces-Zimmerman et al., 2014; Shlush et al., 2014). Pre-leukemic HSCs have competitive advantage over normal HSCs without affecting hematopoietic differentiation (Corces-Zimmerman et al., 2014;

Shlush et al., 2014). While *TP53* mutations and/or deletions are thought to be secondary events occurring during leukemic transformation (Harutyunyan et al., 2011), acquired somatic GOF mutations in *TP53* gene have been identified in the blood of aged healthy individuals as well as in patients with MDS (Bejar et al., 2011; Genovese, et al. 2014; Jaiswal, et al. 2014), implying that *TP53* mutations may be early events that initiate MDS.

In this study, I characterized heterozygous GOF mutant p53 knock-in mice and discovered that $p53^{R248W}$ enhanced the repopulating potential of normal HSCs without affecting terminal differentiation. GOF mutant p53 promotes HSC expansion following genotoxic stress. Furthermore, GOF mutant p53 mice developed features consistent with human MDS during aging. Mechanistically, GOF mutant p53 increases the chromatin accessibility to genes important for HSC maintenance. Furthermore, GOF mutant p53 interacts with histone methyltransferase EZH2 and enhances histone H3 lysine 27 trimethylation (H3K27me3) in genes regulating HSC self-renewal and differentiation. Therefore, I demonstrated mutant p53 drive the development of pre-leukemic HSCs, but not leukemia stem cells (LSCs), predisposing aged mutant mice to the pathogenesis of MDS. My studies have uncovered mechanistic and functional links between GOF mutant p53 and epigenetic regulators in pre-leukemic HSC and MDS development.

While *TP53* mutations were identified in patients with MDS, $p53^{-/-}$ and $p53^{R248W/R248W}$ mice did not develop MDS (Song et al., 2007). As MDS is a disease of elderly and most $p53^{-/-}$ and $p53^{R248W/R248W}$ mice develop spontaneous

tumors within 3 to 6 months after birth (Song et al., 2007), it is possible that homozygous mutant p53 mice do not have enough time to develop MDS before death. Indeed, I found that 30% of aged heterozygous *p53*$^{R248W/+}$ mice developed MDS manifested by leukopenia, anemia, and multi-lineage dysplasia in BM and PB. In addition, I found that MDS developed in mutant p53 mice was transplantable. Thus, I, for the first time, provided experimental evidence that a GOF mutant p53 drives MDS development in mice. Given that MDS patients with *TP53* mutations are resistant to conventional chemotherapy (Bejar et al., 2011; Lindsley et al., 2013) and they cannot be cured by allogeneic bone marrow transplantation, the heterozygous mutant p53 mice developed MDS will provide a valuable tool to investigate MDS pathogenesis and test novel therapeutic agents that can target mutant p53.

Notably, I observed that some *p53*$^{R248W/+}$ mice developed lymphoma, sarcoma, and mixed disease with lymphoma and MDS. These findings were consistent with what have been reported in human cases with heterozygote *TP53* mutations. My findings suggest that pre-leukemic HSCs may exist in both myeloid and lymphoid malignancies, and diverse diseases may be initiated from the same recurrent mutation (Corces-Zimmerman, et al. 2014). My findings that *p53*$^{R248W/+}$ mice developed different hematological malignancies highlighted the clinical importance for cancer prevention of pre-leukemic HSCs harboring *TP53* mutations.

Additionally, my observations that mutant p53 affects terminal differentiation during ageing and aged mutant mice developed MDS raise the

question of how mutant p53 drives the pathogenesis of MDS. Given that *TP53* mutations cause genome instability (Brosh et al., 2009), whether p53 mutations interact or cooperate with genetic alterations to drive the pathogenesis of MDS is not clear. Therefore, further studies, such as whole genome sequencing and karyotyping, are needed to figure out what genetic alterations cooperating with mutant p53 in the pathogenesis of MDS.

Clonal hematopoiesis is a process of cell competition (Jan et al., 2017), where normal HSCs are outcompeted by mutated HSCs. Studies on cell competition proposed that the competition event is cooperative, where losers promotes winners while winners suppress losers (Bondar and Medzhitov, 2010; Marusyk et al., 2010). In this study, I have elucidate the cell intrinsic mechanisms by which mutant p53 enhances repopulation potential and promotes clonal expansion of pre-leukemic HSCs. However, whether and how mutant HSCs cause negative impact on WT cells during clonal hematopoiesis still remain elusive. I predict that mutant HSCs may release inflammatory cytokines, causing apoptosis, senescence or pyroptosis of WT HSCs. Further investigation of how mutant HSCs suppress WT HSCs will improve our understanding of how rare clones become dominant and uncover novel gain-of-function mechanisms for mutant p53. Moreover, these findings may facilitate the development of therapeutic approaches that can improve WT HSC function during treatment.

I demonstrated that mutant p53 maintains HSC function, leading to clonal expansion of HSCs in response to chemotherapy and irradiation treatment. As pre-leukemic HSCs may survive chemotherapy and serve as reservoirs for

disease (Corces-Zimmerman et al., 2014; Shlush et al., 2014), the presence of pre-leukemic HSC pool prevents conventional chemotherapy from achieving long-term remissions. Thus, there remains a critical need to develop novel therapeutic approaches that would be effective in targeting pre-leukemic HSCs and improve leukemia treatment.

In Chapter Seven, I discovered that mutant p53 associated with EZH2 and enhanced H3K27me3 in genes important for HSC self-renewal and differentiation. Serial replating assays revealed that the enhanced replating potential of mutant HSCs was dependent on increased EZH2 activity. Considering that EZH2 interacts with GOF mutant p53 proteins, but not wild-type p53, our research has uncovered a novel mechanism by which GOF mutant p53 modulates gene expression in HSCs. These findings indicates that EZH2 may be an attractive therapeutic target for pre-leukemic HSCs bearing *TP53* mutations. In the future, whether pharmacologic inhibition or genetic knock-down of EZH2 would decrease the repopulating potential of mutant HSCs *in vivo* and delays disease development need to be further examined.

Moreover, I found that *Gadd45g* and *MEF/ELF4* genes were downregulated in mutant HSCs due to enhanced H3K27me3 mediated by mutant p53. Gadd45g and MEF/ELF4 are negative regulator of HSC function (Thalheimer et al., 2014; Lacorazza et al., 2006; Liu et al., 2009b). Downregulation Gadd45g and MEF/ELF4 genes lead to resistance of HSCs to chemotherapy and irradiation (Thalheimer et al., 2014; Lacorazza et al., 2006). It is likely that pre-leukemic HSC harboring mutant p53 downregulated *Gadd45g*

and *Mef/Elf4* expression in order to escape from the cytotoxic effects induced by irradiation or chemotherapy. Therefore, whether these targets contribute to the resistance of mutant p53 HSCs to conventional chemotherapy need to be determined.

In summary, my studies will advance our understanding of the initiation and progression of MDS with *TP53* mutations. Ultimately, delineating the effects of GOF mutant p53 and epigenetic regulators in pre-leukemic and MDS stem cells will likely identify novel targets in human MDS that may have therapeutic potential for eliminating drug-resistant pre-leukemic HSCs and MDS stem cells.

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PUBLICATIONS

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4. **Sisi Chen**, Xicheng Liu, Rui Gao, Michihiro Kobayashi, Hao Yu, Chonghua Yao, Zhijian Qian, Rongwen Xi, Yan Liu. *Polycomb Repressive Complex 1 Enhances HSC Self-Renewal through Repressing the Canonical Wnt Signaling Pathway.* The 57th American Society of Hematology (ASH) Annual Meeting, Orlando, FL. December, 2015. (Poster Presentation)

5. **Sisi Chen**, Xicheng Liu, Rui Gao, Michihiro Kobayashi, Hao Yu, Chonghua Yao, Zhijian Qian, Rongwen Xi, Yan Liu. *Polycomb Repressive Complex 1 Enhances HSC Self-Renewal through Repressing the Canonical Wnt Signaling Pathway.* The 11st Annual Midwest Blood Club Symposium, French Lick, IN, Oct. 2015 (Oral Presentation)

6. **Sisi Chen**, Hao Yu, Michihiro Kobayashi, Rui Gao, H. Scott Boswell, Yan Liu. *Gain-of-Function Mutant p53 Enhances Hematopoietic Stem Cell Self-renewal.* The 56th American Society of Hematology Annual Meeting and Exposition, San Francisco, CA, Dec. 2014 (Oral Presentation)