THE ROLE OF STRA13

IN

HEMATOPOIESIS

AVINASH GOVIND BAHIRVANI

(M.Sc., Manipal University, India)

A THESIS SUBMITTED FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

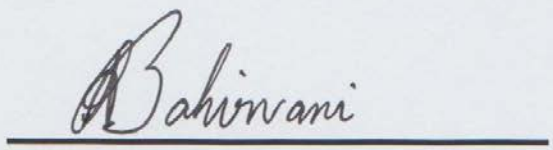
NATIONAL UNIVERSITY OF SINGAPORE

2014

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



Avinash Govind Bahirvani

22nd January 2014

ACKNOWLEDGEMENTS

My stem cell adventure started six years ago in Bangalore, India as a research assistant. However, not being able to pursue a PhD in the field I love in Bangalore, a PhD program at National University of Singapore was soon realized in 2010 and I'm very glad it clicked at the right time. It gave me the opportunity to persevere with my stem cell escapade in the field of hematopoietic stem cell biology for the next four years.

First and foremost, I would like to earnestly thank my PhD supervisor and mentor, Associate Professor **Reshma Taneja**. Working under her guidance has been an enriching experience which I'll cherish for years to come by. The intellectual scientific discussions about my project and the quality training that I have received under her leadership has only instilled more passion and helped me to grow as a better scientist. I can't thank her enough for being extremely patient with me and for motivating me during my lowest phases of my PhD.

To the present and past members of "*Cellular Differentiation and Apoptosis Lab" aka "Taneja Lab"*, it's been a joy sharing the lab space with you all. I am very grateful to **Vinay Kumar Rao**, **Shilpa Rani Shankar, Dr. Narendra Bharathy, Jin Yu**, **Tan Yong Hua, Teng Kai, Dr. Ling Mei Tze Belinda**, **Wang Yaju**, **Jin Rong Ow**, **Chu Chung Yin**, **Kok Wai Kay**, **Suma Gopinadhan, Dr. Dijendra Nath Roy, Sumita Sethi, and Bapat Devaki Dinesh** for providing an excellent, warm and at the same time a competitive atmosphere to work in the lab. The

discussions about our projects over lunch and coffee have also contributed immensely to fine tune our projects. Thank you for being good professional colleagues and great friends outside the lab.

I would like to sincerely thank Professor **Daniel G. Tenen**, Director, Cancer Science Institute of Singapore, my co-supervisor, for accommodating me in his lab for the major part of my PhD journey. I would like to thank him for generously sharing his lab resources and reagents and his contribution to my thesis through great scientific discussions. **Dr. Akira Kawasaki**, with whom I worked in close association on this project, thank you for helping me to learn the basics and the technical skills required in hematology. I would also like to thank former and present members of the "*Tenen Lab*"- Dr. Deepak Bararia, Dr. Vikas Madan, Dr. Senali Abayratna, Swathi Yada and Dr. Akihiko for always being approachable and sharing their technical knowledge. A heart-felt thanks to Dr. Motomi Osato for his invaluable inputs and expertise in the field of hematopoiesis and also his FACS facility team- Michelle Huang and Tan Huifang where I have spent countless hours sorting stem cells. I would also like to express thanks to Dr. Jayapal Manikandan for helping me to analyze the microarray data.

Most importantly, I am very grateful to my family. My Mom **Seema**, has always been a pillar of strength, her love and endless support for me to achieve my dreams has been praiseworthy. My father, **Govind**, I miss him every day in my life. Papa, I hope I've done my best to make you proud. I know you've been my guardian angel showering your love unconditionally and helping me accomplish

my dreams. To my brother, **Mukesh**, who has made the biggest sacrifice one can ask for, giving up his career so that I could realize mine, a big thank you. To my dearest sister, **Shalini**, thank you for the love and constant support and your frequent visits to Singapore which made my PhD journey in Singapore quite joyful. An honest thank you to all my close friends for being there during the happy and sad times in these rollercoaster four years.

I would like to dedicate this thesis to my family for believing in my dreams and leaving no stone unturned to help me accomplish this important milestone in my life.

There comes a time when the mind takes a higher plane of knowledge but can never prove how it got there- Albert Einstein

TABLE OF CONTENTS

SUMMARY

Hematopoiesis is a tightly regulated process maintained by a small pool of hematopoietic stem cells (HSCs). Precise lifelong regulation of HSCs is important for self-renewal and differentiation into mature blood cells. Stra13/Sharp2/DEC1, a basic helix-loop-helix orange (bHLH-O) transcription factor plays important roles in cellular proliferation, differentiation and apoptosis. Previous studies have identified the importance of Stra13 function in maintaining homeostasis of T regulatory (Treg) cells as aged Stra13 knockout (KO) mice show defective T cell activation and exhibit a lymphoproliferative autoimmune disease. However, the role of Stra13 in hematopoiesis has not been studied so far. Our study demonstrates that Stra13 is highly expressed in early progenitors Lin^{-} $Sca1^{+}$c-Kit^{+} (LSK) cells and in the long term hematopoietic stem cells (LT-HSCs) /SLAM ($CD150^{+}CD48^{-}$) and its expression is downregulated in the differentiated progenitors. Moreover, Stra13 mRNA expression is elevated in the LSK progenitors in aged mice (22-24 months) compared with LSK fraction of young mice (2-3 months). Our analysis indicates that Stra13 is dispensable during steady state hematopoiesis. However, the LSK fraction of young Stra13 knockout mice is more sensitive to genotoxic stress. Moreover, aged Stra13 knockout mice show decreased percentages of $SLAM^{+}$ and LSK fraction of the bone marrow and skewed progenitor levels. With the help of gene expression profiling studies, we have identified potential target genes that may potentiate the role of Stra13 in hematopoietic stem cell ageing and response to genotoxic stress. Our data uncovers a novel role of Stra13 in ageing and stress hematopoiesis.

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

Shankar SR, **Bahirvani AG**, Rao VK, Bharathy N, Ow JR, Taneja R. G9a, a multipotent regulator of gene expression. Epigenetics. 2012; 8(1):16-22. (Review)

Nekanti U, Rao VB, **Bahirvani AG**, Jan M, Totey S, Ta M. Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. Stem Cells Dev. 2010 Jan; 19(1):117-30.

LIST OF SYMBOLS & ABBREVIATIONS

5-FU	5 fluoro uracil
8- MOP	8-methoxypsoralen
Acadvl	Acyl-CoA dehydrogenase, very long chain
ACK buffer	Ammonium Chloride Potassium buffer
AML1	Acute Myeloid Leukemia 1
ANOVA	Analysis of variance
APC	Allophycocyanin
APC-Cy7	Allophycocyanin Cy7
BATF	B cell activating transcription factor
BCL2	B cell lymphoma 2
Bcorl1	BCL6 co-repressor-like 1
bHLH-O	Basic helix loop helix Orange
BM	Bone marrow
Bmi-1	B Lymphoma Mo-MLV Insertion Region 1
BMP	Bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
Bt2cAMP	N 6,O2'-dibutyryladenosine 3':5'-cyclic monophosphate, a dibutyryl derivative of cAMP
CAFC	Cobblestone area forming cell
cAMP	Cyclic adenosine monophosphate
CAMT	Congenital amegakaryocytic thrombocytopenia
CBF-1	Core binding factor 1
CBX	Chromobox protein homolog
CDC42	Cell division cycle 42
CDK	Cyclin dependent kinases
CDKN2A	Cyclin dependent kinase inhibitor 2A
CFC	Colony forming cell
CFU	Colony forming unit

CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR8	Cytokine Response gene 8
CRA	Competitive repopulation assay
CXN 43	Connexin 43
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for annotation, visualization and integrated discovery
Ddah1	Dimethylarginine dimethylaminohydrolase 1
DEC1	Differentiated Embryo Chondrocyte gene 1
Dhh	Desert hedgehog
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Eip1	E47 interacting protein
Elf4	E74-Like Factor 4
EZH2	Enhancer of zeste homolog 2
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLK2/FLT3	Fetal Liver kinase
FOXO	Forkhead box O
Frz	Frizzled
G-CSF	Granulocyte colony-stimulating factor
Gfi-1	Growth factor independent 1
GFP	Green fluorescent protein
GMP	Granulocyte macrophage progenitor
Gr1	Granulocyte receptor-1 antigen
Gy	Gray
H3K27me3	Trimethylation of H3 on lysine 27
H3K4me3	Trimethylation of H3 on lysine 4
H4K16	Acetylation of histone H4 on lysine 16
HDAC	Histone deacetylase

Hh	Hedgehog
HIF- 1	Hypoxia inducible factor 1
HoxB4	Homeobox B4
HSCs	Hematopoietic stem cells
Hsp8	Heat shock protein 8
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
Igl-v1	Immunoglobulin lambda variable 1
Ihh	Indian Hedgehog
IL6	Interleukin 6
IL-7	Interleukin 7
IRES	Internal ribosome entry site
Lin^-	Lineage negative
Lin^+	Lineage positive
LSK	Lineage- Sca-1+ c-Kit+
LT-HSC	Long term HSCs
LT-IC	Long term culture initiating cell
LTRs	Long terminal repeat
Mac1	Macrophage-1 antigen
Maml	Mastermind like
MAPK	Mitogen activated protein kinase
Mat2b	Methionine adenosyltransferase II, beta
MEP	Megakaryocyte erythroid progenitor
MIG	MSCV-IRES-GFP
MLL	Mixed lineage leukemia
MPP	Multipotent progenitor cells
MSCV	Murine stem cell virus
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MTR3	mRNA transport regulator 3

Nedd4l	Neural precursor cell expressed, developmentally down-regulated gene 4-like
NF κB	Nuclear factor κB
NICD	Intracellular domain of Notch
NOD	Non obese diabetic
PE	Phycoerythrin
PECy-7	Phycoerythrin Cy7
PI	Propidium Iodide
POLG	Polymerase catalytic subunit gamma
PPARγ2	Peroxisome proliferator-activated receptor gamma 2
PRC	Polycomb repressive complexes
Ptch1	Patched 1
PTEN	Phosphatase and Tension analog
RA	Retinoic acid
RANTES	Regulated upon activation, normally T-expressed
Rb	Retinoblastoma
RBCs	Red blood cells
ROS	Reactive oxygen species (ROS)
Runx1	Runt-related transcription factor 1
RXR	Retinoic acid receptor
RXR-LXR	Retinoic acid receptor -liver X receptor
Sca1	Stem cell antigen-1
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SF	Steel factor
Sharp1	Enhancer-Of-Split and Hairy-Related Protein 1
Sharp2	Enhancer of Split and Hairy Related Protein 2
Shh	Sonic Hedgehog
SIRT2	Sirtuin 2
SLAM	Signaling lymphocyte activation molecule

SMARCA4	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily Amember 4
SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1
Smo	Smoothened
SREBP-1c	Sterol regulatory element binding protein 1c
Srgap2	SLIT-ROBO Rho GTPase Activating Protein 2
STAT3β	Signal Transducer and Activator of Transcription
ST-HSCs	Short term HSCs
Stra13	Stimulated with retinoic acid 13
Stra13- Tg	Stra13 transgenic mice
Tctn3	tectonic family member 3
TGF	Transforming growth factor
Tgm2	Transglutaminase 2, C polypeptide
THPO	Thrombopoietin
TNF-α	Tumor Necrosis Factor alpha
TPO	Thrombopoietin
Tpst1	Protein-tyrosine sulfotransferase 1
Treg	Regulatory T cell
TSA	Trichostatin A
Upf1	Up-frameshift suppressor 1 homolog
VEGF	Vascular endothelial growth factor
Wnt	Wingless type
WRPW	Tryptophan-arginine-proline-tryptophan
XPA2	XPA binding protein 2
XPD	Xeroderma pigmentosum group D complementing protein
XRCC1	X ray repair cross completing protein 1
YRPW	Tyrosine-arginine-proline-tryptophan

CHAPTER 1

INTRODUCTION

1.INTRODUCTION

1.1 Hematopoiesis and hematopoietic stem cells

The human body produces billions of red blood cells, white blood cells and platelets every day to replace cells lost during cell turnover, injuries or trauma. Many homeostatic mechanisms within the body respond to stresses such as bleeding, infections or other toxic insults that allow the production of new blood cells and restore it to normal levels when the stress is resolved. This dynamic and highly orchestrated process of blood cell production and homeostasis is defined as hematopoiesis. It involves a tightly controlled expression of important transcription factors, growth factors and growth factor receptors. The sustained blood production of approximately 10^{11}-10^{12} cells daily is the responsibility of very rare cells in the bone marrow i.e. the hematopoietic stem cells (HSCs) (Bryder et al., 2006; McCulloch and Till, 1960). HSCs reside at the top of the hematopoietic hierarchy and replenish all mature blood lineages through a cascade of differentiation steps. Primitive hematopoiesis originates in the yolk sac where the dominant function is the production of red blood cells (RBCs) to aid tissue oxygenation required for rapid embryo growth and development. Both the blood and endothelial cells arise from the hemangioblast of the yolk sac (Ema et al., 2006; Palis and Yoder, 2001). The next site of hematopoiesis is the aorta gonardo mesonephroses (AGM), which form the hemogenic endothelial cells (ECs) in the ventral and dorsal wall of the aorta that bud off HSCs. The placenta also harbors significant numbers of HSCs during this stage of embryo

development (Gekas et al., 2005). Subsequently definitive hematopoiesis occurs in the fetal liver, thymus, spleen and ultimately the bone marrow. In definitive hematopoiesis, long term HSCs (LT-HSCs) form short term HSCs (ST-HSCs). ST-HSCS give rise to multipotent progenitor cells (MPPs) that become restricted in their proliferation potential and differentiate to form progenitors of the myeloid, lymphoid or mixed lympho-myeloid lineage respectively which are Common Myeloid Progenitor (CMP), Common Lymphoid Progenitor or Lymphoid primed Multi Potent Progenitor (LMPP) (Adolfsson et al., 2005; Akashi et al., 2000; Kondo et al., 1997; Mansson et al., 2007). The myeloid progenitors commit further to form into granulocyte macrophage progenitors (GMP) arising from CMP or LMPP or into megakaryocyte/erythroid progenitors (MEP) that originate from CMP. These committed progenitors eventually differentiate into mature blood cells i.e. GMPs form committed precursors of eosinophils, macrophages, mast cells and neutrophils. CLPs are the source of committed precursors of B and T lymphocytes. Megakaryocyte Erythroid Progenitors (MEPs) differentiate to form RBCs and platelets. The differentiation of these committed progenitors into precursor cells and finally to mature blood cells specific for its lineage completes the hematopoietic differentiation cascade (Figure 1.1)

a.

b.

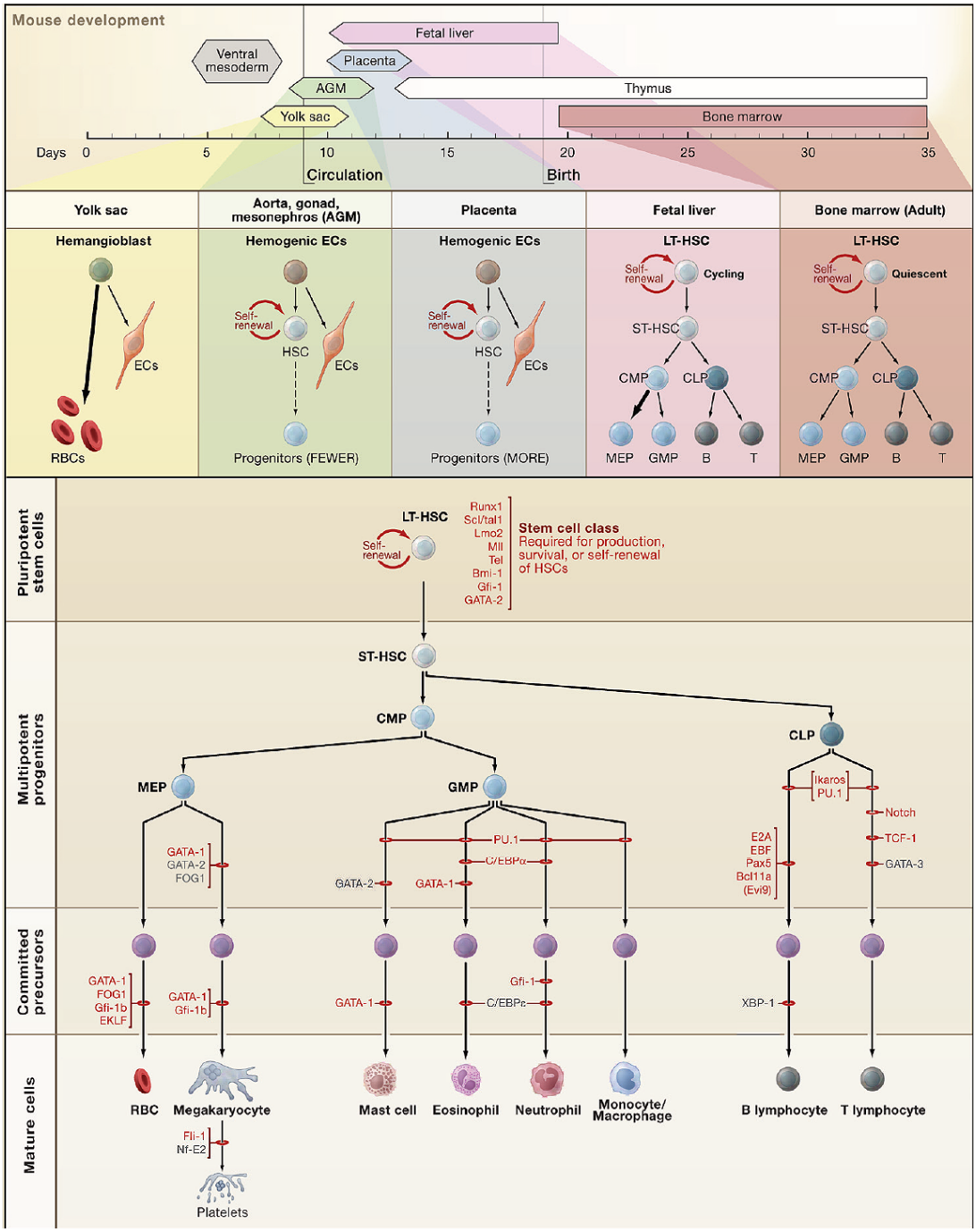


Figure 1.1: Snapshot of hematopoiesis in the mouse and its hierarchical organization with the involvement different transcription factors during the differentiation cascade (Orkin and Zon, 2008).

The upper panel **[a]** depicts the developmental time windows for different sites and stages of hematopoiesis in the mouse. Hematopoiesis originates in the yolk sac (YS) blood islands and soon after at the aorta-gonad mesonephros (AGM)

region, placenta, and fetal liver (FL). Hematopoiesis in each region leads to the production of specific blood lineages. The hierarchical organization and the transcription factors involved in hematopoiesis are depicted in the lower panel **[b].** The red loops signify the stages at which the hematopoiesis is blocked due to deficiency of the given transcription factor. Transcription factors involved in oncogenesis are highlighted in red whereas those in black have not been found mutated or translocated in mouse/human hematological malignancies.

1.2 Characteristics of hematopoietic stem cells

To date, murine HSCs are one of the best studied and characterized stem cells and provide a model for other tissue specific stem cells. They were first discovered by Till and McCulloch more than 50 years ago where they found a clone of murine bone marrow cells capable of reconstituting all the lineages in an irradiated mouse host (Becker et al., 1963; Till and Mc, 1961). Murine HSCs express a wide variety of surface markers which allow easy identification and purification but also to simply characterize them in terms of their functional potential. A true HSC is a considered a rare cell type constituting 1 per 10^5 bone marrow cells (Harrison et al., 1988). The development of tools and selection methods based on cell surface markers has advanced the prospective identification and isolation of HSCs. During the course of differentiation, the changes in cell surface marker expression allow HSCs to be distinguished from its progenitors and differentiated cells. Many studies have demonstrated that murine HSCs are confined within a cell population that lacks the surface marker expression of mature blood cells (lineage markers). HSCs express a subset of markers- Stem Cell Antigen-1(Sca-1) and c-Kit and are termed as LSK population ($Lineage^-$, $Sca\text{-}1^+$ $c\text{-}Kit^+$) (Okada et al., 1992; Spangrude et al., 1988). The LSK compartment is further subdivided into long term (LT) HSC which represents ~2% of LSK population, short-term

(ST) HSC and multipotent progenitors (MPPs) based on the expression of additional surface molecules such as CD34, Flt-3, Signaling lymphocyte activation molecule [SLAM (CD150,CD48)] and Endothelial protein C receptor (EPCR/CD201) (Balazs et al., 2006; Christensen and Weissman, 2001; Kiel et al., 2005; Osawa et al., 1996). The SLAM family is a large group of cell surface glycoproteins in the immunoglobulin superfamily (Wang et al., 2001). From this superfamily, CD150 and CD48 have been used for the isolation and purification of enriched HSCs (Kiel et al., 2005; Yilmaz et al., 2006). Recently, along with CD150 and CD48 cell surface markers, CD229 and CD244 are other new markers of the SLAM family that are used for characterizing HSCs (Oguro et al., 2013). Studies on the SLAM family have shown that a simple combination of $CD150^{+}CD48^{-}$ can give rise to a cell population that is specifically enriched in HSCs. One in five cells is capable of reconstituting a lethally irradiated mouse. The application of these markers complementing with other known HSC markers (Lin^{-} $CD150^{+}CD48^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$) have paved the way for highly enriched and purer HSC population in which almost 50% of single cells can reconstitute lethally irradiated animals.

HSCs are also characterized by their ability to efflux fluorescent DNA binding dyes such as Hoechst 33342 or Rhodamine 123 which is a mitochondrial activity marker. This led to the characterization of different flow cytometric profile of HSCs called the “side population” (SP) (Goodell et al., 1996; Li and Johnson, 1992). The SP cells are capable of long term reconstitution potential express high

level of multidrug resistant pump, the ABC transporter Bcrp1/ABCG2, which mediates the efflux of the Hoechst dye (Zhou et al., 2001).

1.3 *In vitro* and *in vivo* assays to detect HSCs

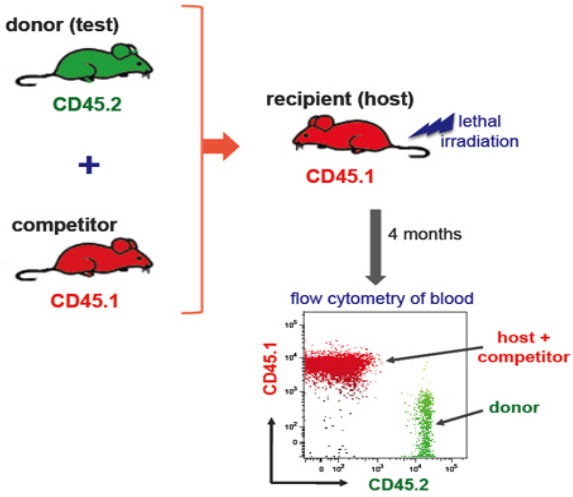
A number of *in vitro* assays have been employed successfully to study the function of HSCs and progenitor cells. One of most commonly used assay is the colony forming cell (CFC) assay to quantify lineage committed progenitor cells. The cells are seeded on methylcellulose semi-solid media dishes containing a rich cocktail of cytokines and grown for 5-7 days. The colonies that arise from one single cell are analyzed based on morphology and scored by inverted light microscopy. Cobblestone area forming cell (CAFC) assay is a scaled down version of long term bone marrow culture setup in 96 well plates in the presence of a stromal cell layer. The primitive cells to be tested are seeded in situ on the stromal layer in a limiting dilution analysis. Each individual well is microscopically screened for the presence or absence of "cobblestone areas" which are defined as colonies of minimum five small non refractile cells that grow on the base the stromal layer at particular sequential time points after the assay has been initiated. The CAFC assay is not reliable as it can overestimate the number of hematopoietic cells and other methods are needed to validate its accuracy (Denning-Kendall et al., 2003). Another variant technique to detect more primitive human and mouse hematopoietic cells *in vitro* is the long term culture initiating cell (LTC-IC) assay (van Os et al., 2008). Similar to CAFC assay, the primitive cells are cultured on stromal layer for 4-5 weeks. The progenitors differentiate, die and disappear and the primitive cells which remain

in the culture are then reseeded on methylcellulose media and grown for 10-12 days. The de novo CFCs that are formed from a LTC-IC are detected and scored. Colonies forming cells with high proliferative potential (HPP-CFC) are measured as dark colonies greater than 0.5mm and more than 50000 cells (McNiece et al., 1990). This is generally used in detecting human HSCs. These *in vitro* assays allow a relatively easier and quicker method to estimate the frequency and function of stem or progenitor cells in a test cell population. It is particularly useful to determine the efficacy of certain drugs or compounds on HSC function.

However the gold standard of studying HSC function is employing the *in vivo* transplantation assay which measures both self-renewal and differentiation potential of test cells after transfer to a myeloablated host. The competitive repopulation assay (CRA) is a relative measurement of the repopulation ability of the test cells compared to a reference standard of normal non fractionated bone marrow cells. Genetically marked test cells from the donor mice are transplanted into myeloablated mice along with a fixed number of competitor cells (Harrison et al., 1993; Szilvassy et al., 1990). The donors and hosts are chosen and distinguished based on the expression of hematopoietic cell marker CD45.1 or CD45.2 alloantigens which allow selective tracking of test cells and competitor cells in congenic mice. Competitor cells are syngenic to the recipient strain. For studying the function of human HSCs, xenogeneic immunodeficient nonobese diabetic- scid/scid (NOD/SCID) mice are used as recipients (Conneally et al., 1997). However, it does not permit absolute or rigorous quantification of HSC frequency and number in the test cell population (Figure 1.3). Another variant, the

limiting dilution assay involves the titrating various doses of test cells against a fixed number of competitor cells (Harrison et al., 1993; Taswell, 1981). For each dose, the percentages of hosts which fail to engraft the test donor cells are determined. A best fit line is generated by Poisson statistics taking into account the positive and negative recipients from at atleast three different doses. The frequency of competitive repopulation units (CRUs) and the number of HSCs are then calculated. However, the most stringent measure of stemness is the serial transplantation assay (Lemischka et al., 1986). It may utilize any one of the above assays. First, the test cells are transplanted into primary recipients, allowed to engraft for two months followed by harvesting of the cells from primary recipients. They are then transplanted into secondary recipients and further from secondary to tertiary hosts. Only the true primitive HSC can lead to long term multilineage repopulation.

a.



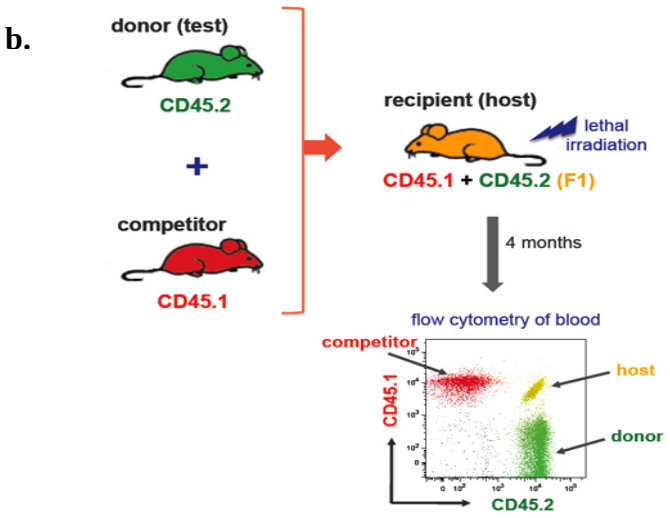


Figure 1.3: Schematics of Competitive Repopulation Transplantation Assay (Adapted from Bersenev 2011, Stem Cell Assays)
a. The use of two different host strains for the donor (CD45.2) and competitor (CD45.1) mice allows tracking of the donor and the competitor population by flow cytometry. However, the host and competitor contribution toward reconstitution cannot be determined as they belong to the same congenic strain. **b.** By use of a heterozygous host strain CD45.1/CD45.2, reconstitution ability between the donor and the competitor can be ascertained accurately by flow cytometry.

1.4 Regulation of hematopoietic stem cell homeostasis

One of the most fundamental questions of biology is how stem cells are regulated. A large number of studies have shown that multiple factors are responsible for maintaining HSC renewal. Gene manipulation studies have identified important proteins, transcription factors, cell cycle regulators and epigenetic modifiers that intricately regulate HSC renewal (intrinsic factors). The microenvironment or HSC niche (extrinsic factor) and a complex network of interacting stimuli at various levels are invariably involved in the controlling self-renewal in HSCs. Dysfunctional control of HSCs lead to its decreased and defective self renewal.

a.

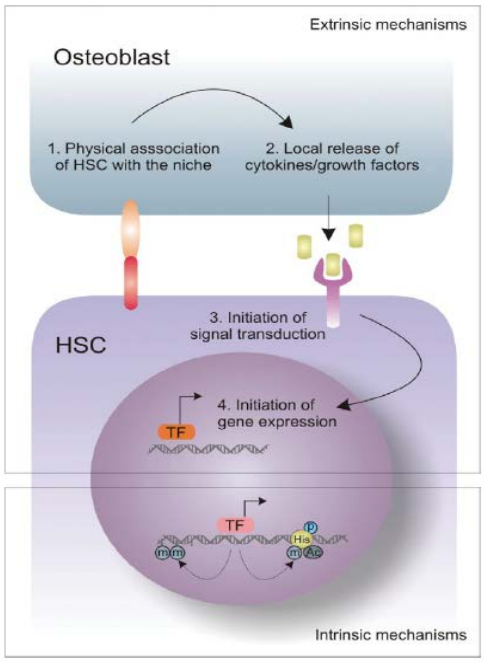


Figure 1.4a: Intrinsic and extrinsic mechanisms determining HSC regulation Intrinsic mechanisms are niche-independent and can alter or influence the epigenetic state of HSC since it is under the control of chromatin remodelers namely the transcription. Extrinsic mechanisms in HSC regulation are controlled by the environment, i.e. niche. A physical connection between the osteoblast and the HSC leads to the release of different growth factors that trigger different and parallel signal transduction pathways initiating the expression of downstream target genes (Rizo et al., 2006)

1.4.1 Intrinsic regulation by key transcription factors

A diverse range of transcription factors are involved in regulating hematopoiesis at different stages of the differentiation cascade. Many of the transcription factors identified are genes that are involved in chromosomal translocations in leukemia or are common integration sites in murine retroviral induced leukemia (Jude et al., 2008). Some of them are essential during developmental process during the emergence of HSCs (Dzierzak and Speck, 2008).

Runx1/AML1 (Runt related transcription factor 1) known to be disrupted by chromosomal translocation in leukemia produces a fusion protein that acts by a dominant interfering mechanism (Meyers and Hiebert, 1995). Conditional deletion of Runx1 in adult bone marrow results in expansion of HSCs and their progenitors by three fold (Growney et al., 2005; Ichikawa et al., 2008). Runx1 deficient HSCs, similar to Myc deficient HSCs, show reduced repopulating ability in competitive and noncompetitive transplantation settings and with progressive loss of Runx1 deficient cells in the peripheral blood of transplanted recipients.

Among the trithorax genes, the **MLL (Mixed Lineage Leukemia)** gene has been characterized extensively with respect to hematopoiesis. Gene disruption studies have identified the role of MLL in maintaining quiescence of HSCs (Jude et al., 2007; McMahon et al., 2007). Acute deletion of MLL gene disrupts quiescence and allows entry of $CD48^-$ LSK (enriched in quiescent cells) to enter the G_1 and shortly thereafter into the S phase leading to increased total number of LSKs. A recent study has highlighted a MLL transcriptional network that extends beyond its Hox targets, contains genes that are dependent and independent of the MLL cofactor, Menin. These genes include Prdm16, Mecom, Pbx1, Eya1, and Hoxa9 involved in regulating HSCs. Prdm16 exhibits the highest activity and can rescue the intrinsic proliferation defect of MLL deficient HSCs (Artinger et al., 2013).

Homeobox B4 (HoxB4), a member of the homeobox family of transcription factors was one of the first genes described to play a role in HSC fate determination. Overexpression of this gene results in strong expansion of HSCs

both *in vitro* and *in vivo* without the induction of leukemia in transplanted recipients (Antonchuk et al., 2002; Sauvageau et al., 1995). However, the loss of HoxB4 results in a mild defect in HSCs due to redundancy with other Hox proteins. Other members of Hoxa and Hoxb clusters (HoxA4, HoxA9 and HoxB6) similarly function to control adult HSC fate determination (Fischbach et al., 2005; Fournier et al., 2012; Lawrence et al., 2005; Thorsteinsdottir et al., 2002).

Gata2, a zinc finger transcription factor is important for definitive hematopoiesis. It is expressed before the emergence of HSCs and marks the hematopoietic specified cells (Minegishi et al., 1999). Gata2 function is essential for the generation of HSCs during the transition from endothelial to hematopoietic cell type and deletion of Gata2 in the Vec (vesicular endothelial cadherin) expressing endothelial cells leads to deficiency of LT-HSCs and intra-aortic cluster cells (de Pater et al., 2013). However, reduction of Gata2 expression or activity is essential for the commitment of HSCs (Minegishi et al., 2003). Gata2 has an overlapping function with Runx1 in definitive hematopoiesis as double heterozygous mice of Gata2 and Runx1 do not survive and demonstrate hematopoietic defects at midgestation whereas the single heterozygous mice are viable with no overt phenotype (Wilson et al., 2010). Studies have also shown that Gata2 is important for maintaining quiescence of human HSCs as enforced expression of Gata2 leads to longer occupancy in the G_0 phase of the cell cycle (Tipping et al., 2009).

Forkhead box O (FoxO) families of proteins participate in many physiologic processes, such as cell cycle arrest, stress resistance, differentiation, apoptosis and metabolism. Conditional knockouts of FoxO1, FoxO3 and FoxO4 in the hematopoietic system demonstrate reduction in the LT-HSCs and a functional decline in long term repopulation in both competitive and non competitive reconstitution assays (Tothova et al., 2007). FoxOs affect integrity of HSCs by regulating ROS levels. Germline loss of FoxO3a leads to increased levels of ROS in the LSK compartment which correlates with reduced levels of superoxide dismutase 2 (SOD2) and catalase expression and increased expression of p38 MAPK in $CD34^-$ LSK cells. FoxO3a KO HSCs show defective maintenance of HSC quiescence and increased sensitivity to cell cycle specific myelotoxic injury. With ageing, there is a reduction in the HSC frequencies and therefore FoxO3a plays a critical role in the maintenance of HSC pool (Miyamoto et al., 2007).

The polycomb protein **Bmi-1 (B Lymphoma Mo-MLV Insertion Region 1)** is involved in the maintenance of leukemic stem cells and HSCs (Iwama et al., 2004; Lessard and Sauvageau, 2003; Ohta et al., 2002; Park et al., 2003). Embryonal development of HSCs is normal in Bmi-1 knockout mice. Adult Bmi-1 knockout mice display progressive reduction of HSCs, engraft poorly and exhaust prematurely in serial transplantation assays leading to postnatal pancytopenia. The loss of self-renewal in Bmi-1 knockouts is due to derepression of its target genes $p16^{lnk4a}$ and $p19^{arf}$ (Park et al., 2003).

Gfi-1(Growth factor independent 1), a zinc finger repressor restricts HSC proliferation, Gfi-1 knockout HSCs proliferate faster than their wild type counterparts and display a reduction in p21 expression but decreased HSC function in transplantation experiments (Hock et al., 2004; Zeng et al., 2004).

Myc, a proto-oncogene first discovered in lymphoma and implicated in various cancers is also involved in HSC regulation. There is transient accumulation of HSCs in the absence of Myc in adult bone marrow. Perinatal deletion of Myc results in increase in of lineage negative, Sca-1 positive, c-kit negative cells displaying aberrant, senescent primitive progenitor phenotype (Baena et al., 2007). These Myc deficient HSCs are functionally defective and fail to engraft in both competitive and noncompetitive repopulation assays (Wilson et al., 2004).

The Ets domain protein **MEF/Elf4 (E74-Like Factor 4)** maintains steady state HSC homeostasis and has also been implicated in leukemia. Elf4 knockouts exhibit increase in LT-HSCs (LSK, $CD34^{-}Flt3^{-}$) and shows a phenotype similar to c-Myc knockouts. El4 knockouts recover faster than wild type mice after myeloablation and outcompete in transplantation settings. However Elf4 knockout HSCs display reduced S/G2/M cells, reduced BrdU uptake and impaired cytokine stimulation *in vitro* but show normal bone marrow cellularity (Lacorazza et al., 2006).

Another important transcription factor for maintaining HSCs in the G_0 phase of the cell cycle is the **phosphatase and tension analog (PTEN)**. Disruption of PTEN increases the entry of HSCs into S phase and finally causes exhaustion of

the HSC stem pool. PTEN inhibits the phosphatidyl inositol-3 kinase/AKT pathway during cell cycle (Zhang et al., 2006).

p53, the guardian of the genome has been to show to play critical roles in the maintenance of cell integrity under stress conditions by controlling its target genes which are involved in cell cycle arrest, DNA repair, apoptosis, senescence and metabolism. Hematopoiesis in p53 KO mice is almost normal during steady state hematopoiesis (Lotem and Sachs, 1993). p53 is preferentially expressed in the HSC compartment compared to the progenitor fractions such as CMP, GMP and MEP (Liu et al., 2009). Loss of p53 in HSC compartment leads to decreased HSC quiescence as monitored by reduction in $CD34^{-}$LSK SP (side population) cells and increased proliferation of $CD34^{-}$LSK cells. Through gene expression profiling studies, Gfi1 and Necdin were identified as p53 target genes important for maintaining HSC quiescence (Asai et al., 2012; Liu et al., 2009). HSCs lacking p53 reconstitute recipient bone marrow in a competitive repopulation assay during the first transplantation (TeKippe et al., 2003), but upon serial transplantation, p53 does not support the long term function of HSCs (Chen et al., 2008). DNA damage by ROS results in upregulation of p53 in ROS^{low} HSCs because increased p53 levels decreases the ROS intracellular levels to protect the genome from ROS induced genomic damage. It does so by upregulating several antioxidant genes in ROS^{low} HSCs and therefore maintains quiescence for the survival of HSCs (Sablina et al., 2005).

1.4.2. Developmental regulators of HSCs

The developmentally conserved pathways such as the wingless-type (Wnt), Sonic hedgehog (Shh), Notch and Fibroblast growth factor (FGF) have been known to play important functions during ontogeny but recently many studies have reported its role in regulating the adult hematopoietic stem cell compartment. They will be discussed in brief here to highlight their functions in HSC maintenance.

Wnt/ β catenin pathway: The Wingless protein and its mouse homolog Integrase-1 comprise a family of 19 soluble secreted glycoproteins that are expressed by fetal and adult HSC environment (Staal and Luis, 2010). The canonical Wnt signaling pathway which involves the immature hematopoietic cells occurs in the BM stroma, where Wnt ligand binds to its receptor complex Frizzled (Frz) and two co-receptors Lrp 5/6 activating the transcription factor β-catenin and its translocation to the nucleus brings about the activation of cyclin D1 and c-Myc. Addition of soluble Wnt proteins like Wnt3a or Wnt5a *in vitro* enhances mouse and human HSC self-renewal, survival, and proliferation measured by engraftment into lethally irradiated recipient mice during transplantation assays (Austin et al., 1997; Murdoch et al., 2003; Willert et al., 2003). In particular, the Bcl2 transgenic LSK cells expanded and maintained the immature phenotype after 7 days of ex vivo exposure to Wnt5a (Reya et al., 2003). Overexpression of the constitutive form of β-catenin resulted in expansion of the HSC pool by up regulation of Notch1 and HoxB4 thus supporting the fact that the two pathways cooperate to maintain HSC homeostasis. Similarly ectopic

expression of constitutive β-catenin in lineage committed myeloid and lymphoid progenitors have conferred multilineage potential to these cells *in vitro* (Baba et al., 2006). However, the role of Wnt signaling *in vivo* is still unclear. Deletion of β-catenin or its homolog γ catenin has little effect on HSC renewal and reconstitution during transplantation assays using the Mx-Cre conditional systems (Jeannet et al., 2008; Koch et al., 2008). Vav-Cre conditional knockouts of β-catenin show defects in HSC renewal in transplantation settings but the frequency of LSK $Flk2^-$ and downstream progenitors are not changed.

Hedgehog Pathway: The three secreted proteins of the hedgehog (Hh) family- the Sonic hedgehog (Shh), the Desert hedgehog (Dhh) and Indian hedgehog (Ihh) are major players in embryonic development. Hh ligand binds to its receptor Patched 1 (Ptch1) and releases a second surface protein Smoothened (Smo) allowing Smo mediated activation of two key Glioblastoma proteins Gli1 and Gli2 which act as positive activators of downstream target genes like Cyclin D1, Cyclin E, c-Myc and Vegf (Trowbridge et al., 2006). Shh has been shown to activate human $CD34^+$ cells proliferation via bone morphogenic protein (BMP) signaling and Ihh expression on human stromal cells increases colony formation and engraftment function of human $CD34^+$ progenitors (Bhardwaj et al., 2001; Kobune et al., 2004). However, contradictory findings from conditionally deleted Vav-Cre Smo knockout mice showed no global defects in lineage cell production but only impaired colony formation and reduced reconstitution in serial replating and serial transplantation assays respectively (Zhao et al., 2009). Interestingly, Gli-1 knockout mice show increased numbers of LSK $CD34^-$ cells and enhanced

reconstitution when compared to its wild type counterparts *in vivo* transplantation experiments (Merchant et al., 2010). Thus Gli-1 possesses antagonistic function in mediating Smo signaling for HSC renewal and maintenance (Warr et al., 2011). Likewise, using the Mx-Cre system, Smo deleted cells have no apparent phenotype during steady state hematopoiesis or upon 5-FU treatment thereby providing contradictory conclusions for the role of Hh in maintenance of HSC (Gao et al., 2009; Hofmann et al., 2009).

Notch Pathway: The notch pathway genes consists of four notch members (1-4), which interact with 5 ligands, Jagged 1 and 2, and Delta 1,3 and 4. Upon binding to its ligand, the intracellular domain of notch (NICD) is cleaved by the γ secretase complex which then translocates to the nucleus and interacts with other coactivator proteins such as Core binding factor 1 (CBF-1) and Mastermind like (Maml) to bring about transcription of key target genes like Notch itself, Hey1, Hes1, and Hes5 (Bugeon et al., 2011; Schwanbeck et al., 2008; Weber and Calvi, 2010). There is defective embryonic HSC development in Notch-1, Jagged-1 or CBF-1 knockout mice (Kumano et al., 2003; Robert-Moreno et al., 2005; Robert-Moreno et al., 2008). The bone marrow niche, which consists of osteoblasts and endothelial cells, expresses Jagged and Delta ligands and contribute to signaling between the immature hematopoietic compartment and essential components of the BM niche leading to expansion of the wild type (WT) LSK cells but not Notch 1/2 deficient LSK cells (Butler et al., 2010). Retroviral expression of activated Notch in $Lin^{-}Sca1^{+}$ cells leads to expansion of LSK cells population *in-vivo* through competitive transplantation assays while concurrently favoring

differentiation towards the lymphoid lineage and reduced differentiation towards the myeloid lineage (Stier et al., 2002). Conditional deletion of Jagged-1 or Notch-1 in the bone marrow compartment or stromal cells driven by Mx-Cre showed no apparent phenotype at steady state, transplantation and 5-FU treatment conditions (Mancini et al., 2005). Therefore, there is much debate on the role of Notch on HSC maintenance by limiting cell proliferation and differentiation due to redundancy and compensatory roles by other Notch ligands and their receptors but it maybe only important during certain stress conditions.

TGF-β Pathway: The transforming growth factor (TGF) superfamily- TGF-β, activin and bone morphogenic protein (BMP) have diverse functions both during embryogenesis and in adult organisms. TGF-β type 1 receptor knockout mice do not have any hematopoietic defects during steady state as well as under stress conditions (Larsson et al., 2003). However, *in vitro*, TGF-β inhibits HSC proliferation and expansion and causes cell cycle arrest by the induction of cyclin dependent kinase inhibitors p21 and p57 (Cheng et al., 2000a; Cheng et al., 2000b). But *in vivo* deletion of Smad4 in HSCs results in decreased repopulation activity by transplantation into recipient mice demonstrating that Smad4 has a crucial role in regulation of HSCs (Karlsson et al., 2007). Ectopic expression of Smad7 in HSCs leads to disrupted Smad signaling and TGF-β responsiveness *in vitro* (Blank et al., 2006).

FGF signalling: Murine HSCs express high levels of FGF receptors and their role in HSC fate regulation has been recently investigated. FGF-1 and FGF-2 when

added to cytokine and serum-free cultures have been reported to maintain/expand multilineage, serially transplantable, long-term repopulating HSCs of unfractionated mouse BM (de Haan et al., 2003; Yeoh et al., 2006). However, addition of standard cytokines to these *in vitro* cultures and stimulation of proliferation dominated FGF-dependent stem cell preservation. In addition, FGFs appear to have indirect effects as they can only support the maintenance of an undifferentiated state of purified murine HSC in culture when active stromal elements are present.

1.4.3 Cell cycle regulation of HSCs

The cell cycle machinery plays an important function maintaining the balance between proliferation and quiescence within HSCs to prevent the exhaustion of the stem cell pool. The four phases of the cell cycle G_1 (gap 1), S (synthesis), G_2 (gap 2) and M (mitosis) are required for cell division and proliferation. Cells sometime temporarily stop dividing and exit the cell cycle through the G_1 phase to become non dividing and quiescent. This state is referred to as the G_0 phase. Upon certain external stimulation or cues, G_0 cells re-enter the cell cycle for further proliferation. Positive regulators stimulate cell cycle progression by inhibiting cell cycle exit or by enhancing cell cycle reentry. Negative regulators slow down cell cycle progression by accelerating cell cycle exit and inhibiting cell cycle reentry (Wang et al., 2010). There is insufficient data to support if these regulators act at the G_0/G_1 transition or on the cell cycle directly. There is a distinct cell cycle activity in fetal, adult and aged HSCs. During fetal life, 95-100% of HSCs actively cycle in the fetal liver with a cell cycle transit time of 10-14 hours

(Bowie et al., 2006). In adulthood only 5% of the HSCs are actively cycling in the BM with 95% reaching quiescence by 4 weeks of age (Kiel et al., 2007). Once in the cell cycle, fetal and adult HSC transit at the same slow rates when compared to it more differentiated progenitors due to an extended passage through the G_1 phase of the cell cycle (Nygren et al., 2006). HSCs occur in a hierarchical organization on the basis of quiescence with the most primitive stem cells existing in the G_0 phase and possessing the highest stem cell activity. 5-bromo-2-deoxyuridine (BrdU) labeling of dividing hematopoietic cells *in vivo* in normal adult mice has shown that 50% of all LT-HSCs divide every 6 days. At any given time, 75% of LT-HSCs are quiescent and after 57 days 99% of all LT-HSCs enter the cell cycle (Cheshier et al., 1999). Through label retention studies using BrdU and histone H2B-GFB, a highly dormant HSC (d-HSC) population within the Lin^- $Sca1^+cKit^+CD150^+CD48^-CD34^-$ have been identified that possesses stem cell activity validated by serial transplantation assays. These d-HSCs divide every 145 days or five times per lifetime. But these d-HSCs are rapidly and efficiently activated by G-CSF stimulation or in case of BM injury. These activated HSCs return back to dormancy once the injury is repaired (Wilson et al., 2008).

The function of cyclins, cyclin dependent kinases (CDKs) and members of the retinoblastoma (Rb) family have been well documented with respect to differential expression within the hematopoietic system (Passegue et al., 2005). In eukaryotic cells, the cyclin-CDK complexes drive the progression of cell cycle. The CDK inhibitors of the KIP family (p21, p27 and p57) inhibit CDK2 complexes whereas those of INK family (p15, p16 and p18) inhibit CDK4/6.

Since the CDK4/6 complex is active at the G1/S boundary and the CDK2 complex during the S phase, the loss of inhibition by CDK inhibitor differentially affects the cells especially at G_0 phase (HSCs) when compared to rapidly cycling cells (progenitors). p21 knockout mice show increased HSC proliferation and increased absolute number of HSCs under homeostatic conditions. Premature death occurs in these knockouts on exposure of the animals to myelotoxic injury due to HSC depletion. The self-renewal of HSCs from these mice is impaired in serially transplanted bone marrow. Therefore, p21 acts as a molecular switch controlling the entry of HSCs into the cell cycle and its loss leads to increased cell cycling and stem cell exhaustion (Cheng et al., 2000b). Interestingly, deletion of p18, an early G_1 phase inhibitor leads to increased number of HSCs with self-renewal potential (Yuan et al., 2004). The loss of p18 is epistatic to p21 loss and the double mutant shows no stem cell exhaustion and HSCs proliferate better than p21 null HSCs (Yu et al., 2006a). In contrast, p27 knockouts exhibit normal HSC numbers and proliferation but outcompete wild type cells during transplantation. p57 is expressed predominantly in quiescent HSCs and conditional deletion of p57 in the hematopoietic compartment leads to loss of quiescence in HSCs in the G_0 phase with severe defect in self-renewal capacity and reconstitution ability after transplantation. Double knockouts of p21 and p57 displayed a severe phenotype of HSC loss and knockin of p27 at the p57 locus rescued the defects of p57 deficient HSCs which suggests a functional overlap between p57 and either p21 or p27 (Matsumoto et al., 2011). It has also been reported that quiescence of HSCs by TGF-β and Thrombopoietin (THPO) is mediated by p57 upregulation

(Qian et al., 2007; Scandura et al., 2004). p16 is highly expressed in LT-HSCs and is a downstream target of Bmi-1 and plays a role in HSC senescence and is upregulated during ageing (Lessard and Sauvageau, 2003; Passegue et al., 2005). The HSCs of ageing p16 knockouts function better in functional assays suggesting p16 may negatively regulate HSC numbers during ageing (Janzen et al., 2006).

1.4.4 Critical signaling molecules

One of the first important growth factor-receptor interactions involved in hematopoiesis is **stem cell factor (SCF) or steel factor (SF**), a ligand for **c-Kit**, a transmembrane tyrosine kinase signaling molecule whose functions have been documented in late fetal and adult hematopoiesis. The dominant white spotting in mouse reported in the early 1900s was found to be encoded by c-Kit (Chabot et al., 1988; Geissler et al., 1988). The two different splice forms of c-Kit are transmembrane bound and the soluble form SCF. Stimulation of the transmembrane form lacking a proteolytic cleavage site leads to sustained activation of the c-Kit receptor whereas the soluble form brings about rapid and transient activation of the same receptor. The mechanistic and functional differences between the two isoforms suggest signaling differences where the membrane bound SCF leads to prolonged activation of Erk1/2 and p38 mitogen activated protein kinase (MAPK) in comparison to the soluble form. The spontaneous mouse mutant harboring the Sl^d allele and encoding the secreted SCF is involved in hematopoiesis only after bone marrow injury. The engineered mice that lacked the protease site showed delayed hematopoietic recovery and

increased mortality after sublethal radiation (Tajima et al., 1998). Sl mutants devoid of both forms of SCF exhibit reduced steady state hematopoiesis which demonstrated that only the membrane bound form is sufficient for maintaining HSC homeostasis. Signaling through the c-Kit receptor is essential for erythropoiesis, lymphopoiesis, megakaryopoiesis, melanogenesis, gametogenesis and melanogenesis in mice (Lyman and Jacobsen, 1998; Ronnstrand, 2004). c-Kit is highly expressed in HSCs where it acts as a survival factor and acts in synergy with other cytokines to augment HSC proliferation (de Sauvage et al., 1996). Studies have shown that c-Kit plays a redundant role in steady state hematopoiesis but functions as an important signaling molecule for HSC self-renewal and/or regulation of committed progenitors after transplantation. Humans with c-Kit deficiency do not have any hematopoietic defects which suggest its redundancy in function when compared to mouse hematopoiesis (Lyman and Jacobsen, 1998).

Fetal liver kinase (Flk2 or Flt3) is a type III class receptor similar in biological functions to c-Kit *in vitro*. Flt-3 has a transmembrane ligand and the soluble form of the ligand is generated through alternative splicing (Christensen and Weissman, 2001). Recent studies have reported the surface expression of Flt3 is not observed in LT-HSCs and high expression of Flt3 is infact associated with loss of stem cell renewal (Adolfsson et al., 2001). The surface expression of Flt3 is important in the LSK population which is actively cycling and provides short term multipotent engraftment activity. The $Flt3^-$ population is dominated by the quiescent LT-HSCs (Christensen and Weissman, 2001; Passegue et al., 2005). Flt3 may play a redundant role as Flt3 null mice have normal levels of HSC

(Sitnicka et al., 2002). The differentiation of dendritic cells and lymphocytes is also brought about by Flt3 signaling (Lyman and Jacobsen, 1998).

Thrombopoietin (THPO) is a cytokine that was initially identified to maintain exclusive regulation of megakaryocytic and platelet production (de Sauvage et al., 1996; Kaushansky, 2005). But over a period of years many groups have identified unique roles of THPO during developmental hematopoiesis and maintenance of HSCs (Ku et al., 1996; Petit-Cocault et al., 2007). Indirect studies have suggested that mice deficient in THPO or its receptor c-Mpl have reduced numbers and/or functions of HSCs (Fox et al., 2002; Kimura et al., 1998). THPO regulation of HSCs has also been implicated due to high expression of its receptor c-Mpl in HSCs and THPO responsiveness of highly purified HSCs (Solar et al., 1998). When present with other cytokines, THPO supports the expansion of colony forming units, competitive repopulation units and cell accumulation *in vitro*. For example, in the presence of THPO alone, the LSK cells proliferate very slowly or not at all but by the addition of another cytokine SCF, the clonal efficiency and cell accumulation increases *in vitro* as THPO functions by increasing the number of cells and reducing the time delay to enter the cell cycle (Ku et al., 1996; Sitnicka et al., 2002). However some reports also suggest that THPO is not effective in maintaining HSC potential under defined conditions *in vitro* (Uchida et al., 2003). Thus THPO has opposing functions on LT-HSC when compared to its differentiating progeny. Patients with loss of functional mutations in c-Mpl receptor and congenital amegakaryocytic thrombocytopenia (CAMT) exhibit a

multilineage deficiency and aplastic anaemia within 2 years of birth resembling a stem cell deficiency (Ballmaier et al., 2003).

b.

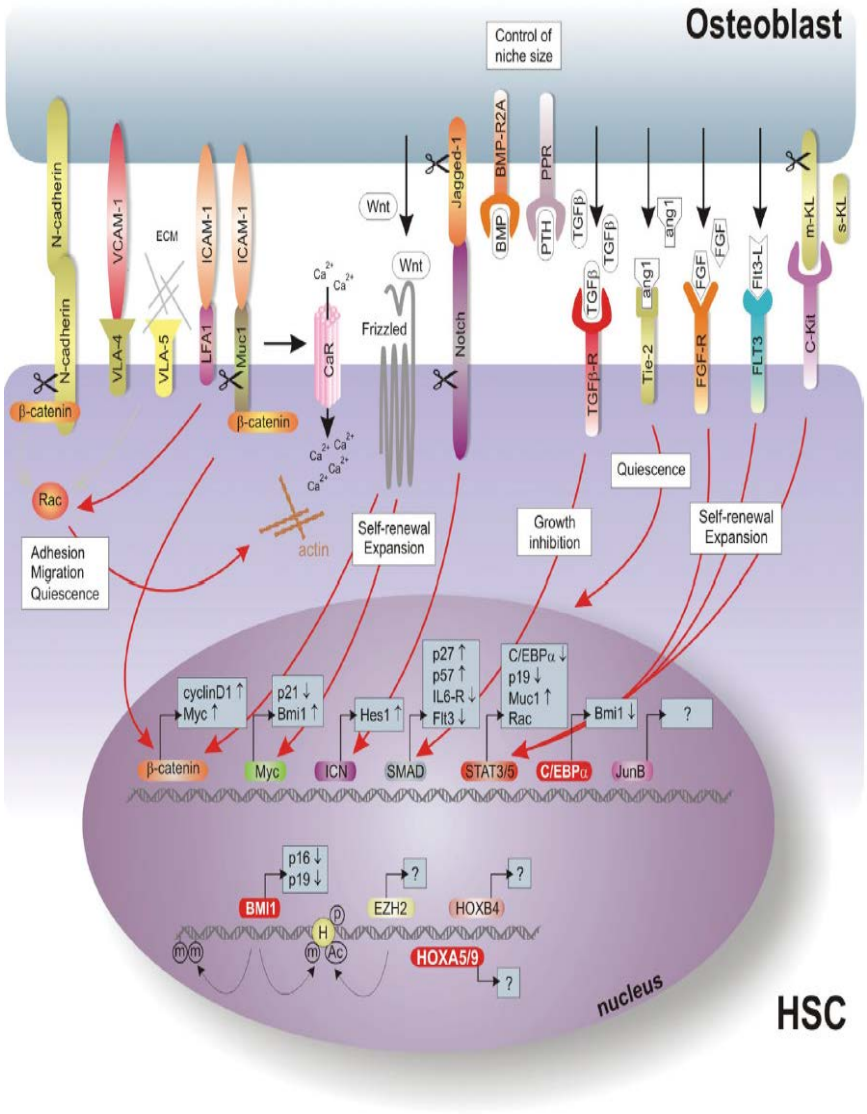


Figure 1.4.b: Summary of the extrinsic and intrinsic mechanisms of hematopoietic stem cell (HSC) regulation

Convergence of signal transduction pathways and involvement of different transcription factors, developmental genes, epigenetic modifiers and critical signaling molecules (Rizo et al., 2006).

1.5 The ageing hematopoietic stem cell compartment

Stem cells are essential for maintaining homeostasis of mature functional tissues and organs. The unique ability of a stem cell is to self renew and replenish damaged and dying cells throughout an organ's lifetime and hence stem cells are thought to be immortal and free from ageing. However, this is not true as there is an age specific functional decline of stem cells which leads to defective ability to repair tissues and increased susceptibility to cancer and other age related diseases. Gatekeeping tumor suppressor genes like $p16^{lnk4a}$, $p19^{Arf}$ and p53 negatively regulate cellular proliferation and survival (Kinzler and Vogelstein, 1997; Signer and Morrison, 2013). They negatively regulate stem cell function ageing as their expression and function increases with age in multiple tissues (Janzen et al., 2006; Krishnamurthy et al., 2006; Krishnamurthy et al., 2004). In many stem cells, the expression of let-7 micro RNA increases with age. Let-7 upregulation inhibits the expression of high mobility group transcriptional regulator, Hmga2, a proto-oncogene expressed in stem cells in old mice and a target of let-7 (Nishino et al., 2008). This loss of function of Hmag2 in adult stem cells like neural stem cells increases the expression of $p16^{lnk4a}$ and $p19^{Arf}$ reducing the frequency and self renewal potential of the stem cells. Ageing of the hematopoietic system not only causes tissue attrition but also increased incidence of many hematologic malignancies including myeloid and lymphoid leukemia (Henry et al., 2011). Ageing is accompanied by an overall reduction in hematopoietic competence despite the enormous proliferative and regenerative capacity of HSCs. Immunosenescence which refers to global changes or alterations that have an

effect on the immune system are among the factors associated with ageing. Increase in ageing and associated changes in the immune system predispose the elderly to infectious diseases, autoimmunity, vaccine failure, anemia and leukemia. Many reports have demonstrated that ageing of the innate and adaptive immune system originates at the top of the hematopoietic hierarchy i.e. dysfunction of HSCs. For example, B cell lymphopoiesis is impaired during ageing as there is a decreased regeneration of B cell precursors linked to defective function of aged HSCs (Kuranda et al., 2011; Miller and Allman, 2005). The old B cells produce antibodies with less affinity and diversity. In contrast, there is an increase in the numbers of myeloid precursors in the myeloid compartment but there is a decrease in phagocytic ability and oxidative burst in neutrophils and macrophages (Plowden et al., 2004).

1.5.1 Phenotype of aged HSCs

1.5.1.1 Increased HSC frequency and numbers but reduced regenerative potential

HSCs have been defined by a specific set of cell surface markers in both mice and humans. With ageing, the numbers of these defined HSCs increase two to ten fold in humans and mice (Table I). However, under stress conditions and regeneration (serial transplantation assays), these aged HSCs are impaired in function and demonstrate reduced regenerative potential. The increase in HSC numbers cannot compensate for the loss of function and as a result it leads to diminished regenerative capacity of the aged HSC pool (Sudo et al., 2000). The increase in HSC numbers has been proposed to be a compensatory mechanism during ageing

to overcome its decreased regenerative function. Other mechanisms propose symmetric cell divisions for increased frequency of aged HSCs and impaired function. Similarly, age specific changes in expression, response to extrinsic cues and secretion of niche factors may also contribute to increased HSC numbers (Mercier et al., 2012; Wagner et al., 2008).

HSC population (mouse)	**Changes upon ageing**
$LIN^-THY1^-SCA1^+$	Unknown
$LIN^-SCA1^+ KIT^+CD34^-$	Unknown
Side population (high Hoechst efflux) $LIN^- SCA1^+ KIT^+$	Increase of $LIN^-SCA1^+KIT^+$ cells with high Hoechst efflux activity (myeloid-biased)
$LIN^-SCA1^+KIT^+CD34^-$ cells with the highest Hoechst efflux activity	Unknown
$LIN^-SCA1^+KIT^+CD34^- FLK2^-$	Increased frequency of $LIN^-SCA1^+KIT^+CD34^-FLK2^-$ (LT-HSCs) and decreased frequency of $LIN^- SCA1^+KIT^+CD34^+FLK2^+$cells (lymphoid committed progenitors
$CD244^-CD48^-CD150^+$	Unknown
$LIN^-SCA1^+KIT^+CD34^- FLK2^-CD48^-CD41^-CD150^+$	Increased frequency of $LIN^-SCA1^+KIT^+CD34^-FLK2^- CD48^-CD41^-CD150^{hi}$ cells (myeloid-biased LT-HSCs)
$CD45^+EPCR^+CD48^-CD150^+$	Unknown
$LIN^-SCA1^+KIT^+CD48^- CD150^+EPCR^+CD34^-$	Increased frequency of $LIN^-SCA1^+KIT^+CD48^- CD150^+EPCR^+CD34^-$ cells

Table I. Ageing associated changes in mouse HSCs [Adapted from (Geiger et al., 2013)].

1.5.1.2 Skewed differentiation potential

The most distinct hallmark of an ageing hematopoietic system is the expansion of the myeloid compartment. Other features include anaemia and decreased fitness of the adaptive immune system. There is skewing of hematopoietic stem cell

differentiation with increased outputs of the myeloid lineage but compromised quality of myeloid cells and decreased output of the lymphoid and erythroid lineage when compared to young HSCs (Beerman et al., 2010). Lineage specification in aged HSCs is also disrupted with decreased numbers of CLP cells and increased numbers of CMP cells in comparison with young mice (Rossi et al., 2005). This skewing effect from early lymphoid to myeloid lineage may be caused by changes in HSC differentiation and the proliferative ability and survival of CMP. Another explanation is the functional heterogeneity of the long term HSCs in terms of differentiation potential, some HSCs have low tendency to differentiate into lymphoid cells and consequently are myeloid biased. Some HSCs show a reverse trend and hence are lymphoid biased whereas others are balanced HSCs with a balanced equal output of myeloid and lymphoid cells (Benz et al., 2012; Dykstra et al., 2007; Muller-Sieburg et al., 2002). Few reports suggest that the composition of the HSC pool changes with ageing with no alterations in its differentiation potential. The number of myeloid biased HSC clones increases in comparison with lymphoid biased or balanced clones upon ageing (Morita et al., 2010; Muller-Sieburg et al., 2004). As a consequence all HSC subtypes age by a combination cell intrinsic and extrinsic mechanisms that result in lineage skewing.

1.5.1.3 Differences in homing and mobilization to and from the bone marrow

The HSC niche plays an important role in maintaining HSC quiescence, self renewal, differentiation and mobilization. Young HSCs home to the bone marrow and restrict themselves in close proximity to the endosteum whereas aged HSCs

localize themselves away from the endosteal stem cell niche after transplantation (Kohler et al., 2009). Mobilization, a phenomenon for recruitment of HSCs to the peripheral blood through the use of stimulatory signals like cytokines and chemotherapy is also enhanced in aged HSC when compared to young HSCs (Geiger et al., 2007; Liang et al., 2005; Xing et al., 2006). These data demonstrate the differences in the adhesive properties of aged HSCs and their interaction with the niche cells in the aged bone marrow. However, a better understanding of the crosstalk between aged HSCs and the niche cells and the expression of adhesion molecules *in vivo* is still lacking due to difficulties in experimentally determining the complex interactions *in vivo*.

1.6 Mechanisms involved in HSC ageing

1.6.1 Cell intrinsic mechanisms

Genome wide expression studies comparing the HSCs from young and aged mice may not only identify novel biomarkers of stem cell ageing but also provide useful mechanisms of ageing in HSCs and other stem cells. Transcriptome profiling between young and aged HSCs have identified a general downregulation of lymphoid genes and an upregulation of myeloid genes in aged HSCs. For example; the expression of Runx1 is upregulated in aged HSCs and one of the factors responsible for skewing of HSCs towards the myeloid lineage (Rossi et al., 2005). Furthermore, genes associated with proinflammatory state like nuclear factor κB (NF-κB), Interleukin 6 (IL-6), Tumor Necrosis Factor alpha (TNF-α) and Interleukin 1β (IL1-β) are also upregulated in aged HSCs along with molecules involved in cell to cell interactions such as P selectin and intercellular

adhesion molecule 1(ICAM-1) (Franceschi et al., 2000) (Figure1.6a). Consistently, expression of genes related with protein folding like heat shock protein 8 (hspa8) and HSP40 are highly expressed in aged HSCs which shows that protein integrity maybe deregulated in aged HSCs similar to increase protein damage and misfolding in aged fibroblasts (Baraibar and Friguet, 2013).

1.6.1.1 Oxidative damage, mitochondrial DNA mutations and metabolism

Oxidative damage, which is primarily carried out by the production of reactive oxygen species (ROS) causes a decline in mitochondrial function by affecting mitochondrial DNA (mtDNA) replication and transcription. A feedback loop is created which results in increased ROS production and a cumulative damage to the mitochondria which leads to telomere attrition and replicative senescence and ageing as observed in differentiated fibroblasts. Transgenic mice with increased mtDNA mutations due to deficiency of mtDNA polymerase catalytic subunit gamma (POLG) develop anemia and lymphopenia, which demonstrates that mtDNA damage, mutations, ROS and hematopoiesis are coupled. HSCs with elevated ROS production demonstrate increased activation of mammalian target of rapamycin (mTOR) and p38 mitogen activated protein kinase (MAPK) that leads to exhaustion of HSCs during serial transplantation assays *in vivo* (Jang and Sharkis, 2007). Similarly, mice lacking the Forkhead box O (FOXO) family of transcription factors have higher production of ROS in HSCs when compared to wild type control mice with short term HSC hyper proliferation but increased HSC apoptosis (Miyamoto et al., 2007; Tothova et al., 2007). Contrary to this, there have been reports that suggest that the levels of ROS production and levels

are not elevated in physiologically aged HSCs when compared to POLG knockout mice. mtDNA mutations may not directly impact HSC function although mitochondrial function maybe required for multilineage stem cell differentiation (Norddahl et al., 2011). This warrants further study to directly link mtDNA mutations and oxidative stress with HSC ageing (Figure 1.6a).

1.6.1.2 Loss of cell polarity and connection with CDC42

Aged HSCs have changes in cell adhesion molecule expression and therefore are linked with changes in cell polarity. Gene expression studies have shown that there is increased expression of Cell division cycle 42 (CDC42), a small RHO GTPase cell division control protein. It is known to be expressed in mononuclear hematopoietic cells and has been strongly associated with ageing and morbidity in humans (Kerber et al., 2009). CDC42 is turned on to a GTP bound state from a GDP bound state in the presence of various HSC stimuli like growth factors, cytokines, extracellular matrix (ECM) proteins etc. Recent data suggests that as HSC age there is substantial increase in the expression of CDC42 which causes disruption of cell polarity in aged HSCs and random distribution of tubulin, other planar cell polarity markers and indiscriminate nuclear distribution of acetylated H4K16 (epigenetic polarity) (Florian et al., 2012) (Figure 1.6a).

1.6.1.3 DNA damage and telomere attrition

Cells are confronted with multiple insults to their DNA every day. As discussed earlier, ROS a metabolic and natural by product can cause oxidative damage in cells. Other forms of DNA damaging agents include ultra violet radiation and the natural error rate upon DNA replication. Moreover, thousands of lesions arise in the genome of each cell on a daily basis due to constant depurination and hydrolysis of DNA (Lindahl, 1993). Thus the DNA repair machinery plays an enormous importance in ensuring that the integrity of the DNA content is intact and free from errors. Therefore, it is imperative that HSCs are equipped with robust repair mechanisms and response to prevent its progeny from inheriting error prone genomic alterations. As majority of HSCs reside in G_0 phase of the cell cycle they are less prone to replication errors (Cheshier et al., 1999). In addition, HSCs produce low levels of ROS compared to its progenitors and are capable of fluxing out toxic compounds (Goodell et al., 1996; Tothova et al., 2007). HSCs are resistant to irradiation and formation of double strand breaks whereas the myeloid progenitors undergo apoptotic cell death (Mohrin et al., 2010). As HSCs are quiescent, they employ an error prone repair mechanism or non homologous end joining whereas the progenitors use the high fidelity homologous recombination (HR) repair pathway. Thus HSCs are somewhat protected from DNA damage as they are quiescent but utilize a suboptimal and error prone DNA repair pathway which presents a scenario of DNA damage accumulation during ageing. Many studies have reported that it is the accumulation of DNA lesions and mutation which are responsible for age related

phenotypes in different stem cells (Park and Gerson, 2005). In this regard, HSCs are in no way different from other stem cells. Both mouse and human HSCs harbor increased levels of DNA damage highlighted with increased H2AX foci formation in the nucleus indicative of double stranded breaks (Rossi et al., 2007b; Rube et al., 2011; Yahata et al., 2011). Several transgenic mice show defective HSC function and DNA maintenance. Transplantation of HSCs with compromised DNA integrity has shown an age related decline in HSC function leading to reduced self renewal, repopulation capacity and increased apoptosis (Navarro et al., 2006; Nijnik et al., 2007). DNA damage trigger various signaling molecules leading to various cell cycle check point activation, apoptosis and differentiation. Major molecules include the tumor suppressor p53, cyclin dependent kinase inhibitor 2A (CDKN2A or $p16^{INK4A}$), anti apoptotic members of the family BCL2 and B cell activating transcription factor (BATF) which enhances lymphoid differentiation of HSCs in response to gamma radiation (Mandal and Rossi, 2012; Wang et al., 2012a). The importance of p53 in HSC ageing is still controversial. On the other hand $p16^{INK4A}$ expression is high in aged HSCs and its absence in aged HSCs modestly rescues the compromised numbers and defective self renewal capacities during transplantation settings. Other studies have reported no role of $p16^{INK4A}$ during ageing of HSCs under steady sate conditions (Attema et al., 2009). Only a handful of genes are differentially expressed in young and aged HSCs function in DNA repair and stress response, some of which include genes like Bloom syndrome protein (Blm), XPA binding protein 2 (Xab2) and X ray repair cross completing protein 1

(Xrcc1). Contrary, knockouts of mice for xeroderma pigmentosum group D complementing protein (Xpd) and mRNA transport regulator 3 (Mtr3) do not exhibit any function HSC defects during ageing under steady homeostatic conditions (Rossi et al., 2007a). These observations reinforce that the decreased regenerative potential of aged HSCs results not only from accumulating DNA damage during ageing but is also linked to telomere shortening.

Telomeres are regions of repetitive DNA sequences that characterize the ends of chromosomes and prevent DNA damage response and DNA repair pathway activation. Telomeres are maintained by the enzyme telomerase that prevents them from shortening after each round of DNA replication. Shortening of telomeres gives rise to unprotected ends of chromosomes which are recognized as chromosome breaks or DNA damage. Telomeres in humans shorten with age. In murine studies, telomere shortening occurs in HSCs during serial transplantation assays and HSCs which lack telomerase have reduced serial transplantation potential (Allsopp et al., 2003b). In addition, third generation of mice lacking telomerase enzyme show premature ageing of HSCs as well as other type of stem cells (Choudhury et al., 2007). Murine HSCs express low levels of telomerase and ectopic expression of telomerase maintains telomere length but does not increase the self renewal capacities of HSCs in transplantation assays (Allsopp et al., 2003a). Mice with telomere dysfunction display impaired mitochondrial biogenesis and function, decreased gluconeogenesis and increased productions of ROS levels as discussed earlier. Treatment with antioxidant does not rescue the transplantation defects of telomere disrupted HSCs (Sahin et al., 2011).

1.6.1.4 Epigenetic regulation during ageing

The role of epigenetic stability in stem cell ageing has recently come into highlight. The transmission of epigenetic information to daughter cells ensures proper self renewal and function of HSCs. Epigenetic marks that maintain HSC decline and become altered with ageing with changes in DNA methylation signatures due to failure of DNA methyltransferases, histone methyl transferases and acetyl transferases to copy HSC specific DNA and histone modification to daughter strands (Beerman et al., 2013; Bocker et al., 2011; Rando and Chang, 2012). Downregulation of genes involved in chromatin remodeling and chromatin dependent transcriptional silencing are observed in aged HSCs. These genes include SWI/SNF complex such as Smarca4 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4), Smarcb1, histone deacetylase 1 (Hdac1), Hdac2 and Hdac6. Other genes like sirtuin 2 (Sirt2), Sirt3, and Sirt7 are also downregulated upon ageing in HSCs (Chambers et al., 2007). Similarly the level of acetylation of histone H4 on lysine 16 (H4K16) is reduced in aged HSCs and may be one of the important factors contributing to the ageing process (Florian et al., 2012). Epigenetic modifications by the polycomb group of proteins are best studied during blood development. The combined functions of polycomb repressive complexes PRC1 and PRC2 are involved in replication of histone modifications and bring about chromatin compaction and gene repression. Polycomb mediated gene repression is through the formations of H3K27me3 (trimethylated H3K27) by histone methyltransferase enhancer of zeste homolog 2 (EZH2). The chromobox protein homolog (CBX) in BMI-1 containing PRC1

recognizes this H3K27me3. Aberrant expression of BMI1, MEL18, RING1, EZH2 and other members of PRC1 and PRC2 complex are involved in deregulated function of blood formation in mice (Klauke and de Haan, 2011). Of these genes, Bmi-1 is important as it binds to genomic regions marked by H3K27me3 (repressive) and H3K4me3 (active) in the hematopoietic progenitors which indicate that these genes are transcriptionally inclined to be expressed during differentiation (Bernstein et al., 2006; Oguro et al., 2010). Deregulation of Bmi-1 gene results in the aberrant expression of the splice variants of the INK4a locus $p16^{INK4a}$ and $p19^{Arf}$ that inhibit cell proliferation and cause apoptosis. Coincidently, derepression of B cell lineage genes have been reported in Bmi-1 knockout HSCs which leads to reduction of H3K27me3 and increase in B cell generation and thus Bmi-1 has been considered as one of the genes regulating lineage specification in HSCs during ageing (Pollina and Brunet, 2011). With the exception of H4K16 acetylation and DNA methylation large scale screening, there is limited data on the genome wide analysis of other epigenetic modification in young and aged HSCs. If epigenetic changes have a key role in ageing of HSCs, intervention of pharmacologic drugs that alter epigenetic modification could be used therapeutically to reverse the effects of HSC ageing such as valproic acid and decitabine which are already in clinical use (Pollina and Brunet, 2011; Rando and Chang, 2012).

1.6.1.5 Switch from canonical to non canonical Wnt signaling

Expression of multiple members of Wnt signaling in both hematopoietic cells as well as in non-hematopoietic stromal cells has been documented (Malhotra and Kincade, 2009). The canonical Wnt protein Wnt3a and the non canonical Wnt protein Wnt5a have been reported to play important roles in hematopoiesis (Luis et al., 2011; Nemeth et al., 2007). There is an increase in the expression of Wnt5a in middle aged (10 months) and aged (20-24 months) LT-HSCs and Lin^- cells from C57BL/6 and DBA/2 mice but absent in young (2-3 months) LT-HSCs (Florian et al., 2013). Aged LT-HSCs present lower levels of β-catenin and are cytoplasmic in localization when compared to young LT-HSCs which are predominantly nuclear. Reduced transcript levels Axin2, a downstream target of canonical Wnt pathway was reported in aged LT-HSCs (Brack et al., 2007). Young LT-HSCs treated with Wnt5a causes ageing associated stem cell apolarity, features of ageing myeloid-lymphoid differentiation skewing through the activation of Rho GTPase Cdc42 and reduction in regenerative potential. This shift from canonical to non canonical Wnt signaling during the ageing of LT-HSCs initiated by Wnt5a is cell intrinsic but how the expression of Wnt5a in ageing LT-HSCs is elevated is not known but may involve epigenetic mechanisms.

a.

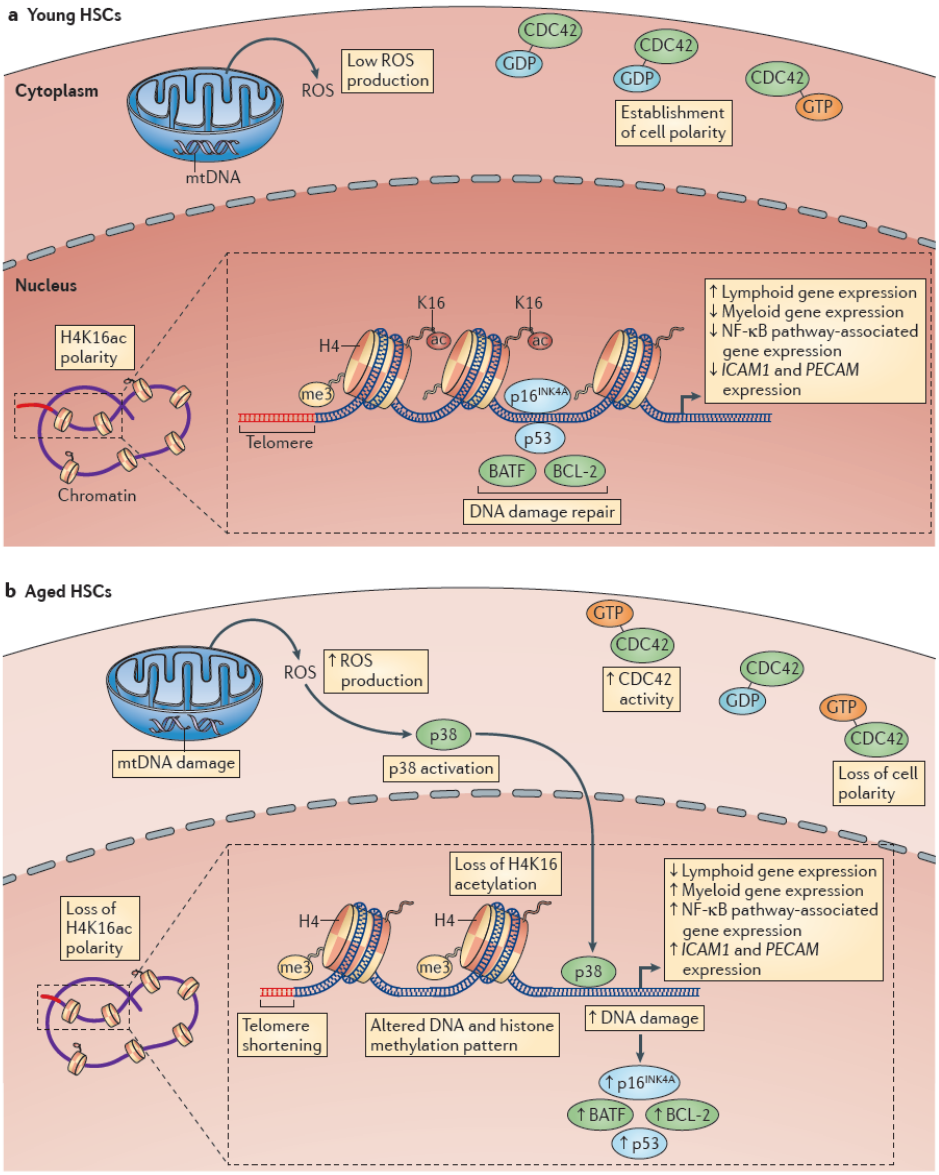


Figure 1.6a: Cell intrinsic mechanism of HSC ageing

Young HSCs generally produce low ROS and establish cell polarity by Cdc42. Along with telomere maintenance, a robust DNA repair machinery exists in young HSCs. Aged HSCs have higher Cdc42 activity which causes loss of H4K16ac in the nucleus depolarization of planar cell polarity markers in the cytoplasm. Higher ROS production, telomere shortening and high DNA damage lead to activation of p16, BCl2, VATF and p53 leading to senescence, apoptosis or differentiation. Changes in epigenetic marks such as H4K16ac lead to reduced genome wide level of acetylation upon ageing [(Geiger et al., 2013); Nat Rev Immunol. 2013 May; 13(5):376-89)].

1.6.2 Cell extrinsic mechanisms

1.6.2.1 The importance of the niche in HSC ageing

The hematopoietic stem cell (HSC) niche undergoes several changes with ageing. Osteogenesis is reduced with a higher production of ROS. There is increase in the number of adipocytes which is a result of the skewed differentiation of aged mesenchymal stem cells and changes in the extracellular matrix (ECM) composition of proteins (Bellantuono et al., 2009; Freemont and Hoyland, 2007). The HSC niche consists of a vast array of locally secreted factors like activators of notch signaling, WNT ligands, stem cell factor (SCF) and TPO which have been discussed earlier and their contribution towards HSC homeostasis is significant (Lo Celso and Scadden, 2011). The skewing of aged HSC towards a myeloid lineage is also through the secretion of increased levels of the pro-inflammatory CC-chemokine ligand 5 (CCL5 or RANTES). These findings demonstrate that the regenerative and differentiation potential of aged HSCs are influenced by aged niche components. An aged HSC microenvironment favours the expansion of pre-leukemic HSCs presumably leading to different kinds of myeloproliferative disorders (Vas et al., 2012a; Vas et al., 2012b). Increased adiposity and decreased osteogenesis may also be linked with decreased CXC-chemokine 12 (CXCL12/ SDF1) levels in the bone marrow niche upon ageing (Tuljapurkar et al., 2011). Reduction of hypoxic conditions in the osteoblast niche through unknown mechanisms also contributes to early age associated changes in the HSC niche. Therefore, an aged niche may have a negative influence on HSC function and homeostasis. Connexin 43 (CXN 43) which forms important gap

junctions between HSCs and stromal cells keep the ROS levels low in HSCs to preserve HSC function (Cancelas et al., 2000; Gonzalez-Nieto et al., 2012). During genotoxic stress response which produces ROS, majority of the ROS are eliminated by the CXN 43 gap junction channels and are deposited in the niche cells. This prevents HSC senescence as the p38 MAPK and $p16^{INK4A}$ pathway are not activated thereby preserving HSC function. There might be an involvement of other niche factors, unidentified cytokines and adhesion molecules that crosstalk between the aged HSC niche and HSC intrinsic mechanisms to augment HSC ageing (Figure 1.6b).

1.6.2.2 The contribution of systemic factors on HSC ageing

The sympathetic nervous system regulates the HSC niche coordinating interactions of HSCs with nestin-positive (NES+) mesenchymal stem cells (MSCs) important for HSC mobilization and may show a different activation status upon ageing, although there are no reports so far, the number of NES+ MSCs may changes with age. Altered cell–cell communication and changes in adhesion receptor-mediated interactions in the aged niche and gap junction channels might lead to an aged phenotype in HSCs. Unidentified sympathetic signals promote high reduction in the function of osteoblasts following granulocyte colony-stimulating factor (G-CSF) (Katayama et al., 2006). Osteoblasts also express receptors for several neuropeptides and integrate with several other neuronal signaling molecules. A decrease in the number of osteoblasts and neuropeptides with ageing may also affect the self renewal and turnover of HSCs (Figure 1.6b).

b.

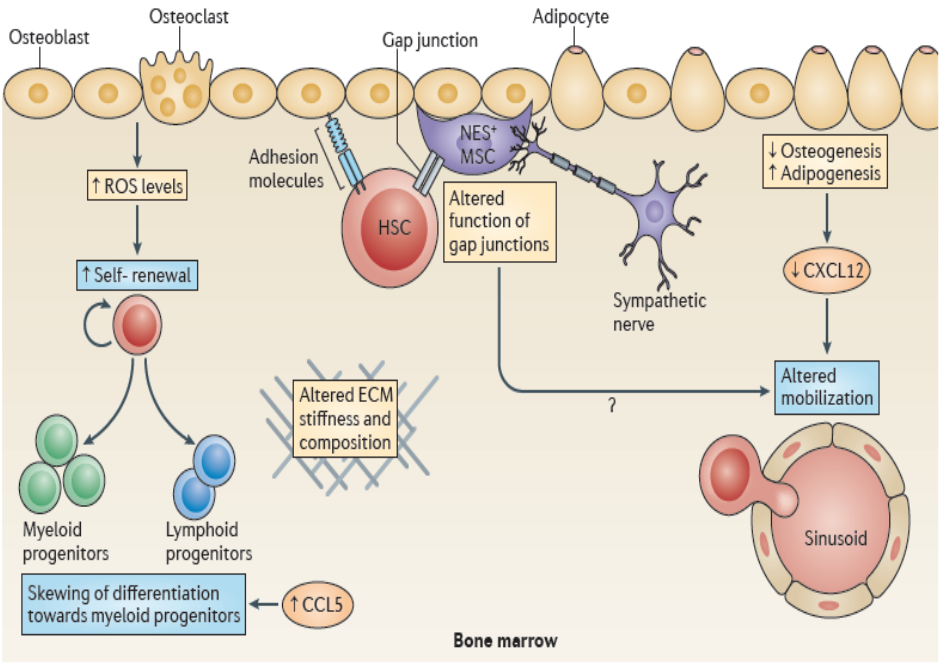


Figure 1.6b: Cell extrinsic mechanisms involved in HSC ageing.
Many changes are associated in the HSC niche with ageing. Skewed differentiation of aged MSCs leads to reduced osteogenesis, increased adipogenesis partly due to decreased levels of CXC chemokine 12 levels in the niche upon ageing. Higher production of ROS and alteration of ECM composition induce p38 MAPK signaling in aged HSCs. Secretion of several chemokines and cytokines in the niche like RANTES causes skewed differentiation of aged HSCs towards myeloid lineage. Nestin-positive (NES+) MSCs, might contribute in the regulation of HSC mobilization and display an altered activation status upon ageing, however this is yet to proven [(Geiger et al., 2013) Nat Rev Immunol. 2013 May; 13(5):376-89)].

1.7 Stra13, a basic helix loop helix Orange (bHLH-O) transcription factor

Stimulated with retinoic acid gene 13 (Stra13) is member of a family of transcription factors called the basic helix loop helix orange (bHLH- O) factors. In general, bHLH proteins are a superfamily of transcription factors that function in various physiological processes like cellular differentiation, cell cycle arrest and apoptosis (Bhawal et al., 2011; Shen et al., 2002; Thin et al., 2007). bHLH-O group of transcription factors which constitute the repressor family of bHLH proteins are structurally and phylogenetically grouped into 4 mammalian subfamilies; the Hey, Hes, Helt and Stra13/Dec. Stra13 along with a homolog gene Sharp 1 belongs to Stra13/Dec under the bHLH-O superfamily (Figure 1.7). The bHLH-Orange transcription factors differ from other helix-loop-helix factors by the presence of a proline residue in their 22 basic motifs, and an additional orange domain for additional protein-protein interaction (Dawson et al., 1995).

Stra13 is ubiquitously expressed during mouse embryogenesis and in adult tissues but its expression is regulated in a cell type specific manner (Azmi and Taneja, 2002; Boudjelal et al., 1997; Fujimoto et al., 2001; Shen et al., 2002; Sun et al., 2001). It was independently identified by four different groups and has been assigned several names; Differentiated Embryo Chondrocyte gene 1 (DEC1) in humans, Enhancer of Split and Hairy Related Protein 2 (Sharp2) in rat, Stimulated by Retinoic Acid gene 13 (Stra13) or Clast5 in mouse, E47 interacting protein (Eip1) and Cytokine Response gene 8 (CR8) in humans (Beadling et al., 2001; Boudjelal et al., 1997; Dear et al., 1997; Rossner et al., 1997; Seimiya et al., 2002). Stra13 is induced by several extracellular stimuli such as TGF-β, retinoic

acid, hypoxia, serum starvation, insulin and gonadotropins in many cells and tissues (Boudjelal et al., 1997; Choi et al., 2008; Ehata et al., 2007; Li et al., 2002; Yamada et al., 2004; Yamada et al., 2003; Yamada and Miyamoto, 2005) .

1.7.1 Stra13 structure and domains

Stra13 in mouse and Sharp2 in rat are made up of 411 amino acids while DEC1 consists of 412 amino acids. Stra13 contains a bHLH motif at the N terminal region, a central orange domain and a proline rich domain at the C terminal region. The tyrosine and serine/threonine residues of Stra13 protein are phosphorylated and it also contains some putative phosphorylation sites which are acted upon by different protein kinases including casein kinase II (Boudjelal et al., 1997; Ivanova et al., 2005). Similar to all bHLH-Orange transcription factors, Stra13 has a bHLH motif situated at the N terminal region of the protein. However, a proline residue in the basic region is displaced by two amino acid residues closer to the N terminus in comparison with the Hes proteins, the founding members of the bHLH-Orange subfamily which suggest differences in DNA binding affinities. The bHLH motif of Stra13 consists of the basic region and the helix-loop-helix region. The basic region is the DNA binding region and the HLH region is the dimerization domain. The HLH domain comprises of two α helices separated by a flexible loop, allowing Stra13 to form homodimers and heterodimers with other bHLH proteins and function as a nuclear transcriptional repressor. Stra13 recognizes and binds canonical E-box sequence, a hexanucleotide sequences of 5’-CANNTG-3’ (N represents any nucleotide) located in the transcriptional regulatory regions of its target genes. Stra13 binds to

sequence 5'-CACGTG-3' in a high affinity manner and 5'-CATGTG-3', 5'-CACGTN-3', 5'-CACGCG-3' in a low affinity manner (St-Pierre et al., 2002; Zawel et al., 2002). The transcriptional repression activity of Stra13 is decreased by deletion or introduction of mutation in the basic region which demonstrates that the E box sequence is critical for repressive function of Stra13. The orange domain of Stra13 at its central region is a motif of ~35 amino acids and is a characteristic feature of all bHLH-Orange transcriptional factors. The orange domain mediates additional protein-protein interactions of Stra13 and other proteins (Dawson et al., 1995). The C terminus of Stra13 contains a proline rich region and very little is known about the function of this domain. Stra13 lacks tryptophan-arginine-proline-tryptophan (WRPW) tetrapeptide motif or tyrosine-arginine-proline-tryptophan (YRPW) motif which is present in the proline rich region at the C terminus of Hes and Hey proteins respectively. The WRPW motif of Hes protein is essential for the recruitment of corepressor transducin-like enhancer of split (TLE), the mammalian counterpart of the Drosophila corepressor Groucho to enhance its transcriptional repression of genes (Fisher et al., 1996; Grbavec and Stifani, 1996). Stra13 which lacks WRPW motif does not recruit Groucho, but instead interacts with co-repressors such as HDAC1, NCoR and mSin3A at its C terminus to bring about gene repression (Boudjelal et al., 1997; Sun and Taneja, 2000). C terminal truncated forms of Stra13 exhibit decreased transcriptional activity, indicating that both the bHLH and C terminal regions of Stra13 are essential for its transcriptional activity (Boudjelal et al., 1997; Li et al., 2004; Sato et al., 2004; St-Pierre et al., 2002).

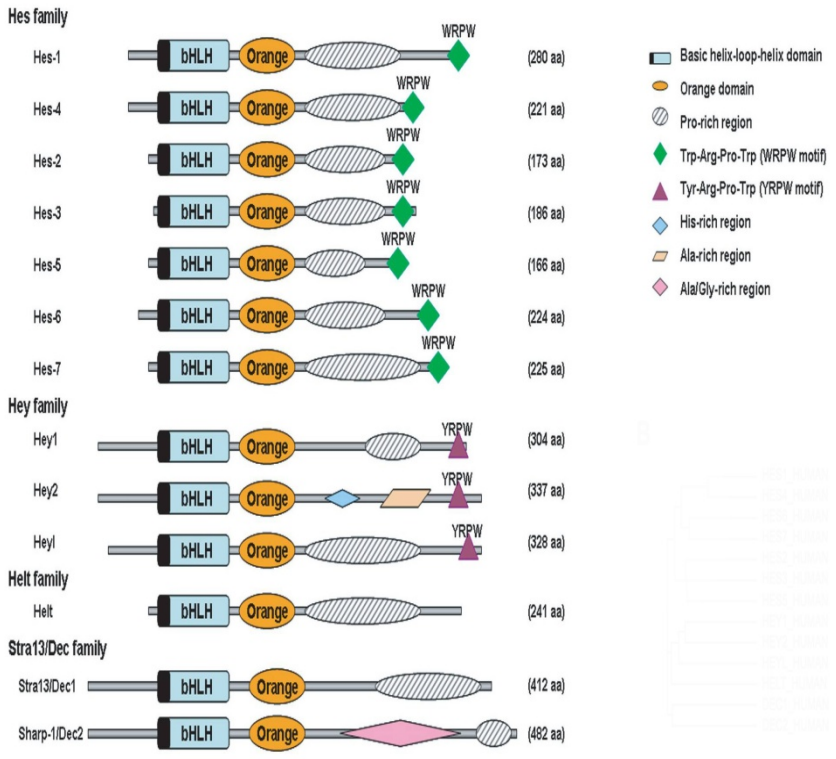


Figure 1.7: Schematic diagram showing the classification of the bHLH-O Transcription Factors and their domain structure. Stra13 falls under the Stra13/Dec sub family. It consists of a bHLH motif, an orange domain and proline rich C terminal domain. A unique feature of this family is the absence of the WRPW and YRPW motifs which are present in other members of the bHLH-O family [(Sun et al., 2007); Translational Oncogenomics. 2007, 2: 105-118].

1.7.2. Functions of Stra13

Since Stra13 is ubiquitously expressed during development and in adult tissues, it participates in a wide variety of physiological functions from cellular differentiation, T cell regulation, genotoxic stress, adipogenesis, muscle regeneration, circadian rhythm, cancer, apopotosis and senescence. In particular,

the role of Stra13 in the immune system in the regulation of T and B cells will be disussed in greater detail.

1.7.2.1 Role of Stra13 in the immune system

Stra13 is expressed in resting B cells and its mRNA expression is down regulated upon activation of B cells by IL-4, CD40 ligand, lipopolysaccharides and other stimuli. Stra13 acts a negative regulator of B cell activation by delaying its entry into S phase of the cell cycle and repressing Fas mediated apoptosis and colony formation (Seimiya et al., 2002). In Stra13 transgenic mice (Stra13-Tg) ectopic expression of Stra13 in B and T lineage cells leads to impaired development of the bone marrow and thymus. The cellularity of the thymus and spleen is reduced when compared to their control littermates. Endogenous Stra13 expression is up regulated in pro B cells, down regulated in pre B cells and elevated in immature B cells. Nevertheless, the germinal center which contains activated B cells show reduced expression of Stra13 even though the expression of Stra13 is sustained in mature splenic B cells (Seimiya et al., 2004). However, in Stra13-Tg mice, Stra13 expression is higher in all stages of B cell development. Similarly, its transcript is elevated in $CD4^+CD8^+$, $CD3^-CD4^-CD8^-$, $CD4^+$ and $CD8^+$ cells when compared to control mice in the thymus. Stra13-Tg mice exhibit an inhibition of early T cell development in the thymus with a reduction of $CD44^+CD25^+$ and $CD44^-CD25^+$ pro T cell population. The frequency of interleukin 7 (IL-7) responses of the progenitor cells of the bone marrow especially the pro B cells shows a significant reduction in colony number in colony forming unit (CFU) –IL7 assay.

In another study published by our lab, Stra13 knockout mice were generated, the knockouts were born normal, fertile with no obvious phenotype when compared to their wild type littermates. Young Stra13 knockouts showed a defective activation of T cells after being stimulated with anti-CD3 and anti-CD28 with decreased production of IL-2, IL-4 and IFN-γ both *in vitro* and *in vivo*. However, as the knockouts aged, massive lymphoid organ hyperplasia was observed within 6-8 months in gender dependent manner where 90% of the females and 50% of the males developed enlargement of the thymus, spleen and lymph nodes. The T and B cells were spontaneously activated with increased production of auto immune antibodies and immune complexes. The hyperactivated T and B cells infiltrated organs like the lungs and kidney and caused a systemic autoimmune lymphoproliferative disorder similar to systemic lupus like disease (Sun et al., 2001). $CD4^+$ T cells from Stra13 KO mice show reduced expression of FasL and were resistant to Fas mediated apoptosis and this resulted in the accumulation of activated T and B cells.

In a recent report, Stra13 was found to play an important role in maintaining regulatory T cell (Treg) homeostasis and function. Stra13 was highly expressed in immature $CD4^-8^-$ (double negative) population and its expression reduced in $CD4^+8^+$ (double positive) cells during thymocyte development. Stra13 expression was higher in the Treg cells ($CD4^+CD25^+$) when compared to its their non Treg cell compartment ($CD4^+CD25^-$). As the mice developed the autoimmune phenotype with ageing, a marked reduction of $CD25^+$ or FoxP3 Treg cells among the $CD4^+$T cells was observed in comparison to their wild type controls (Miyazaki

et al., 2010). Stra13 is required for the maintaining Treg cells in the periphery and for the suppression of effector T cell mediated autoimmunity and its enforced expression leads to increased numbers of Treg cells *in vivo* and upregulation of CD25. Further experiments showed that Stra13 binds to the positive regulatory regions of the IL2RA together with Runx1 in Treg cells and maybe required for its long term expression.

Stra13 was one of the genes identified when naïve $CD4^+$ T cells were costimulated with anti-CD28 in order to determine genes that play an important role in T cell activation and differentiation (Martinez-Llordella et al., 2013). Microarray analysis of Stra13 deficient T cells further supported that Stra13 controls a subset of CD28 dependent genes and the expression of these genes is essential for the function of $CD4^+$ conventional T cell in a mouse model of autoimmune encephalomyelitis (EAE) which is a $CD4^+$ T cell mediated and CD28 dependent inflammatory disease of the central nervous system. Immunization of Stra13 WT and Stra13 KO with MOG_{35-55} peptide in CFA to induced EAE demonstrated that Stra13 KO mice were completely protected from EAE when compared to Stra13 WT controls. Thus Stra13 is required for efficient T cell priming during the development of EAE. Stra13 is important for the production of proinflammatory cytokines during the effector phase of EAE as Stra13 KO T cells show reduced IL-2, GM-CSF and IFN-γ (Martinez-Llordella et al., 2013).

Stra13 was one of the target genes identified post translationally modulating myc activity in human B lymphocytes by using modulator inference by network

dynamics (MINDy) algorithm. MINDy identified Stra13 as a negative regulator of myc activity. To assess the effect of Stra13 gene silencing on expression of myc targets, gene expression profiling was carried out. There was a significant enrichment of canonical myc targets within the differentially enriched genes. However, myc mRNA and protein levels were unchanged indicating a post translational effect. This established Stra13 as an antagonist of myc activity (Wang et al., 2009).

Stra13 is induced and up regulated in primary human gingival epithelial cells by the pro inflammatory interleukin IL-1β. The induction of Stra13 by IL-1β is dependent upon PI3K-Akt signaling pathway linking Stra13 function to inflammation (Bhawal et al., 2012).

1.7.2.2 Role of Stra13 in DNA damage stress response

Stra13 is induced in response to genotoxic stress in the form of ionizing gamma radiation and genotoxic agents- etoposide and cisplatin both *in vitro* and *in vivo* in a p53 independent manner. Thymocytes from Stra13 knockout mice are resistant to ionizing radiation and show impaired apoptosis and express reduced levels of p53 and its downstream target Puma and Noxa. The defective apoptosis in Stra13 thymocytes is accompanied by a decrease in Bim and an increase in Bcl-2 levels compared with wild type thymocytes (Thin et al., 2007).

1.7.2.3 Role of Stra13 in differentiation and as a growth repressor.

Stra13 was initially identified as one of the novel retinoic acid responsive genes in a cDNA differential screening of P19 embryonal carcinoma cells treated with

retinoic acid (RA) in monolayers (Boudjelal et al., 1997). Ectopic expression of Stra13 in P19 cells in monolayer cultures results in neuronal differentiation as opposed to wild type P19 cells that generally differentiate into mesodermal and endodermal lineages. The decreased expression of activin, a mesodermal marker and increased expression of neuronal marker Wnt1 in Stra13 overexpressing cells showed Stra13 to function as transcriptional repressor of mesodermal differentiation pathway and activator of neuronal pathway under these conditions. Through subtractive hybridization technique, Stra13 was found to be one of the most important genes involved in chondrocyte differentiation when stimulated with Bt2cAMP, an analog of cAMP (Shen et al., 2002). In another study, Stra13 acts as a growth suppressor of cells when activated with different stimuli like RA, trichostatin A (TSA) or serum starvation. Stra13 causes growth arrest by down regulation of c-myc expression and arrest of cells at G2/M phase of the cell cycle. Stra13 negatively auto-regulates its own expression through a histone deacetylase (HDAC) dependent mechanism where the C terminal region containing the alpha helices interact with the components of the HDAC corepressor complex. A Stra13 mutant (1-127 amino acids) for this C terminal region fails to interact with HDAC corepressor complex and hence cannot repress Stra13 expression. Stra13 also represses c-myc transcription through its promoter activity by interaction with the basal transcriptional machinery independent of HDAC pathway. Stra13 plays an important function in regulation of adipogenesis under hypoxic conditions, where it inhibits the PPARγ2 promoter activity and functions as an effector of hypoxia inducible factor 1(HIF-1) mediated inhibition of adipogenesis (Yun et al., 2002).

In a similar study under hypoxic conditions, Stra13 along with Sharp1 represses Sterol regulatory element binding protein 1c (SREBP-1c), a bHLH homodimeric transactivator involved in inducing several enzymes of the lipogenesis including fatty acid synthase. Stra13 inhibits SREBP1c by competing with SREBP1c for its binding to the E box located in the SREBP1c promoter or by direct protein interaction (Choi et al., 2008).

Using the yeast two hybrid system, Stra13 was identified as the binding partner of STAT3β. Stra13 was found to bind to both STAT3β and STATα isoforms in its phosphorylated state. Stra13 is implicated in JAK-STAT signaling pathway and Stra13 activates transcription from STAT dependent cis elements. Overexpression of Stra13 leads to apoptosis via STAT3 dependent regulation of the Fas promoter and co expression of STAT3β and Stra13 results in antagonizing its pro-apoptotic effect (Ivanova et al., 2004).

Stra13 transcriptionally regulates ΔNp63, the predominant isoform of p63 gene during keratinocyte differentiation. Stra13 physically interacts with HDAC2 which reduces its ability to regulate ΔNp63 by preventing it from binding to the p63 P2 promoter. By using HDAC inhibitors or knockdown of HDAC2, the ability to Stra13 to modulate ΔNp63 expression is enhanced. Forced expression of Stra13 suppresses keratinocyte differentiation whereas knockdown of Stra13 favors differentiation by modulating ΔNp63 regulation (Qian et al., 2011).

Stra13 along with Sharp1 also act as repressor or the retinoic acid receptor (RXR) and retinoic acid receptor -liver X receptor (RXR-LXR) by interacting with RXR

and represses target genes meditated by RXR-LXR heterodimer. The repression of RXRα transactivation by Stra13 is HDAC dependent but HDAC independent for Sharp1. This regulatory loop between Stra13, Sharp1 and RXR heterodimers is important for hepatic clock system and function (Cho et al., 2009).

Stra13 and Sharp1 (DEC family) are repressors of the miss match repair gene MLH1 under hypoxic conditions. It not only represses MLH1 through HDAC dependent mechanisms but also by direct binding to DEC response element on the promoter of MLH1. However, knockdown of Stra13 cannot attenuate MLH1 repression during hypoxia as Sharp1 protein is increased due to decreased Stra13 expression. This links the HIF-DEC pathway in controlling the expression of mismatch repair genes (Nakamura et al., 2008).

1.7.2.4 Stra13 in cancer, apoptosis, cell cycle arrest and senescence

The role of Stra13 has been widely studied in the field of cancer biology. Stra13 is highly expressed in a number of human cancers such as breast, colorectal, gastric and hepatocellular carcinomas. The endogenous expression of Stra13 or DEC1 (human counterpart) is high in these neoplastic tissues in comparison to its non neoplastic ones which correlates with increased invasiveness of breast cancer and decreased differentiation of gastric cancer. This demonstrates Stra13 role in promoting tumor progression (Chakrabarti et al., 2004; Jia et al., 2013; Shi et al., 2011). However, the differentiation status of hepatocellular carcinoma positively correlate with high DEC1 expression suggesting varying roles DEC1 plays in different cancers.

The mechanism of how Stra13 promotes and regulates cancer development is poorly understood. A large number of correlative studies suggest its involvement in diverse cancers but mechanistic studies are limited. A small number of studies demonstrate that Stra13 regulates apoptosis and/or cell cycle arrest in neoplastic and non-neoplastic cells. For example, Stra13 knockdown in MCF-7 cells upon paclitaxel treatment decreases cleaved poly (ADP-ribose) polymerase (PARP), an indication of decreased apoptotic activity (Wu et al., 2011). Stra13 knockout thymocytes also have impaired apoptosis upon irradiation as discussed earlier (Thin et al., 2007). Both studies suggest Stra13 functions as a proapoptotic molecule.

Interestingly, Stra13 also functions as an anti-apoptotic protein. Stra13/DEC1 was identified as a downstream target of TGF-β signaling pathway promoting the survival of mammary carcinoma cells (Ehata et al., 2007). The antiapoptic function of Stra13 has also been studied in colon carcinomas where Stra13 selectively decreases the activation of procaspases 3, 7 and 9 (Li et al., 2002). Its antiapoptic role is also mediated by the induction of survivin which has been identified as a transcriptional target of Stra13 (Li et al., 2006). Stra13/DEC1 is also implicated in inducing cell cycle arrest. DEC1 brings about cell cycle arrest by repression of Cyclin D1, an essential component required for G1/S transition of the cell cycle. Cyclin D1 contains a DEC response element in its promoter and DEC1 binds to this DEC-response element and represses transcription of Cyclin D1 (Bhawal et al., 2011; Wang et al., 2012b). Inactivation of DEC1 rescues its repression and facilitates proliferation. Stra13/DEC1 is down-regulated in HepG2

cells after treatment with an apoptotic inducing compound 8-methoxypsoralen (8-MOP), and ectopic expression of DEC1 antagonizes 8-MOP induced apoptosis (Peng et al., 2012). Stra13 expression is also induced by hypoxia and regulated by hypoxia inducible factor 1α (HIF-1α) thus promoting the survival of tumours (Chakrabarti et al., 2004). High Stra13/DEC1 mRNA expression is also apparent in various leukemic cell lines (Ivanova et al., 2001). Together these studies suggest a role of Stra13 in oncogenesis.

1.7.2.5 Stra13 and senescence

Since Stra13 regulates both cell cycle arrest and apoptosis, it has been implicated that this function of Stra13 is mediated by p53, a well known regulator of cell cycle and apoptosis. Reports have demonstrated that Stra13 via its bHLH domain physically interacts with C terminal region of p53 and regulates the expression of P53 and enhances its stability. This is brought about by shielding the C terminus of p53 protein from Mdm2 mediated ubiquitination, preventing its nuclear export and degradation (Thin et al., 2007).

Other studies have reported Stra13 as one of the downstream effectors of p53 in facilitating senescence. In response to DNA damage, p53 binds to p53 binding site on the Stra13 promoter to activate its transcription. Stra13 then mediates p53 dependent premature senescence independent of the p21 senescence pathway (Qian et al., 2008).

1.7.2.6 Role of Stra13 in circadian rhythm

Stra13/DEC1 along with Sharp1/DEC2 forms the fifth clock gene family and are regulators of the mammalian clock genes along with other members such as Bmal1, Cry and Per genes. Stra13 is expressed in the suprachiasmic nucleus in a circadian fashion. Through direct protein-protein interactions or competitive binding to E boxes with Clock/Bmal1, Stra13 represses the Clock/Bmal1 induced activation of the Per1 promoter (Honma et al., 2002). The circadian expression of Stra13 in peripheral tissues has also been observed in lungs, kidney, liver, heart and monocytes (Boivin et al., 2003; Grechez-Cassiau et al., 2004; Noshiro et al., 2004).

1.8 Perspectives and Aims of this Study

The major aim of my thesis is to study the role of Stra13 in hematopoiesis. Reports from our lab and others have shown that Stra13 plays an important role in the regulation and homeostasis of regulatory T cells (Miyazaki et al., 2010; Sun et al., 2001). Other reports suggest Stra13 acts as a negative regulator of B cell activation by delaying its entry into the S phase of the cycle and repressing Fas mediated apoptosis (Seimiya et al., 2002). However, the role of Stra13 in hematopoiesis has not been investigated so far. The first part of my thesis demonstrates that Stra13 is expressed in the long term (LT)-HSCs and short term (ST)-HSCs. Using knockout mice, we have identified that Stra13 is dispensable during steady state hematopoiesis. With ageing, Stra13 knockout mice show a reduction in the percentages of both LT-HSCs and ST-HSCs and altered progenitor cell levels. With the help of microarray analysis we have identified

potential target genes which may play a role in hematopoietic stem cell ageing in Stra13 KO mice.

The second objective of my thesis is to study the role of Stra13 in hematopoiesis during genotoxic stress. Genotoxic stress in the form of ionizing radiation or 5-Fluoro Uracil, a myeloablative drug has been well used in clinical settings for bone marrow disorders. Through our studies in hematopoietic cells we have demonstrated that the LSK cell compartment of Stra13 knockout mice is sensitive to both forms of genotoxic stress. Through gene expression profiling studies we have identified potential target genes which could potentiate the role of Stra13 in stress response in HSCs by ionizing radiation. Our data uncovers a novel role of Stra13 in stress response and provides further evidence of the emerging role of Stra13 in ageing and stress hematopoiesis.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIAL AND METHODS

2.1 Stra13 Knockout Mice

Stra13 knockout mice (KO) have been described previously (Sun et al., 2001). Stra13 KO mice were maintained in C57BL/6 background in a SPF mice facility; MD2 Vivarium at National University of Singapore (NUS) and all mice experiments followed strict procedures set according to Institutional Animal Care and Use Committee (IACUC) guidelines at NUS. In general Stra13 heterozygous mice were crossed to generate Stra13 KO and Stra13 wild type (WT) littermate controls which were used for experiments as described below.

2.2 Isolation of cells from the bone marrow, spleen, thymus and peripheral blood

Bone marrow (BM cells) was isolated from 8-12 weeks old C57BL/6 CD45.2 WT mice from the hind limbs (femur and tibia), forelimbs (humerus) and hips (ilium). The muscles and connective tissues were removed and the bones were crushed in a mortar and pestle in PBS containing 2% FBS. Similarly spleen and thymus were minced in PBS with 2% FBS. The cell suspension from the bone marrow, spleen and thymus were passed through a 45 µm filter and RBCs were lysed with Ammonium Chloride Potassium (ACK) buffer. Peripheral blood was collected by retro orbital puncture into EDTA or heparin coated capillary tubes and RBCs lysed with ACK buffer.

BM cells, splenocytes and thymocytes from young (2-3 months) and aged (13-16 months) Stra13 WT and KO mice were isolated in the same manner as above. The

samples were then stained with antibodies for specific surface markers for either flow cytometric analysis or flow cytometric sorting as described below.

2.3 Flow Cytometric analysis of Bone Marrow (BM) cells and Sorting

BM cells were treated with a lineage marker cocktail consisting of PECy-7 conjugated anti-Gr1, Mac1, B220, CD3, CD4, CD8 CD19 and Ter119 monoclonal antibodies (mAbs) and incubated on ice for 30 minutes. For sorting of cells into SLAM, LSK, GMP, CMP and MEP, lineage positive (Lin^+) cells were depleted using Dynabeads Sheep anti-Rat IgG (Invitrogen) and passed through a magnetic column. Lineage negative (Lin^-) cells were stained with FITC conjugated anti-CD34, PE conjugated anti-CD16/32 (FcGRIII/II), APC conjugated anti-CD117 (c-Kit) and APC-Cy7 conjugated anti Sca1 (Ly6A/E) monoclonal antibodies which are markers for progenitors –LSK, CMP, GMP and MEP. For SLAM markers, BM cells were stained with PE conjugated anti CD150 and FITC conjugated anti CD48. The LT-HSCs and progenitors were either sorted or analyzed for their percentages in the bone marrow. Spleen cells were stained for T cells (CD3) and B cells (CD19, CDB220). Thymus was stained for CD4 and CD8 antibodies respectively. Dead cells were excluded by a combination of scatter gates and by propidium iodide or DAPI staining. All mAbs were purchased from BD Biosciences and eBioscience. Multi color sorting and analysis was performed using Becton Dickinson (BD) FACS ARIA and BD LSRII respectively and data analyzed using the FACS Diva and FlowJo Software.

2.4 Cloning of Retroviral Construct

The MIG retroviral vector (MSCV-IRES-GFP), a kind gift from Professor Daniel G. Tenen was used for cloning mouse Stra13. The MIG vector is composed of MSCV (murine stem cell virus) LTRs (long terminal repeat), IRES (internal ribosome entry site) and GFP (green fluorescent protein) gene. MSCV-Stra13-IRES-GFP (pMIG-Stra13) construct to overexpress Stra13 was made by sub cloning mouse Stra13 ORF from pCS2-Myc-Stra13 into the EcoR1 site of MIG vector using standard procedures. The pMIG-Stra13-GFP construct was sequenced to confirm its sequence orientation and amplified by maxi prep for further downstream experiments.

2.5 Bacterial transformation and plasmid DNA extraction

100 ng of plasmid DNA construct was incubated with 50 µl of DH5α competent cells (Invitrogen) on ice for 30 minutes prior to heat shock treatment at 42°C for 90 seconds and snap cooled on ice for 2 minutes and then cultured with 1 ml of Luria-Bertani (LB) broth for 1 hour at 37°C with constant shaking at 250 rpm. The cells in the LB broth were pelleted by centrifugation at 8000 rpm for 1 minute and resuspended in 200 µl of fresh LB broth and plated on LB agar plates supplemented with ampicillin (100µg/ml). The plates were then incubated overnight at 37°C to obtain bacterial colonies containing the transformed DNA plasmid constructs. Individual colonies of transformed bacterial cells were innoculated into 2 ml of LB broth supplemented with ampicillin (100µg/ml) and incubated at 37°C for 7-9 hours in an orbital shaker at 37°C at 250 rpm. The turbid starter culture was then transferred into a 300 ml of LB-ampicillin and

grown overnight in an orbital shaker at 37˚C at 220 rpm for 16 hrs. The following day, the bacterial cells from the overnight culture were pelleted by centrifugation at 5000 rpm for 5 minutes at 4°C. The supernatant was decanted and plasmid DNA extraction from the bacterial pellet was carried with Qiagen Plasmid Midi kit (Qiagen) according to manufacturer's instruction. The bacterial pellet was resuspended completely in 4 ml of buffer P1 (with RNaseA added) and thoroughly lysed. The bacterial cells were lysed using 4 ml of buffer P2 by vigorously inverting the sealed tubes 4-6 times and incubated at room temperature for 5 minutes. Pre-chilled buffer P3 was then added to the lysate mixed thoroughly and was incubated on ice for 15 minutes. The lysate was centrifuged at 15000 rpm for 15 minutes at 4˚C; the clear supernatant was then collected in another 50 ml eppendorf tube. The Qiagen midi column was equilibrated with 4 ml of buffer QBT. The cleared lysate was filtered into the pre-equilibrated column. The column was washed with 10 ml of buffer QC twice to exclude the contaminants. The DNA was eluted using 5 ml of buffer QF, precipitated by the addition of 3.5 ml isopropanol and centrifuged at 13,000 rpm for 30 minutes at 4˚C. The supernatant was carefully decanted and the DNA pellet was washed with 2 ml of 70% ethanol; centrifuged further at 13,000 rpm for 10 minutes at 4˚C. The pellet was air-dried and dissolved in RNase-DNase free water. The concentration of DNA was measured by Nanodrop spectrophotometer (NanoDrop-1000, Thermo Scientific) and stored at -20°C.

2.6 Transfection and production of retrovirus for murine bone marrow infection

BOSC23 cells, a retrovirus packaging cell line cultured in DMEM supplemented with 10% FBS were transfected with the retrovirus constructs pMIG and pPMIG-Stra13 and virus helper structural plasmids- gag and pol with lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. The virus supernatants were collected after 48 hours and 72 hours post transfection and concentrated as described (Zelenock et al., 1997). In brief, the retrovirus medium was collected at 48 and 72 hours, pooled and passed through a 0.45 µm syringe filter to exclude the cell debris. The supernatant were then transferred to a sterile specialized tube and centrifuged at 6000g for 16 hours at 4°C. The supernatant was discarded and the fine pellet was resuspended in 1 ml of serum free DMEM and stored in aliquots at -80°C until further use. The virus titers for the MSCV retroviral supernatants were estimated by transduction of NIH3T3 cells and quantifying the percentage of GFP-positive cells by flow cytometry analysis (Appendix II).

2.7 Retrovirus infection of bone marrow and bone marrow progenitors

8-12 week old C57BL/6 mice (CD45.2, n=3) were intraperitoneally injected with 150 mg/kg of 5-FU. After 5 days, the bone marrow was isolated from the femurs and tibia of treated mice. RBC lysis was performed and the bone marrow was stained with a lineage cocktail of antibodies for PE-Cy7 conjugated anti CD3, CD9, CD4, CD8, Gr1, Mac1, B220 and Ter119 (Lin^+ cells). Lineage depletion was performed with Dynabeads Sheep anti-Rat IgG (Invitrogen) and passed through a magnetic column. Lineage negative (Lin^-) cells were stained with APC

conjugated anti-CD117(c-Kit) and APC-Cy7 conjugated anti-Sca1 mAbs and sorted for LSK cells under aseptic conditions. For infection of LSK progenitors, the retrovirus medium was placed in 35mm non tissue culture dishes for 3 hours precoated with 15 ug/ml of retronectin (Takara Bio) at room temperature for 2.5 hours. The sorted LSK cells were plated for infection for 48 to 60 hours with the coated retrovirus containing pMIG-Stra13 or an empty vector as control. The retrovirus infection was carried out at a multiplicity of infection (MOI) =20 in a rich cytokine cocktail containing IL3 (10 ng/ml), IL6 (10 ng/ml), SCF (50 ng/ml), TP0 (25 ng/ml) and FLT3 (50 ng/ml) (all from Peprotech) in hematopoietic stem cell expansion medium (Stem Cell Technologies) supplemented with 15% FBS (Hyclone).

2.8 Mouse Bone marrow transplantation

Sorted LSK cells (1.5×10^4-2.0×10^4) / mouse isolated from C57BL/6 (CD 45.2) mice infected with Stra13 retrovirus or mock retrovirus were transplanted via tail vein injections into lethally congenic mice C57BL/6-CD45.1 mice. A total radiation dose of 10.5 Gy was given in two doses of 5.75 Gy each separated by 3-4 hours to prevent damage to the gut cells. 2×10^5 of C57BL/6-CD45.1 splenocytes were transplanted together with the bone marrow LSK cells to provide radioprotection. All animal studies were approved by IACUC of National University of Singapore. The animals were monitored closely on a regular basis. Peripheral blood was obtained at different time points after transplantation by puncture of the retroorbital venous sinus using heparin or EDTA coated micro

capillary tube. Peripheral blood count was analyzed using Hemavet 950 auto analyser (Drew Scientific).

2.9 Flow Cytometric Analysis of peripheral blood after transplantation.

RBCs from peripheral blood were lysed with ACK buffer. The cells were washed with PBS and stained with respective antibodies for mature myeloid and lymphoid markers PE-Cy7 Gr-1, APC-Mac1, PE-Cy7 CD19 and APC CD3 at 4°C for 45 minutes. Flow cytometric analysis of the stained cells was performed using BD LSRII and the data analyzed using the BD FACS Diva software.

2.10 Competitive Repopulation Assays in young Stra13 knockout mice

Competitive repopulation assays were carried out as previously described (Capron et al., 2006). C57BL/6 (CD45.1) mice were used as recipients and were lethally irradiated (10.5Gy) in two split doses of 5.75 Gy 3-4 hrs apart. They received different doses of bone marrow test cells from Stra13 WT or Stra13 KO:-5 x 10^6 and 0.5 x 10^5 cells along with a fixed number of competitor cells 1 x 10^6 from CD45.1/CD45.2 mice corresponding to ratios of 5:1 and 0.5:1 respectively. Young Stra13 KO and WT littermate controls (2 months) were used as donors for this assay. Hematopoietic reconstitution was assessed at 4 and 8 weeks through peripheral blood analysis. The mononuclear cells from the peripheral blood were stained with anti PE conjugated CD45.2 and eFlour450 anti CD45.1 mAbs and analyzed by flow cytometry. Reconstitution of ganulocytes (Gr1), B cells (CD19) and T cells (CD3) cells were assessed by flow cytometric analysis.

2.11 Colony forming assays

Stra13 was overexpressed by retroviral transduction in sorted LSK cells isolated from 8-12 weeks old C57BL/6 mice. The infection was carried out on coated retronectin 35 mm dishes with the mock empty vector control for two consecutive days and GFP^+ cells were sorted aseptically after 48 hours. GFP^+ cells were cultured *in vitro* for another 3 days. 1000 GFP^+ were plated in Methocult medium containing the rich cytokine cocktail of human transferrin, recombinant stem cell factor (SCF), IL3, IL6 and erythropoietin (M3434, Stem Cell technologies). Colonies were scored under a microscope after 7 days of culture.

2.12 Real Time PCR

Total RNA was extracted from murine sorted bone marrow progenitors- LSK, CMP, GMP and MEP using the RNeasy micro kit (Qiagen) according to the manufacture's instruction and converted to cDNA using a high fidelity reverse transcriptase (Roche). Real Time PCR was performed using Corbett Rotor Gene 6000 using the two step method and cDNA amplified by using SYBR Green Mix (Applied Biosystems). The following primer pairs were used:

mStra13: Forward 5'-GCCCTGCAGAGCGGTTTACAAA-3'
Reverse 5'-GAGCCGAGTCCAATGGTTTCCTG-3'

mGAPDH: Forward-5'- GACATGCCGCCTGGAGAAAC-3'
Reverse- 5'-AGCCCAGGATGCCCTTTAGT-3'

QPCR reaction was performed in triplicates to obtain CT values. Relative gene expressions were calculated according to the ΔΔCt method. Expression levels of Stra13 were normalized with GAPDH mRNA expression level.

2.13 Genotoxic / DNA damage response in LSK cells by gamma radiation and 5 Fluorouracil (5-FU)

2.13.1 Sublethal Gamma Radiation: Young Stra13 KO mice and its littermate WT control were subjected to a sub lethal dose of 5Gy whole body gamma radiation. After 24 hours, the mice were euthanized and the bone marrow was stained with antibodies for flow cytometric analysis of LSK cells as described under methods 2.3.

2.13.2 Myelotoxic drug 5-FU: Young Stra13 and its wild type littermate control were injected with 150 mg/kg of 5-FU (Sigma Aldrich) intraperitoneally. After 7 days, the mice were sacrificed and the bone marrow was stained with antibodies for flow cytometric analysis of LSK cells as described under methods 2.3.

2.14 Microarray and gene expression analysis

Total RNA was extracted from 2 biological replicates from the sorted LSK cells under each condition (radiation and old age) with RNeasy micro kit (Qiagen). The concentration and the purity of the extracted RNA was obtained using Nanodrop 1000 (Thermo Fisher Scientific) and the integrity of RNA was confirmed using Bioanalyzer (Agilent Technologies, USA). Total RNA was amplified with Target Amp Pico kit for Illumina expression Beadchip (Epientre, Illumina). Reverse transcription to cDNA was done with 500 ng of extracted RNA using Superscript^{TM} II Reverse Transcriptase (Invitrogen) primed with T7-(dT)-24

primer. cRNA was then transcribed *in vitro* from cDNA as biotin-labelled, in the presence of T7 RNA polymerase and biotinylated ribonucleotides (Enzo Diagnostics, USA). 500ng was reverse transcribed into cDNA followed by *in vitro* transcription to biotin-labeled cRNA using the Illumina TotalPrep RNA Amplification kit (Applied Biosystems). 750 ng of each cRNA sample was hybridized to mouse WG-6 v2.0 Expression BeadChip microarrays (Illumina) for 16 hours at 58°C. After the incubation period, the arrays were washed and stained with Streptavidin-Cy3 (GE Healthcare), followed by scanning the arrays using Illumina Bead Array Reader (Illumina) at 25 scan factor 1. The signal values of cRNA bound to each probe was further translated into gene expression values using Partek® Genomics Suite™ version 6.5 (Partek Inc., USA). Background subtraction was applied on raw intensity values and subsequent data was subjected to quantile normalization on the Beadstudio Data Analysis platform (Illumina) with a normalized expression value cutoff at 100. Principal component analysis was performed before analysis of gene expression to ensure quality control. Analysis of variance (ANOVA) was conducted on the complete data set and a list of differentially expressed genes was obtained with $p < 0.05$. Unsupervised two-dimensional average-linkage hierarchical clustering of the genes, differentially expressed was performed for both the cell types by using Spearman's correlation as similarity matrix. Biological significance of the differentially expressed gene list was better realised by classifying the genes based on their biological pathways and molecular functions using DAVID

(**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery v6.7 (Huang da et al., 2009a; Huang da et al., 2009b).

2.15 Stra13 mice genotyping

For genotyping of Stra13 mice, mice tails were clipped during the weaning period (21 days). The mice tails were digested with proteinase K buffer overnight at 55°C (200 µg/ml) and the genomic DNA was isolated by phenol chloroform extraction. A semi-quantitative genomic DNA PCR was carried out to identify the genotypes of the mice using the following Stra13 primers:

Primer 1: 5'-CGTTTTATTCCCCGCCTGGA-3'

Primer 2: 5'-GGAAGCTCAGGCTAGCTCAT-3'

Primer 3: 5'-TCGATTCCACCGCCGCCTTCTATG-3'

Primers 1 and 2 detect the wild type allele. Primers 2 and 3 detect the knockout allele. The PCR conditions were as follows: 94 °C -5 minutes, 94 °C -30 sec, 58°C - 45 sec, 72°C -1 min, 72 °C -5 min for final extension and 4°C cooling step for a total of 29 cycles. The PCR products were run on a 1.5% agarose gel and the gels images were captured using Bio-Rad ChemiDoc™ XRS Gel Documentation system. (Appendix III)

2.16 Statistical Analysis

Statistical significance was determined by a 2-tailed parametric Student's *t* test (Graph Pad Prism software and Microsoft Excel). A *P* value less than 0.05 was considered significant. Data are shown as the mean ± SD unless otherwise indicated in the legend. Different degrees of statistical significances were indicated by asterisks as follows: * is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$.

CHAPTER 3

RESULTS

3. RESULTS

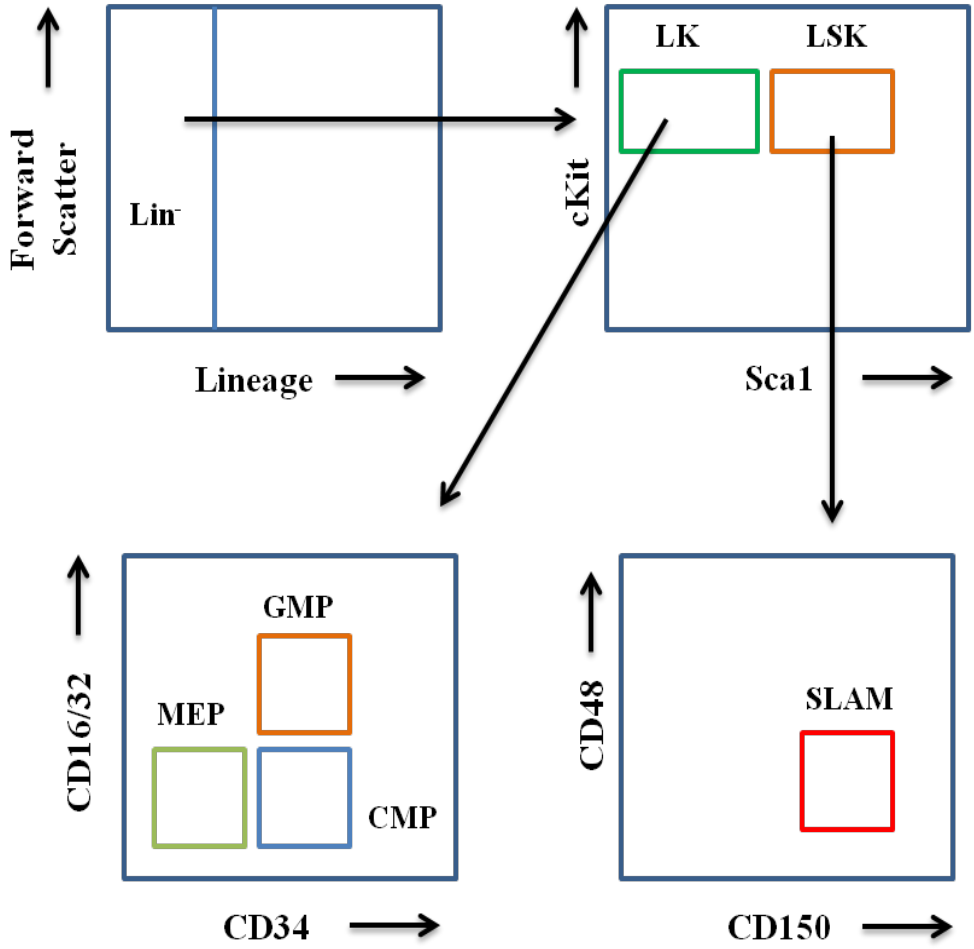
3.1 Role of Stra13 in hematopoietic stem cell regulation

3.1.1. Analysis of expression of Stra13 in the bone marrow progenitors and long term hematopoietic stem cell during steady state hematopoiesis.

Our lab has previously published that Stra13 is induced upon T cell activation (Sun et al., 2001). As a first step in addressing the role of Stra13 in hematopoiesis, we investigated Stra13 expression in the different mouse progenitor populations. Bone marrow cells from C57/BL6 wild type mouse (8-12 weeks) were sorted by flow cytometry into the different progenitor fractions namely LSK, CMP, GMP and MEP based on their surface marker characteristics. A flowchart explaining the FACS sorting strategy of $SLAM^+$ cells, LSK cells and the multipotent progenitor cells- CMP, GMP and MEP are represented in Figure 3.1.1a. After sorting, total mRNA levels were examined by quantitative real time PCR (q-PCR) which showed highest expression of Stra13 in the LSK population and lowest in the MEP fraction of the bone marrow when compared to the other progenitors (Figure 3.1.1b).

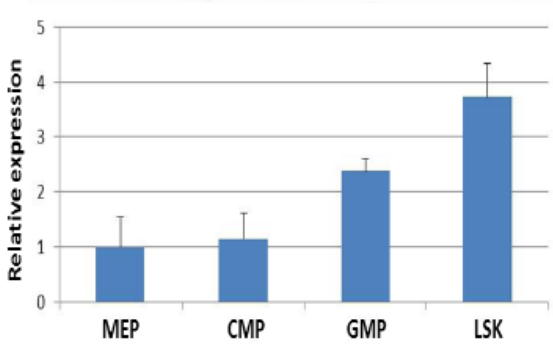
Since the LSK progenitors are heterogeneous and consist of both short term (ST) and long term (LT) HSCs respectively, we checked the expression of Stra13 in the $SLAM^+$ fraction which consists mainly of LT-HSCs which are considered to be the purest form of HSCs characterized by the expression of surface markers $CD150^+$ and $CD48^-$. Stra13 expression was higher in the $SLAM^+$ cells compared to the $SLAM^-$ fraction (Figure. 3.1.1c). This suggests that the Stra13 expression may be essential to regulate HSCs during steady state hematopoiesis.

a.



Lin- - Lineage negative
MEP- Megakaryocyte Erythrocyte Progenitor
LSK - Lineage- cKit+Sca1+
CMP- Common Myeloid Progenitor
GMP- Granulocyte Macrophage Progenitor
LK - Lin-cKit+
SLAM- Signaling lymphocyte activation molecule

b.



c.

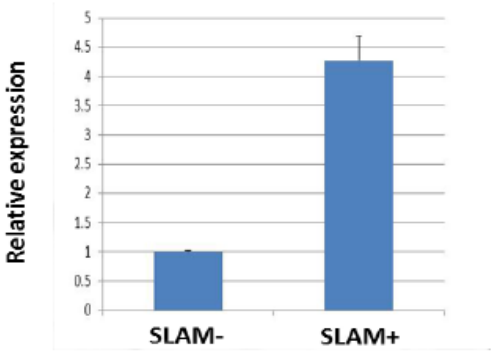


Figure 3.1.1: Stra13 expression in multipotent bone marrow progenitors and hematopoietic stem cells.

a) Strategy for sorting the different stem and progenitor fractions by flow cytometry with their respective gating based on the expression of the different surface markers used.
b) Quantitative Real Time PCR of Stra13 mRNA expression in the different mouse bone marrow progenitors; LSK, CMP, GMP and MEP of 8-12 weeks old wild type C57BL/6 mice. c) Quantitative real time PCR for Stra13 expression in the long term HSC compartment- $SLAM^+$ cells ($CD150^+$ $CD48^-$) and $SLAM^-$ cells ($CD150^-$ $CD48^+$). The expression of Stra13 mRNA is normalized to mouse GAPDH which was used as a control. Data is represented as mean ±SD for two independent sort experiments from 3-4 pooled C57BL/6 bone marrow.

3.1.2 Expression of Stra13 in HSCs during ageing

Knowing that Stra13 is expressed in the LSK and $SLAM^+$ fraction of the bone marrow during steady state hematopoiesis in young mice, we were interested to analyze its expression during ageing. Old aged (22-24 months) wild type

C57BL/6 mice (a kind gift from Professor Lim Kah Leong) were obtained and bone marrow was isolated and sorted for SLAM^{+} fraction (CD150^{+} CD48^{-}) along with young (2-3 months) as controls. Stra13 mRNA expression was ~2.5 fold higher in the LSK progenitor fraction of old aged mice compared to young mice (Figure 3.1.2). These results suggest that Stra13 expression may be modulated and may play an important role in regulating hematopoietic stem cell function during ageing.

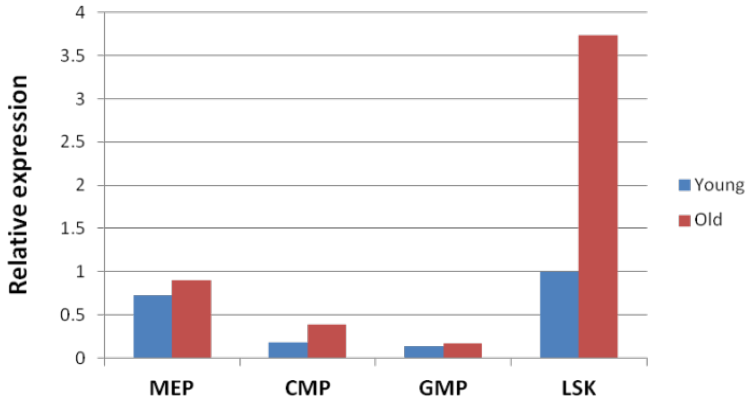


Figure 3.1.2: Stra13 expression is upregulated in the progenitor fraction of aged mice.
Comparison of Stra13 expression by real time PCR in the bone marrow progenitors of young and aged wild type C57BL/6 mice. The expression level of Stra13 mRNA was normalized to mouse GAPDH expression. Data is represented as a pool of 2-3 sorted fractions of bone marrow from young and aged wild type mice.

3.2 Stra13 is dispensable in promoting differentiation of progenitors by colony forming assays

From the above data as Stra13 was expressed in the LSK progenitor fraction, we proceeded further to study the clonogenic capacity and differentiation potential by overexpression of Stra13 in the LSK progenitors. Stra13 was overexpressed in the

LSK fraction by retroviral infection along with its mock control as described (section 2.7) and the GFP^+ cells were flow-sorted and cultured *in vitro* for 3 days before subjecting them to methylcellulose assay. Colonies were counted after 7 days of culture in the semi-solid medium containing a rich cocktail of hematopoietic cytokines, IL3, IL6, SCF and erythropoietin. No drastic changes were seen in the number of colonies in Stra13 expressing cells though the numbers were moderately higher (not significant) compared with the empty vector control (Figure 3.2)

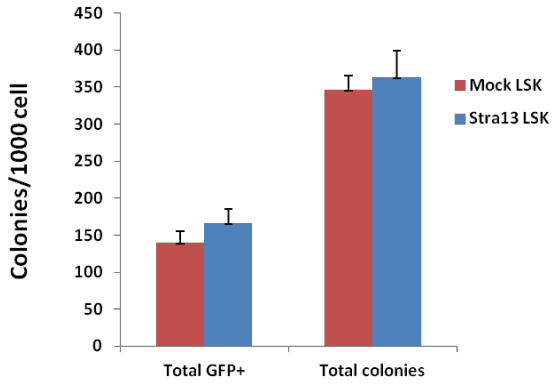


Figure 3.2: Colony forming assay with Stra13 overexpressed in LSK progenitors.
Colony forming assay from LSK cells infected with Stra13 retrovirus or mock virus. Total colonies and GFP+ colonies were counted at the end of 7 days. No significant changes in the number of colonies between Stra13 and mock control (n=3).

3.3. Stra13 is dispensable during steady state hematopoiesis

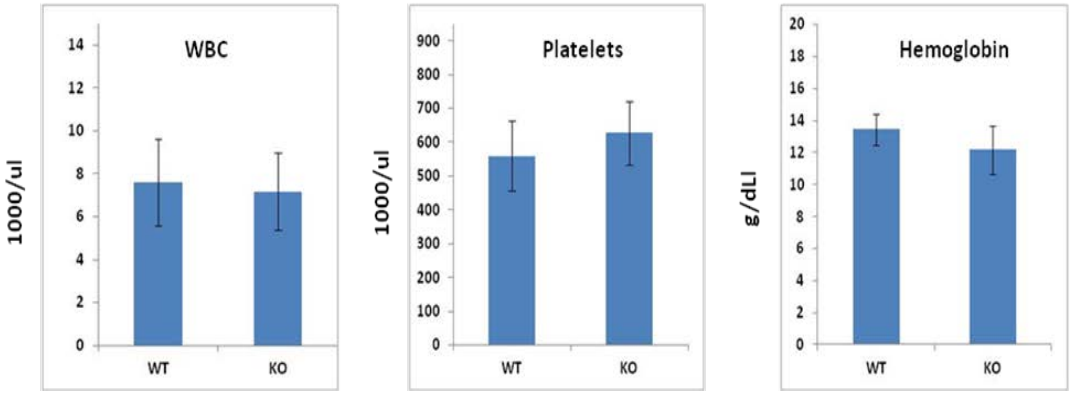
3.3.1 Mature differentiated markers of all hematopoietic lineages is similar in young Stra13 KO mice

We used the Stra13 KO mice to investigate whether its loss affects hematopoiesis. Stra13 KO mice are born normal with no apparent phenotype when compared to its littermate control. Our first step to study if any changes occur in the

hematopoietic system in Stra13 KO mice was to measure the blood counts for any visible differences in different blood parameters. Peripheral blood was isolated from the retroorbital sinus and the different blood parameters like WBCs, platelets and hemoglobin levels were analyzed. No significant differences were observed between young (2-3 months) Stra13 KO and WT littermate peripheral blood levels. (Figure 3.3.1a)

We then proceeded further to analyze the bone marrow of Stra13 KO mice. The differentiated populations of i.e. the granulocytes, lymphoid cells T and B cells were examined in the bone marrow, spleen and thymus. They were stained with the respective mAbs and were analyzed for their percentages by flow cytometry. Consistent with our previous studies (Sun et al., 2001) no changes in lymphoid (Figure 3.3.1b&c) and myeloid (Figure 3.3.1c) cells were apparent in Stra13 KO mice when compared to its wild type control.

a.



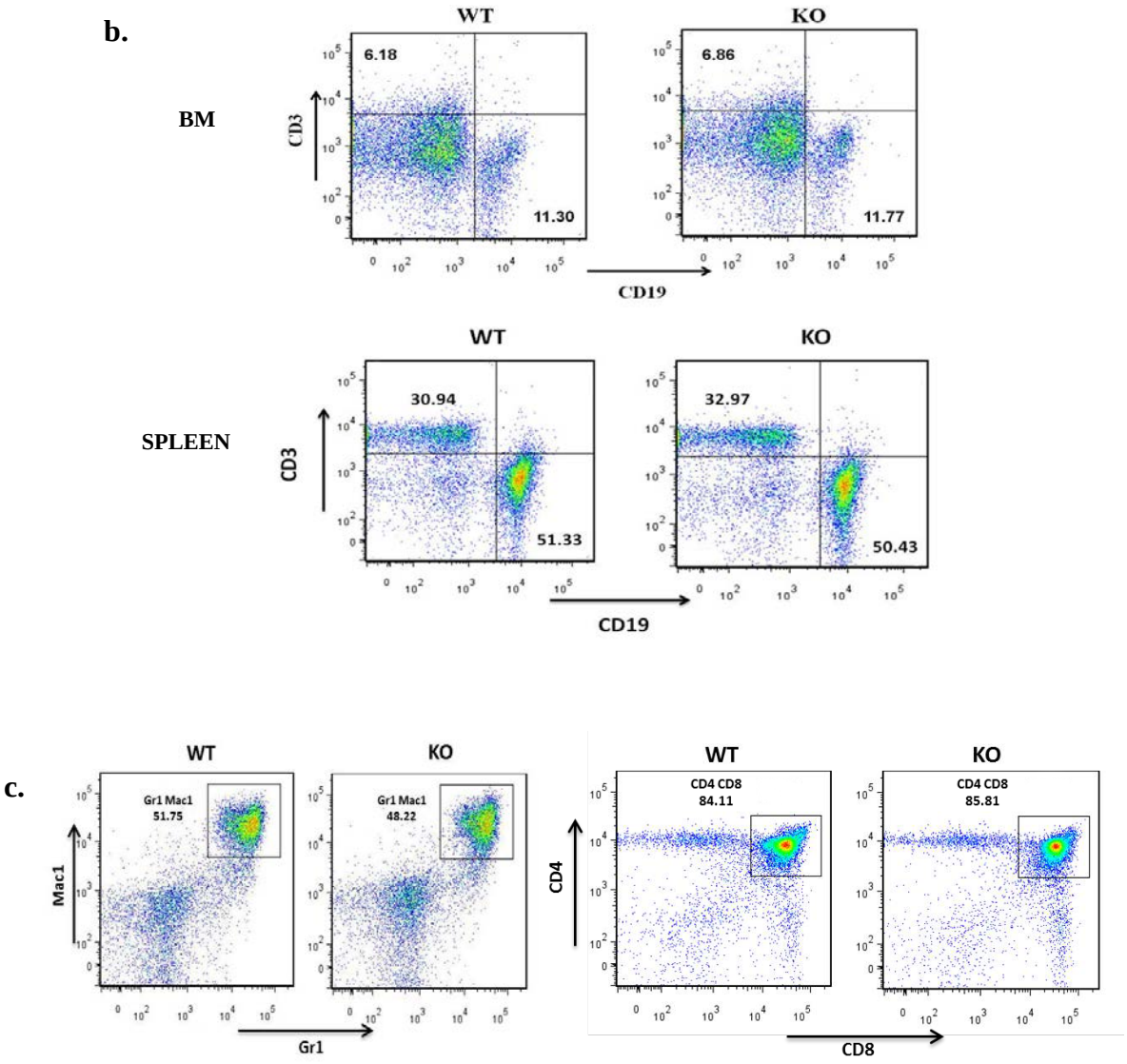


Figure 3.3.1: Similar percentages of lymphoid and myeloid cells and T cells in the bone marrow, spleen and thymus by flow cytometry in young Stra13 KO mice.

a. Peripheral blood analysis of Stra13 KO and WT control isolated from the retrorbital venous sinus using automated blood analyzer. (n=6 mice for each genotype).

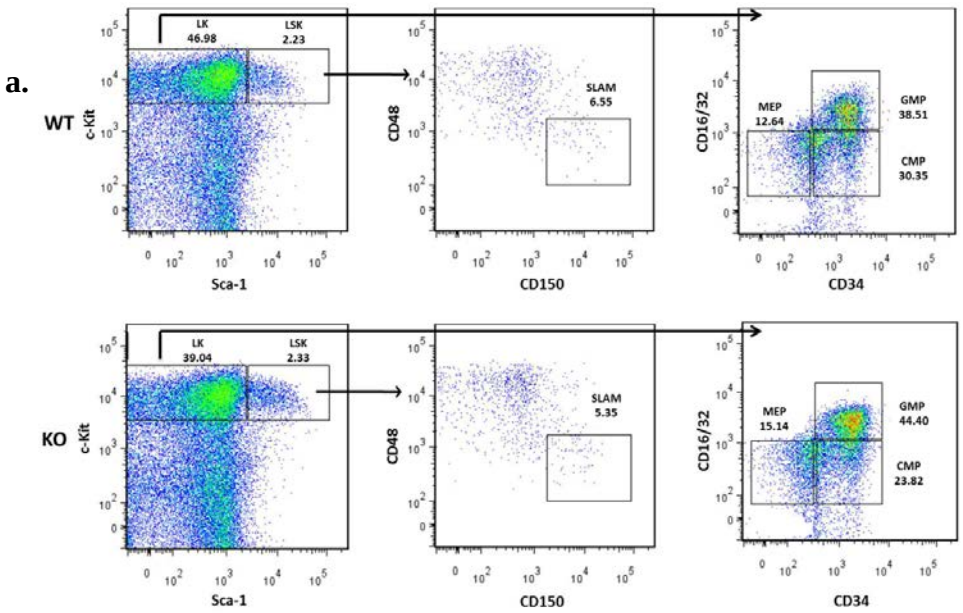
b. The bone marrow and spleen of Stra13 KO and WT control was stained with antibodies specific for B cells (CD19) and T cells (CD3) and analyzed by flow cytometry. No significant differences were observed between them. (n=3 mice for each genotype).

c. The bone marrow was stained with markers for myeloid cells -granulocytes (Gr1/Mac1) and the thymus for T cells (CD4 and CD8). No apparent differences in percentages were observed between Stra13 KO and WT littermate mice. (n=3 for each genotype).

3.3.2 Normal percentages of hematopoietic progenitors and LT-HSCs in young Stra13 KO mice

Since we did not observe any significant differences in the percentages of mature lineage makers of the myeloid and lymphoid lineages, we investigated further to study the bone marrow progenitor fraction namely the GMP, CMP, MEP, LSK and CLP of Stra13 KO mice. After lineage depletion, the bone marrow was stained with antibodies specific for the progenitors. We went ahead further to analyze the LT-HSCs i.e. $SLAM^{+}$ represented by the expression of surface markers $CD150^{+}$ $CD48^{-}$. The percentage of the all the progenitors and $SLAM^{+}$ cells in Stra13 null mice was similar to their wild type controls (Figures 3.3.2a & 3.32b.). This suggests that the loss of Stra13 has no effect on maintaining the numbers of HSCs or differentiation of their progenitors and hence Stra13 might be dispensable during steady state hematopoiesis.

a.



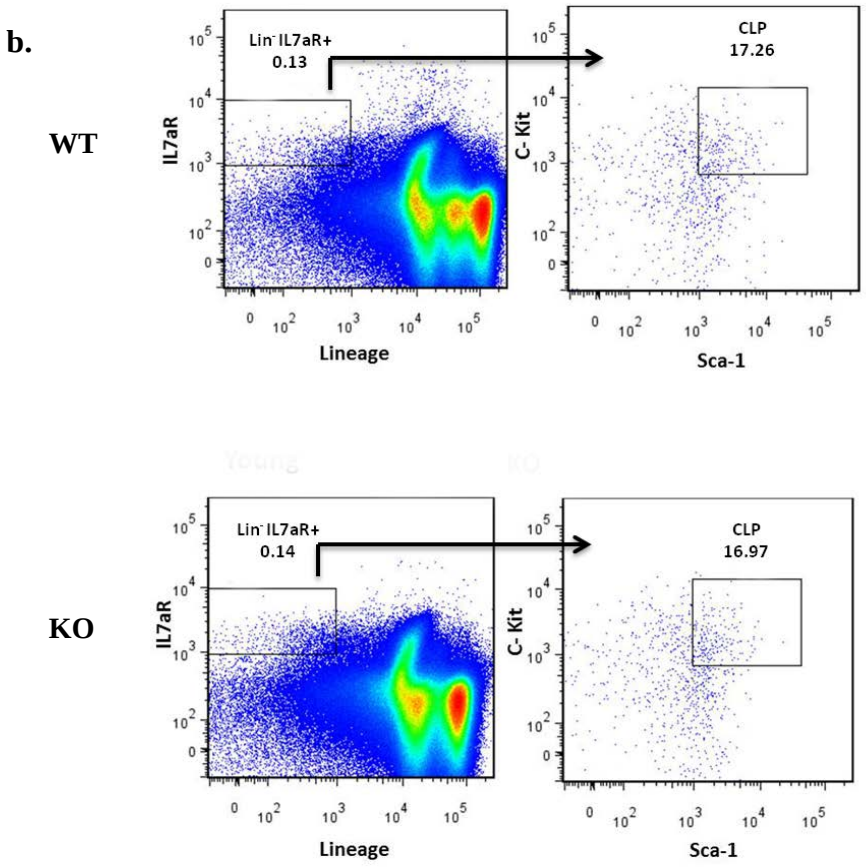


Figure 3.3.2 Stra13 loss has minimal effects on hematopoietic progenitors and LT-HSCs in young mice.

a. Lineage depleted cells are stained with fluorochrome conjugated antibodies specific for LSK, SLAM (LT-HSCs), GMP, CMP and MEP and analyzed by flow cytometry. Loss of Stra13 has no overt differences in the percentages of the progenitors and LT-HSCs in young mice. Flow cytometry plots are referred to the $Lin^{-}cKit^{hi}Sca1^{-}$ gate, in which CMPs are defined as $CD34^{+/lo}CD16/32^{int}$, GMPs as $CD34^{+}CD16/32^{+}$, and MEPs as $CD34^{-}CD16/32^{-}$. LSK cells are Lin^{-} $Sca1^{+}cKit^{+}$. LT-HSCs are defined as LSK, $CD150^{+}CD48^{-}$. Representative plot of n=3 mice.

b. Lineage negative/depleted cells were gated for IL7aR surface marker and subgated for cKit-Sca1 positive population for CLP. As seen, no major changes or differences in CLP population is observed in young Stra13 KO mice. Representative plot of n=3 mice.

3.4 Competitive Repopulating Assay (CRA) is similar in Stra13 KO mice to WT control

The gold standard for studying hematopoietic function *in vivo* is the competitive reconstitution assay as described (section 2.10). To determine if Stra13 is required for the function of primitive hematopoietic cells, we carried out competitive repopulation transplantation to assess the repopulating ability of Stra13 KO bone marrow cells in comparison with its wild type control. Donor bone marrow cells (C57BL/6-CD45.2) from wild type and Stra13 KO mice in the following ratios (0.5:1, and 5:1) were transplanted into lethally irradiated C57BL/6 CD45.1 (congenic) recipient mice. A fixed dose of competitor cells from CD45.1/CD45.2 mice were transplanted together with the donor cells. Chimerism and repopulation of the bone marrow donor cells was assessed by flow cytometry. The different time points of analysis were 4 and 8 weeks. At 4 and 8 weeks after transplantation, the donor cell ratio calculated as (donor cells/donor cells+competitor cells) were comparable between the WT and Stra13 KO donor cells. At 8 weeks post transplantation, flow cytometric analysis of the transplanted recipients revealed that Stra13 KO and WT BM cells were equally capable of reconstituting the bone marrow.

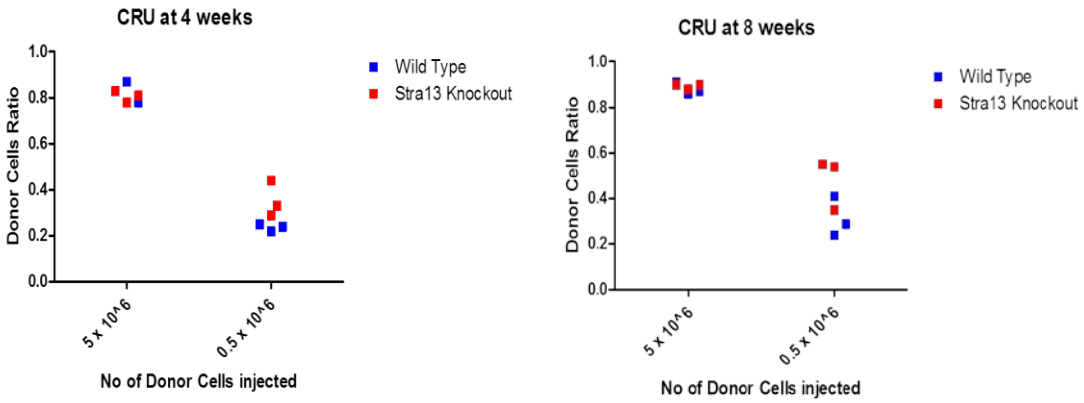


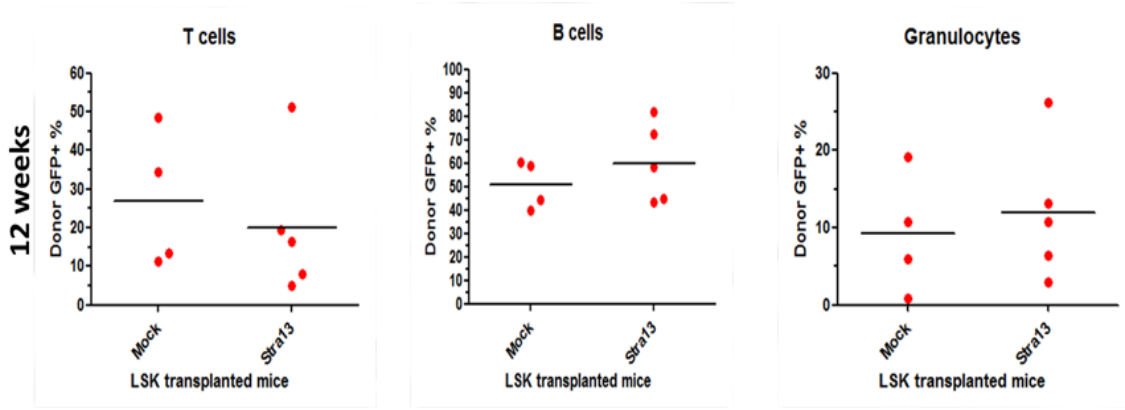
Figure 3.4 Competitive Repopulation Assay is similar in Stra13 KO and WT bone marrow cells.
The graph depicts the ratio of donor cells WT (blue dots) and Stra13 KO donor cells (red dots) at 4 weeks and 8 weeks after transplantation with different BM cell numbers $5x10^6$ and $0.5x10^6$ along with a fixed number of competitor cells $1x10^6$ transplanted. Graphs represent data for two different cell doses $5x10^6$ and 0.5×10^6 bone marrow cells transplanted into 3 recipient mice for each cell dose. (n=3 mice for each genotype)

3.5 Overexpression of Stra13 in LSK and transplantation has little effect on self renewal and differentiation

Since loss of function of Stra13 did not reveal any discernible hematopoietic phenotype, we overexpressed Stra13 in the LSK population and transplanted into recipient bone marrow to investigate if it has any effect on short term and long term HSC renewal. Stra13 was cloned into a retrovirus vector MSCV-IRES-GFP. LSK ells from a pool of 2-3 WT mice were flow sorted under aseptic conditions, infected with Stra13 retrovirus or with its mock control twice on retronectin coated plates with MOI=20. They were transplanted into lethally radiated (10.5 Gy) congenic (CS45.1) mice. Splenocytes from congenic mice were transplanted together to provide short term radio protection. Mouse peripheral blood from the transplanted mice was analyzed at different time points to check for transduction efficiency by monitoring GFP^+ cells and surface marker expression of mature

myeloid and lymphoid markers. At 12 weeks post transplantation, high GFP^+ cells in both B cells and granulocytes were observed in Stra13 overexpressing mice compared to their mock control but the overall percentages of B cells and myeloid cells did not vary much with respect to the mock transplanted mice (Figure 3.5a). At 18 weeks post transplantation, the GFP^+ cells remained high in the B cells which might suggest better reconstitution and differentiation towards B cell lineage in Stra13 overexpressing mice (Figure 3.5b).

a.



b.

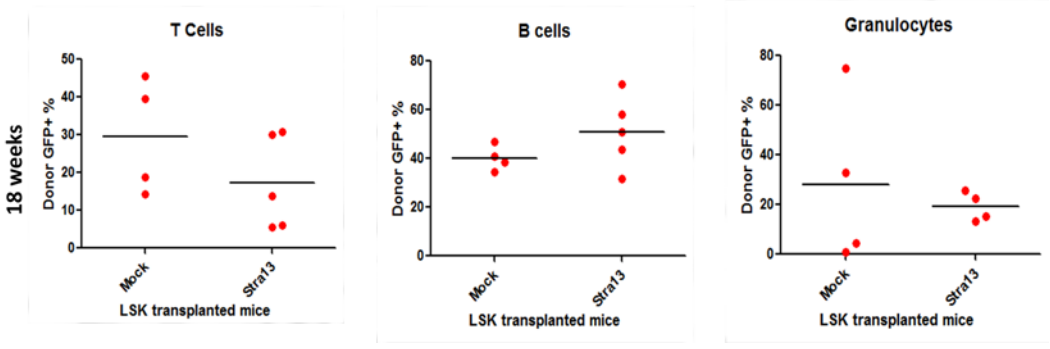


Figure 3.5: Similar reconstitution of myeloid and lymphoid cells in Stra13 overexpressing mice compared to WT control carried out by flow cytometric analysis of peripheral blood.

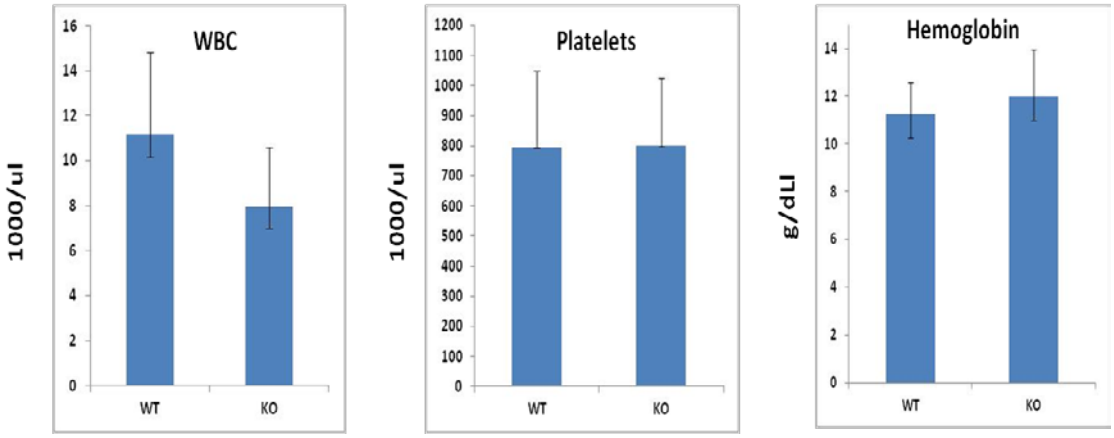
a&b. Mouse peripheral blood was isolated by retroorbital puncture and analyzed by flow cytometry for mature myeloid (Gr-1 Mac-1) and lymphoid markers (CD19 for B cells) and (CD3 for T cells) by flow cytometry at 12 weeks (a) and 18 weeks (b) post transplantation. (n=4 for WT and n=5 for Stra13 KO mice analyzed)

3.6 Ageing Stra13 knockout mice exhibit reduced percentages of long term HSCs and progenitors

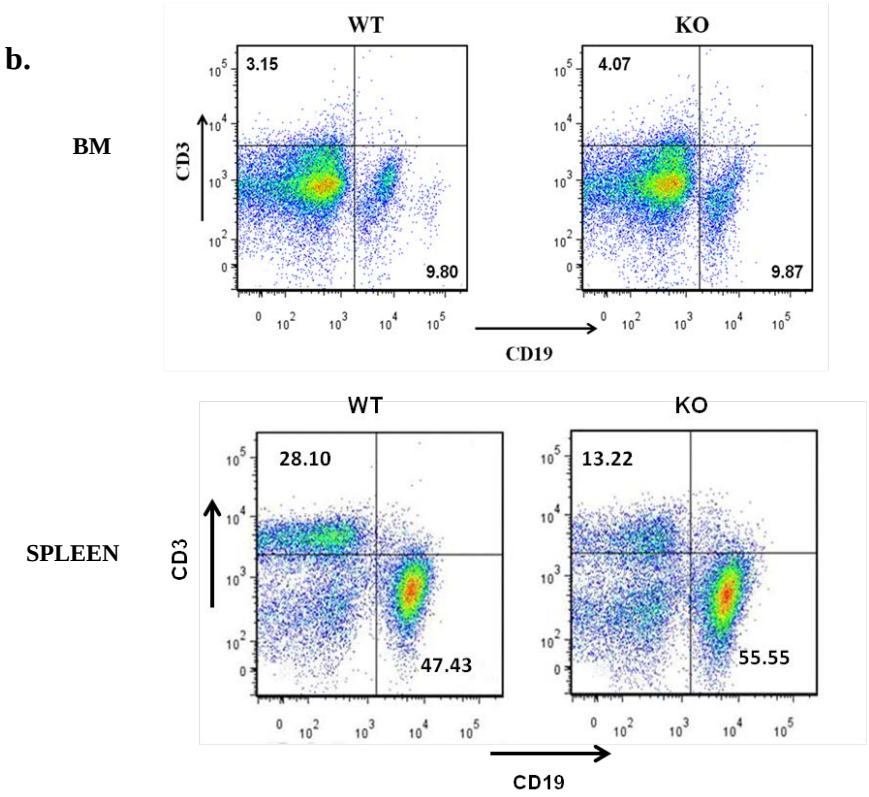
3.6.1 Mature differentiated markers of all hematopoietic lineages are similar in aged Stra13 KO mice

Our previous results have demonstrated that the loss Stra13 has minimal effects on the hematopoietic system with no visible phenotype. As Stra13 expression is upregulated in the LSK progenitors during ageing, we were interested to understand whether loss of Stra13 has any role to play during ageing. Stra13 KO and their WT littermate control were aged for 12-14 months. Similar to young Stra13 mice, the peripheral blood was isolated to check the levels of WBCs, platelets and hemoglobin. The mature markers of the myeloid and lymphoid lineage were analyzed by flow cytometry in a similar way as the young Stra13 KO mice. Interestingly, similar to young Stra13 young knockouts, aged Stra13 KO had very little changes in the peripheral blood counts with respect to WBCs, platelets and hemoglobin levels. Similarly the percentages of lymphoid cells- T and B cells and the myeloid cells- granulocytes in ageing Stra13 KO mice were similar to WT controls.

a.


WBC
1000/ul
16
14
12
10
8
6
4
2
0
WT
KO
Platelets
1000/ul
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
WT
KO
Hemoglobin
g/dLl
14
12
10
8
6
4
2
0
WT
KO

b.


BM
WT
KO
3.15
4.07
9.80
9.87
CD3
CD19
SPLEEN
WT
KO
28.10
13.22
47.43
55.55
CD3
CD19

c.

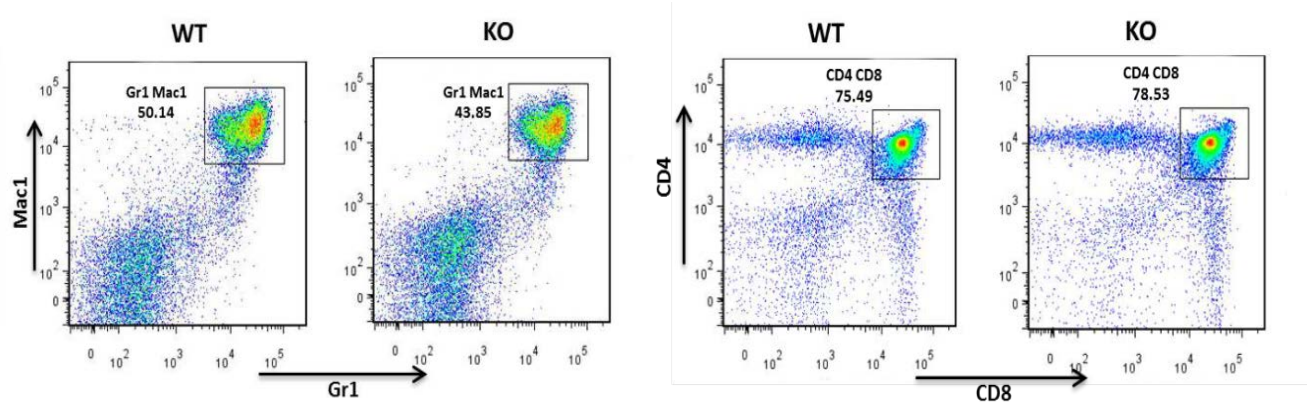


Figure 3.6.1: Similar percentages of lymphoid and myeloid cells and T cells in the bone marrow, spleen and thymus by flow cytometry in aged Stra13 KO mice.

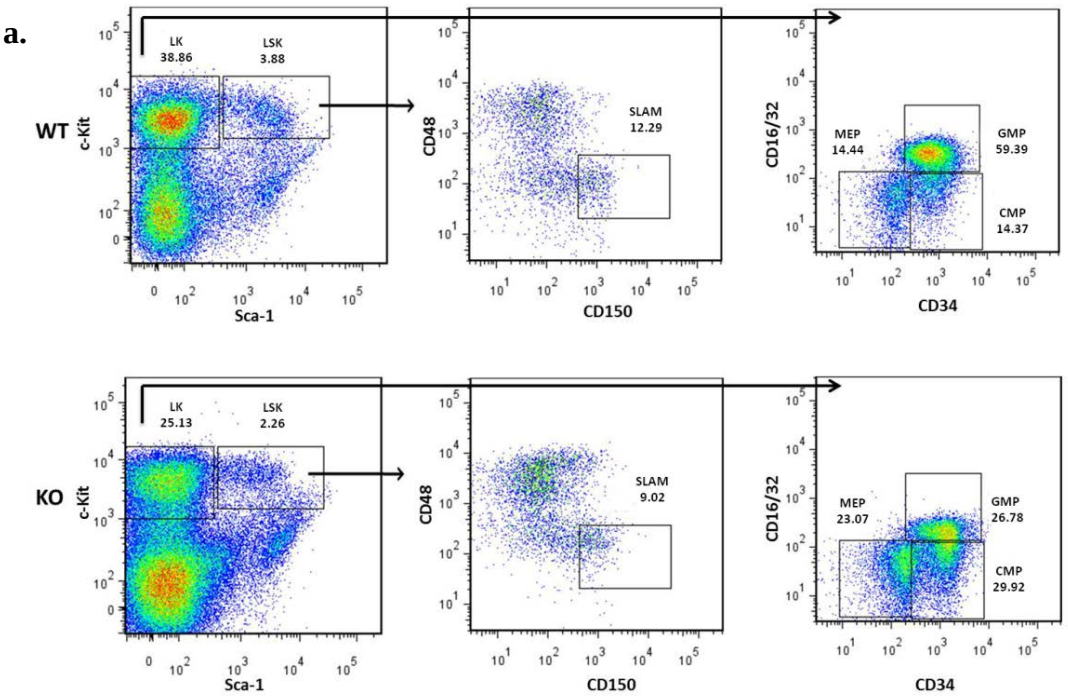
a. Using an automated blood counter, the different blood parameters were measured in ageing (12-14 months) Stra13 KO mice and ageing WT controls (n=5 for each genotype).
b. The bone marrow and spleen of aged 14 months Stra13 KO and WT aged littermate controls were stained with antibodies specific for B cells (CD19) and T cells (CD3) and analyzed by flow cytometry. No significant differences were observed between them. Representative plots for n=3 mice analyzed.
c. The bone marrow cells were stained with markers for myeloid cells -granulocytes (Gr1/Mac1) and the thymus for T cells (CD4 and CD8). No apparent differences in percentages were observed between aged Stra13 KO and WT littermate mice. Representative plots for n=3 mice analyzed.

3.6.2. Decreased bone marrow stem and progenitor cells in aged Stra13 KO mice

Further investigation into the bone marrow progenitors of ageing Stra13 KO mice showed a reduction in the percentages of the LSK by approximately 1.8 fold. It has been reported that differentiation is skewed towards the myeloid lineage in ageing mice (Beerman et al., 2010). Compared to WT ageing mice which clearly demonstrated myeloid bias as observed by the increase in GMP percentage, Stra13 KO mice infact showed reduction in the percentage of GMP by almost 50%. Likewise there was a 50% increase in the percentage of CMP and a moderate increase in the MEP progenitor fraction in Stra13 null bone marrow

compared to WT (Figure 3.6.2a). The SLAM^{+}/LT-HSCs which are subgated from the LSK fraction in Stra13 KO mice also reduced by 1.3 fold compared to littermate control (Figure 3.6.2a). These results indicate that the loss of Stra13 has its dominant effect on the HSC and progenitor population in aged animals. The reduction of different progenitor cell populations predominantly originated from a reduced number of LT-HSCs. The described stem and progenitor abnormalities were progressive as the mice aged, being undetectable in young Stra13 KO mice.

a.



b.

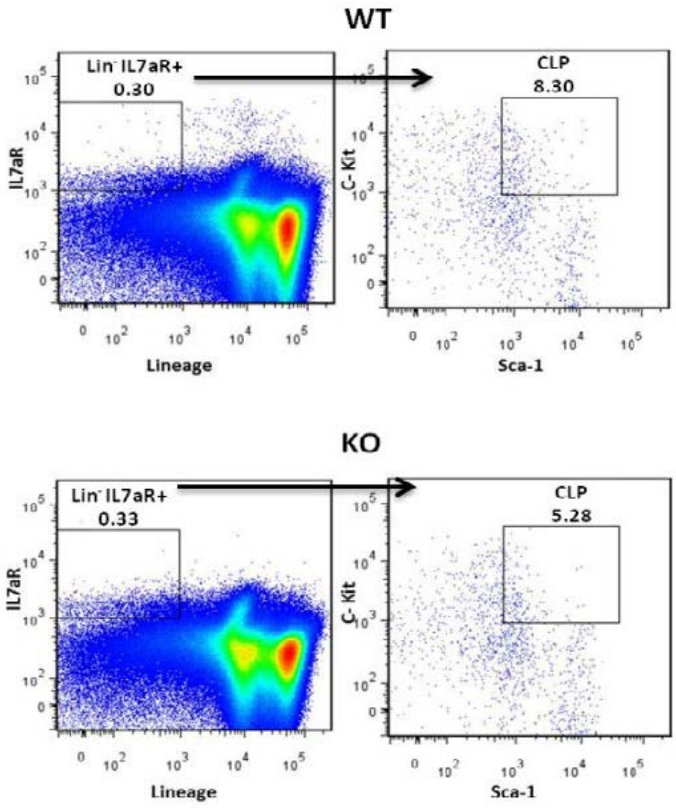


Figure 3.6.2 Decreased HSC pool and progenitor cells in aged Stra13 KO mice

a. Lineage depleted cells are stained with fluorochrome conjugated antibodies specific for LSK, SLAM (LT-HSCs), GMP, CMP and MEP and analyzed by flow cytometry. Ageing causes a decline in the numbers of LSK and $SLAM^{+}$ cells and an alteration of the progenitor fractions in Stra13 null bone marrow when compared to their littermate aged controls. Representative plots of n=3 mice analyzed.

Flow cytometry plots are referred to the $Lin^{-}cKit^{hi}Sca1^{-}$ gate, in which CMPs are defined as $CD34^{+/lo}CD16/32^{int}$, GMPs as $CD34^{+}CD16/32^{+}$, and MEPs as $CD34^{-}CD16/32^{-}$. LSK cells are Lin^{-} $Sca1^{+}cKit^{+}$. LT-HSCs are defined as LSK, $CD150^{+}CD48^{-}$.

b. Lineage negative/depleted cells were gated for IL7aR surface marker and subgated for cKit-Sca1 positive population specific for CLP. Moderate reduction in CLP population in Stra13 KO is seen in comparison to WT CLP. Representative plots of n=3 mice analyzed.

3.7 LSK fraction of Stra13 KO mice is sensitive to genotoxic stress

Our previous results demonstrated that ageing Stra13 KO mice have reduced LSK percentages; hence we investigated further whether the stress response in the hematopoietic compartment in the absence of Stra13 has any effect on the stem and progenitor cell compartment. Young Stra13 KO mice and their WT littermate controls were subjected to a single sublethal dose of whole body irradiation (5Gy) and the HSC compartment was analyzed after 24 hours (shorter duration of 6 hrs and 12 hrs did not reveal differences between KO and WT mice) by flow cytometry. Similarly, the myelotoxic drug 5-FU (150mg/kg) was intraperitoneally injected and the HSC compartment was analyzed by flow cytometry after 7 days. Pilot experiments were performed to record peripheral blood counts every 3 days for up to one month after 5 FU injections (data not shown). We found delayed recovery of blood counts in Stra13 KO mice by day 7 when compared to WT control and hence this time point was chosen to analyze the BM compartment. Changes in HSC quiescence correlate to reciprocal changes in chemoresistance and radioresistance. Interestingly, we found that the lack of Stra13 in HSCs sensitizes them to genotoxic stress. There was a drastic reduction of LSK progenitors in Stra13 KO mice both by radiation and 5FU treatment in comparison to their littermate controls after 24 hours and 7 days respectively (Figure 3.7).

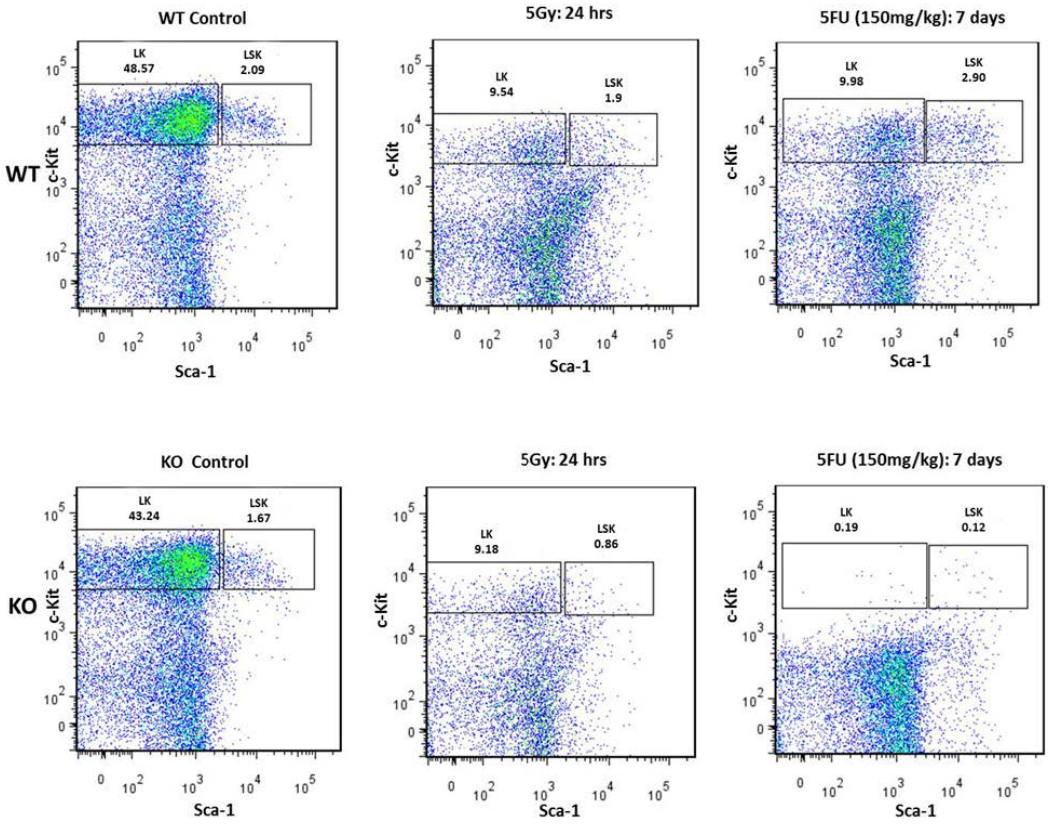


Figure 3.7: Stra13 KO LSK fraction is sensitive to genotoxic stress.
Stra13 WT and KO mice were subjected to genotoxic stress in the form of sub lethal whole body gamma irradiation (5Gy) and after 24 hours were analyzed for the LSK population gated on Lin^- population (middle panel). The myelotoxic drug 5FU (150mg/kg) was injected and LSK population analyzed after one week by flow cytometric analysis (right panel). In both cases, Stra13 WT and KO mice served as controls without any genotoxic treatment (left panel). Representative plots of n=3 mice analyzed.

3.8 Gene expression profiling highlights genes potentially involved in hematopoietic stem cell ageing in Stra13 KO mice.

Knowing that the aged Stra13 KO mice show reduced percentages of LSK and $SLAM^+$ cells, we were interested to identify Stra13 target genes that could account for the reduced HSC numbers in Stra13 KO mice. We performed gene expression profiling using microarray on LSK cells isolated from ageing Stra13

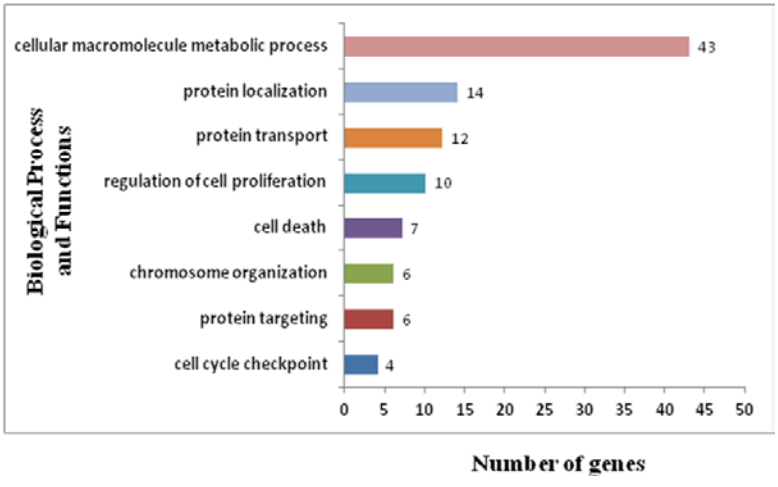
KO and ageing littermate WT controls (12-13 months) [that did not develop the autoimmune phenotype]. Sorted LSK cells from young WT and Stra13 KO mice (2-3 months) were also hybridized and served as controls to identify the genes responsible for ageing phenotype. From these cells, total RNA was isolated, reverse transcribed, labeled and subsequently hybridized to Illumina mouse WG-6 v2.0 array. The microarray identified 133 genes that were differentially expressed between ageing Stra13 KO and WT control. The top 6 genes that were upregulated and downregualted in Stra13 KO LSK vs ageing WT LSK were as follows:

Table II: Top genes differentially regulated in ageing Stra13 KO LSK vs WT LSK cells

Gene Symbol	Gene Name	P <0.05
Upregulated genes in Stra13 KO LSK (old) vs Stra13 WT LSK (old)		
Igl-V1	immunoglobulin lambda variable 1	0.02
Tctn3	tectonic family member 3	0.03
Tpst1	protein-tyrosine sulfotransferase 1	0.003
Nedd4l	neural precursor cell expressed, developmentally down-regulated gene 4-like	0.02
Bcorl1	BCL6 co-repressor-like 1	0.03
Tstd2	thiosulfate sulfurtransferase (rhodanese)-like domain containing 2	0.03
Downregulated genes in Stra13 KO LSK (old) vs Stra13 WT LSK (old)		
Ywhab	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta	0.04
Stx12	syntaxin 12	0.01
Trib3	tribbles pseudokinase 3	0.02
Fam168b	family with sequence similarity 168, member B	0.001
Tgm2	transglutaminase 2, C polypeptide	0.03
Ddah1	dimethylarginine dimethylaminohydrolase 1	0.01

Using DAVID functional analysis tool (Version 6.7), the biological significance of the differentially expressed genes list was better realised by classifying them based on Gene Ontology and with relevance to biological processes, molecular functions and pathways (Figure 3.8a). The top biological processes were cellular macromolecule metabolic process, cellular nitrogen metabolic process, gene expression, protein transport and localization, positive and negative regulation of cell proliferation, cell cycle checkpoints etc. Unbiased clustering between all the samples from young and ageing WT young and ageing Stra13 KO LSKs revealed four distinct clusters. Cellular metabolic process was the top biological process that was common in all the three clusters except in cluster 2. In cluster 1, the top genes that were upregulated were Igl-v1, Nedd4l, Tctn3, Bcorl1, Tpst1. Interestingly; these same genes were significantly upregulated in ageing Stra13 vs ageing WT LSK and may be directly or indirectly involved in ageing Stra13 HSC phenotype.

a.



b.

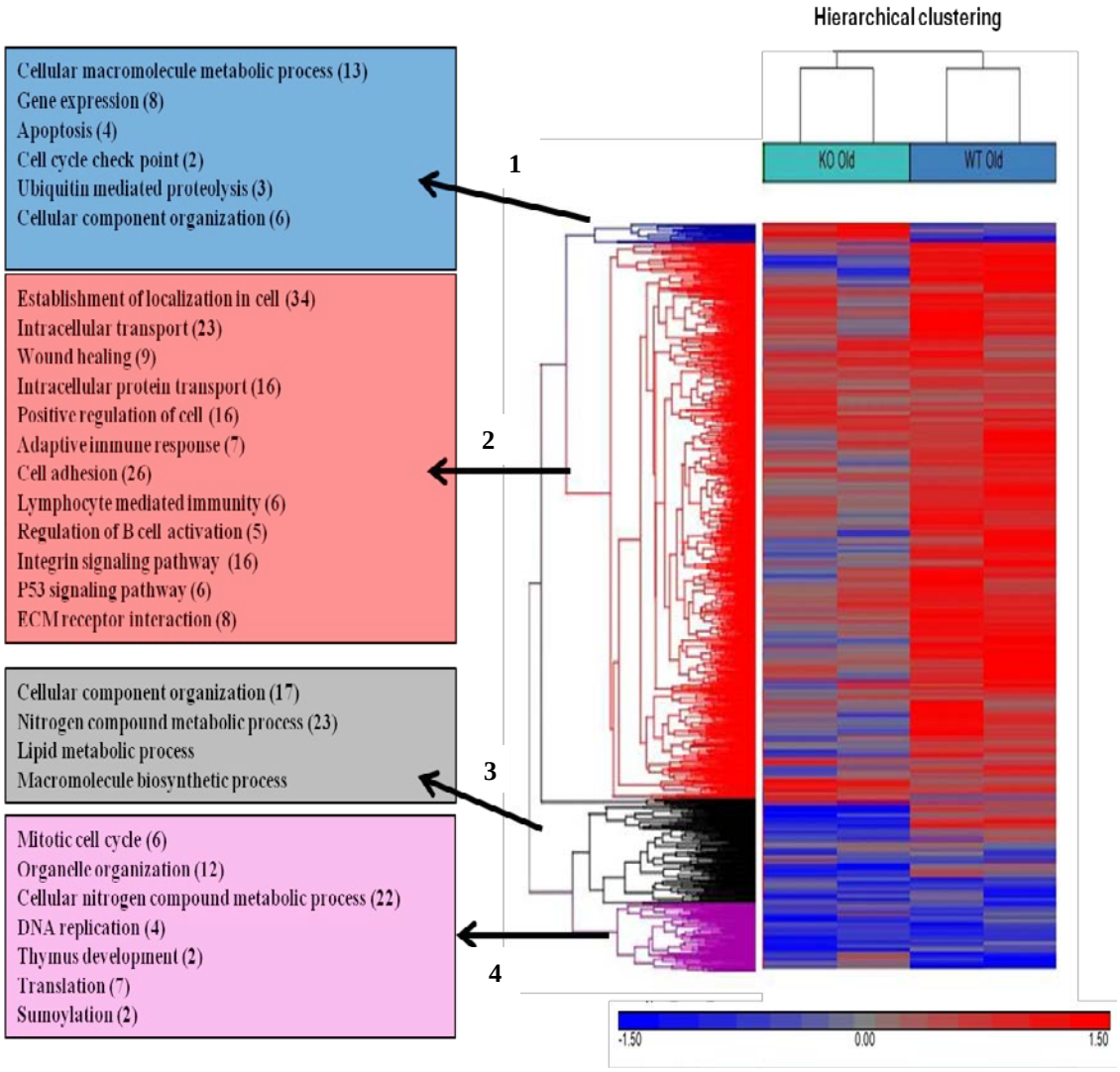


Figure 3.8: Heat Map, biological processes and pathway analysis of the differentially expressed genes in ageing Stra13 KO LSK vs WT LSK cells.
a. Biological processes and functions altered between old Stra13 KO LSK vs. aged WT littermate control cells. Using DAVID annotation tool based on gene ontology, the top biological processes and functions were analyzed and schematically represented.
b. A heat map showing the hierarchical unsupervised clustering of significantly deregulated genes. 4 patterns of concentrated deregulation were picked out using Partek Genomics Suite and plotted along with their associated molecular and cellular functions using DAVID analysis tool. Number of genes involved in the differential regulation of molecular and cellular functions is highlighted within brackets and the appropriate functions mentioned in each cluster is based on the significance of the differential regulation; the colour gradient represents fold change.

3.9 Gene expression profiling reveals genes potentially involved in stress hematopoiesis in Stra13 KO mice

Our previous results have highlighted that the Stra13 KO LSK compartment is sensitive to genotoxic stress i.e. sub lethal doses of gamma radiation. In order to elucidate the potential target genes that could be involved in this phenotype we took a similar approach of gene expression profiling. We performed gene expression profiling using microarray on LSK cells that were sorted after 24hrs from young Stra13 KO mice and their WT littermate subjected to sub lethal dose of whole body gamma radiation (5Gy). Sorted LSK cells from young WT and Stra13 KO mice (2-3 months) without radiation exposure were also hybridized and served as controls. From these cells, total RNA was isolated, reverse transcribed, labeled and subsequently hybridized to Illumina mouse WG-6 v2.0 array. The data were imported into Partek Genomics Suite using RMA (Robust Multi-Chip Average) normalization. Differential expression and its statistical significance were calculated using linear contrasts with an ANOVA (analysis of variance) model. We identified 80 differentially expressed genes that were deregulated between Stra13 KO LSK and WT LSK upon radiation treatment. The top genes that were upregulated and downregualted in Stra13 KO LSK vs ageing WT LSK were as follows:

Table III: Top genes differentially regulated in Stra13 KO vs WT LSK cells upon sub lethal radiation treatment (5Gy)

Gene Symbol	Gene Name	P<0.05
Upregulated genes in Stra13 KO LSK (5Gy) vs Stra13 WT LSK (5Gy)		
Zfyve21	zinc finger, FYVE domain containing 21	0.04
fit3	interferon-induced protein with tetratricopeptide repeats 3	0.03
Slc25a19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	0.01
Rdh11	retinol dehydrogenase 11	0.02
Ralgps2c	Ral GEF with PH domain and SH3 binding motif 2	0.01
Sapcd2	Sapcd2 suppressor APC domain containing 2	0.03
Cd59a	CD59a antigen	0.02
Car12	carbonic anyhydrase 12	0.01
Narfl	nuclear prelamin A recognition factor-like	0.03
Pfkfb4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.03
F10	coagulation factor X	0.04
Srgap2	SLIT-ROBO Rho GTPase activating protein 2	0.02
Downregulated genes in Stra13 KO LSK (5Gy) vs Stra13 WT LSK (5Gy)		
Upf1	UPF1 regulator of nonsense transcripts homolog	0.02
Tmed7	transmembrane emp24 protein transport domain containing 7	0.03

Zfp930	zinc finger protein 930	0.04
Pdgfb	platelet derived growth factor, B polypeptide	0.03
Srxn1	sulfiredoxin 1 homolog	0.02
Zdhhc19	zinc finger, DHHC domain containing 19	0.04
Jarid1a	Kdm5a lysine (K)-specific demethylase 5A	0.042

Using DAVID functional analysis tool (Version 6.7), the differentially expressed genes were annotated based on gene ontology. The top biological processes and pathways relevant to these genes were regulation of cell division, regulation of signal transduction, platelet derived growth factor (PDGF) signaling pathway, and actin cytoskeleton reorganization. For a clear understanding of other pathways important in LSK in response to radiation, we grouped the differentially expressed genes and unbiased clustering between all the samples revealed two distinct cluster using DAVID annotation tool (Figure 3.9). In cluster 1, the genes that were upregulated were Ifit-3, Zfyve21, Slc25a19, CD59 were similar to genes upregulated in Stra13 KO LSK (5Gy). Cluster 2 generally showed downregulation of many genes similar in Stra13 KO LSK (5Gy) like Jarid1a, Upf1, Pdgfb, Tmed7 which may play a role in the sensitivity of the LSK cells in Stra13 KO mice towards irradiation.

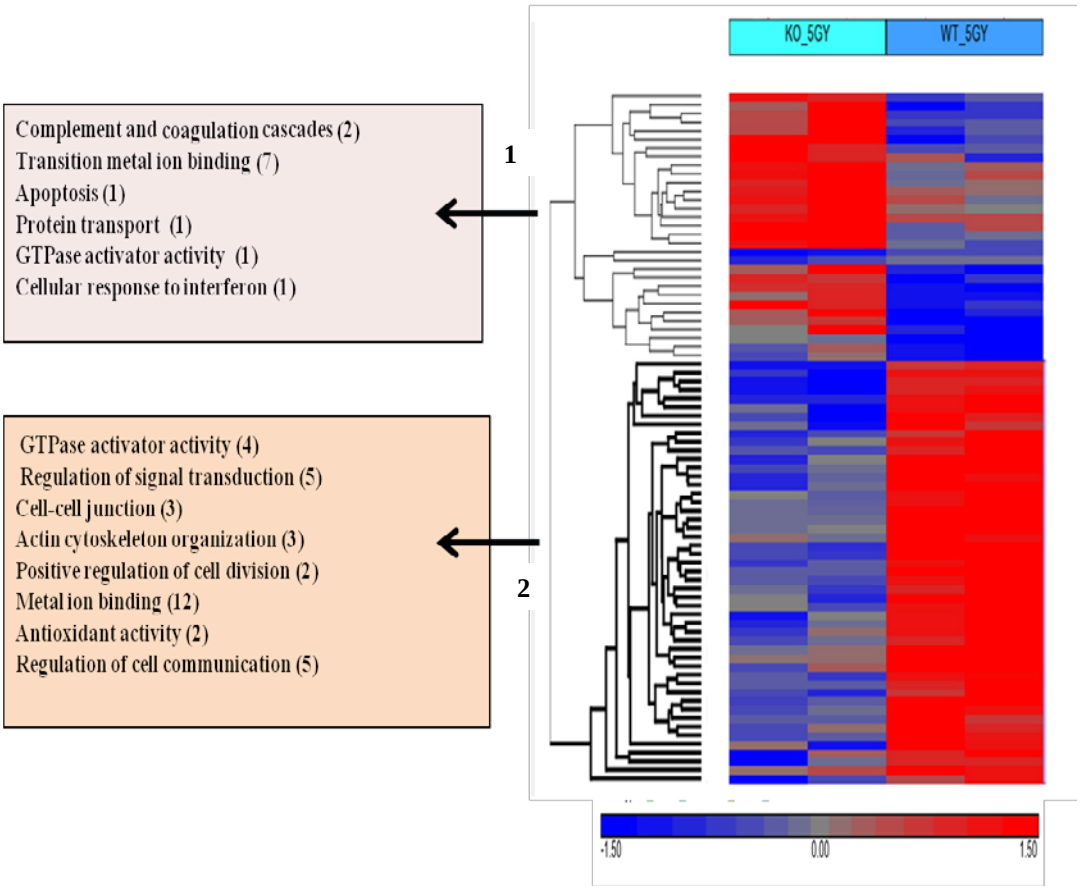


Figure 3.9: Heat Map, biological processes and pathway analysis of the differentially expressed genes in Stra13 KO LSK vs WT LSK cells upon sub lethal gamma radiation.

Unsupervised clustering of significantly deregulated genes in LSK cells treated with radiation between Stra13 KO LSK and WT LSK. 2 patterns of concentrated deregulation were picked out using Partek Genomics Suite and plotted along with their associated molecular and cellular functions using DAVID analysis tool. Number of genes involved in the differential regulation of molecular and cellular functions is highlighted within brackets and the appropriate functions mentioned in each cluster is based on the significance of the differential regulation; the colour gradient represents fold change.

CHAPTER 4

DISCUSSION

4. DISCUSSION

The hematopoietic system serves as a paradigm to understand the biology of hematopoietic stem cells (HSCs) and the processes involved in HSC ageing and disease. It is arguably the best characterized system among all other tissues of the human body due to its unique biological properties allowing easy experimental manipulation for pre-clinical studies. Transcriptional regulation of hematopoiesis has been studied in many organisms, which govern whether HSCs will self-renew or differentiate towards the myeloid, lymphoid or erythro-megakaryocytic lineages. However, the critical signals and transcription factors that control HSC fate are still not clearly defined. Over the years it has become evident that disruption of HSC homeostasis brings about qualitative and quantitative changes that affect the ability of HSCs to self renew and maintain normal blood levels and functions that eventually affect the ageing process and longevity of organisms.

The main goal of my dissertation was to study the role of Stra13 in adult hematopoiesis as there have been no reports on its role in the hematopoietic compartment. Following a classical reverse genetic approach, we have demonstrated that Stra13 is highly expressed in LT-HSCs i.e. $SLAM^{+}$ ($CD150^{+}CD48^{-}$) cells and LSK progenitors and its expression is downregulated in differentiated progenitor subsets CMP, GMP and MEP which indicates that Stra13 expression maybe important for self renewal of HSCs during steady state hematopoiesis. However, over-expression of Stra13 in LSK cells *in vitro* did not promote the self-renewal of HSCs or affect the clonogenic potential of

hematopoietic progenitor populations by colony forming assays. We employed Stra13 knockout mice to understand whether its loss had any effect on the hematopoietic compartment. In accordance to our previous report, there were no significant differences in the major hematopoietic compartments - myeloid and lymphoid lineages in the bone marrow, spleen and thymus of young (2-3 months) of Stra13 KO mice (Sun et al., 2001). Stra13 KO bone marrow showed similar percentages of $SLAM^{+}$, LSK and other progenitors CMP, GMP, MEP and CLP compared to their wild type littermate control. Likewise, *in vivo* competitive repopulation transplantation assay confirmed that Stra13 null bone marrow can reconstitute the recipient bone marrow similar to its wild type control at 4 and 8 weeks respectively. Our results demonstrate that loss or gain of Stra13 does not alter the number, function and repopulating ability of hematopoietic stem and progenitor cells and therefore Stra13 is dispensable during steady state hematopoiesis. One possibility that may explain the lack of phenotype is functional redundancy with other related transcription factors. Stra13 is highly related to Sharp-1 and both proteins mutually regulate each other (Azmi et al., 2003; Li et al., 2003). The loss of Stra13 function may be compensated by Sharp-1. However, the role of Sharp1 in hematopoiesis has not been investigated and it would be interesting to study if loss of both Stra13 and Sharp1 affect steady state hematopoiesis using Stra13-Sharp1 double knockout mice. Nonetheless, it is possible that other more distantly related bHLH-O transcription factors like Hes1, Hes5 and Hey 2 whose role in hematopoiesis have already been established may show some degree of functional redundancy with Stra13 in HSCs or compensate

for the loss of Stra13 function (Guiu et al., 2013; Rowlinson and Gering, 2010; Yu et al., 2006b). This still remains to be explored.

Stra13 expression is upregulated in the LSK progenitors in ageing mice compared to young mice. These studies suggest that Stra13 expression may be modulated to provide a protective mechanism counteracting HSC ageing in order to maintain a balanced HSC composition. With ageing, normal wild type mice show elevated numbers of LT-HSCs and myeloid bias (Dykstra et al., 2011; Muller-Sieburg et al., 2004). However, we observed decreased percentages of $SLAM^{+}$ and LSK cells and skewed myeloid and lymphoid progenitor fractions in ageing Stra13 KO mice. The GMP percentages were significantly lower compared to WT littermate control. On the other hand, we did not detect any changes in the relative composition of the different mature lineages in peripheral blood, bone marrow, spleen and thymus. This is not surprising as it has been previously reported that in spite of clear changes in HSC composition, the blood lineage ratio is not significantly altered (Cho et al., 2008). There exists a homeostatic mechanism that can overcome the differentiation bias of HSCs. There was an increase in $CD48^{+}$ population in Stra13 KO mice compared to WT control (Figure 3.6.2.a). Previous reports have demonstrated the upregulation of CD48 on enriched HSCs isolated from 5FU treated bone marrow (Venezia et al., 2004). CD48 was preferentially expressed by restricted colony forming progenitors but not HSCs or transiently reconstituting multipotent progenitors in young adult bone marrow and hence they were considered as non HSC-contaminating cells. (Kiel et al., 2005; Yilmaz et al., 2006). Competitive repopulation assays in which $CD48^{+}$ and $CD48^{-}$ cells from

old, reconstituted, or mobilized mice were transplanted into lethally radiated recipients confirmed that $CD48^-$ cells have the multilineage reconstitution potential when compared to $CD48^+$ cells which showed little or no HSC activity in fractions of bone marrow cells, reconstituted bone marrow cells and mobilized splenocytes (Yilmaz et al., 2006). Hence, the increase in $CD48^+$ percentages in aged Stra13 KO mice is another indicator of the decreased self renewal potential of Stra13 KO mice where majority of LT-HSCs become committed to form multipotent progenitors when compared to WT control. It would be interesting to study if $CD48^+$ cells in Stra13 KO mice are lymphoid primed multipotent (LMPP) progenitors. Since the CLP compartment of Stra13 KO mice has not changed in both young and aged mice when compared to WT littermates we can speculate that the LMMPs which are precursors of CLP that have lost GMP potential may retain the same lymphoid differentiation capacity between Stra13 KO and WT control although this was not explored in our study. Similarly whether competitive repopulation transplantation assays using ageing Stra13 KO mice will recapitulate decreased myeloid reconstitution (as seen in ageing Stra13 KO) in recipient mice compared to its ageing WT control will be an interesting to explore. Our Stra13 KO phenotype with respect to hematopoiesis is similar to SIRT3 KO and FoxO3 KO mice in which the loss of either gene does not have any impact on stem cell numbers during steady state hematopoiesis. However, upon ageing, these KO mice demonstrate reduced frequency of HSCs due to elevation of reactive oxygen species (ROS) production (Brown et al., 2013; Miyamoto et al., 2007; Yalcin et al., 2008). In order to elucidate the potential

genes that may play a role in HSC ageing in Stra13 KO mice, we employed micro-array analysis. Through gene expression profiling between aged Stra13 KO LSK and aged WT LSK, we have identified potential target genes that were significantly upregulated in Stra13 KO vs WT LSK fractions. Broadly, many biological processes and functions like acetylation, cell proliferation, cell cycle check points and cell death were deregulated in ageing Stra13 KO LSK. In particular cellular metabolic processes were highly deregulated based on DAVID gene ontology tool. HSCs generally have a low metabolic activity that prevents them from damage by ROS. An increase in metabolic activity in aged Stra13 KO may possibly indicate build up of ROS. FoxO3 is considered as a critical regulator of oxidative stress in HSCs (Yalcin et al., 2008), but FoxO3 was not in our list of differentially expressed genes that were upregulated in ageing Stra13 KO LSK. However, some of the mitochondrial genes like Ddah1, Tgm2, Acadvl and Mat2b were significantly downregulated. These results may indicate a disruption of mitochondrial machinery and build up of ROS that may lead to oxidative stress in ageing Stra13 LSK although this was not examined in our study.

Similarly, BCoRL1 a homolog of BCor complex, a known co repressor of BCl-6 was upregulated in ageing Stra13 LSK. In a study published recently, deletion of BCorL1 in Runx1 KO (an important HSC transcription factor) Lin^- cells enhances its replating capacity (Ross et al., 2012). Hence, we may predict that BCorL1 upregulation in ageing Stra13 KO LSK may function by repressing cell proliferation and self renewal of HSCs. Another gene TCNT3 known for its regulation of the Shh pathway (one of the developmental pathways regulating

hematopoiesis) and implicated in apoptosis was also upregulated in Stra13 KO LSK though its role in hematopoiesis is not known (Thomas et al., 2012).

The next objective of my thesis focused on the role of Stra13 in hematopoiesis during genotoxic stress. Stra13 has been reported to be induced upon genotoxic stress and regulates ionizing induced apoptosis (Thin et al., 2007). Our results clearly show that the hematopoietic compartment of Stra13 KO mice is sensitive to both forms of genotoxic stress- namely ionizing gamma radiation and 5-FU. On subjecting to sub lethal doses of whole body gamma radiation of 5Gy we observed that the LSK compartment of Stra13 KO mice were extremely sensitive when compared to WT control after 24 hrs. Likewise intravenous injection of 5-FU in Stra13 KO mice showed sensitivity in the LSK compartment even after 7 days post injection. The LSK progenitor percentages were still significantly lower and showed prolonged myelosuppression compared to their WT LSK controls. It is interesting to note that the phenotype in Stra13 KO mice upon sublethal gamma radiation is similar to Necdin KO mice (Asai et al., 2012). Necdin is p53 target gene that regulates response to genotoxic stress of HSCs (Asai et al., 2012). Similarly, there have been reports that Stra13 is a downstream target of p53 (Qian et al., 2008). We may predict that enhanced sensitivity of Stra13 null LSK cells after genotoxic stress may be p53 dependent. However, this remains to be investigated.

Therefore, to identify Stra13 target genes that account for the enhanced radiosensitivity of Stra13 KO HSCs, we performed gene-expression profiling on

LSK cells isolated from Stra13 null mice and WT mice after a sub lethal dose of 5Gy radiation. Several deregulated genes and signaling pathways were identified in Stra13 KO LSK cells some of which were related to actin cytoskeleton organization, DNA damage, cell to cell junction, metal ion binding and GTPase activator. UPF1, a gene involved in S-phase progression and genome stability was downregualted in Stra13 KO LSK which indicates that Stra13 LSK are more susceptible to DNA damage compared to WT LSK cells (Azzalin and Lingner, 2006). Similarly Srgap2, a gene expressed in LT-HSCs was upregulated in Stra13 KO LSK cells (Forsberg et al., 2005). It is interesting to know some of the differentially expressed transcripts in Stra13 KO LSK fit into pathways and protein complexes not previously recognized in HSCs, possibly providing evidence of new HSC regulatory factors. However, the function of these genes involved in these different pathways in response to irradiation in Stra13 KO HSC remains to be validated. To further corroborate the Stra13 KO hematopoietic stress phenotype, studies can performed to detect the DNA damage by the formation of γ-H2AX foci by flow cytometry and DNA damage response such as activation of p53 dependent cell cycle check points.

The spleen is a major site of extramedullary hematopoiesis. Adult mouse and human spleen retain low percentages of HSCs. When the bone marrow cannot cope with the body's demand in times of stress and disease, the spleen serves a backup to generate all the different lineages of the blood (Chadburn, 2000). During recovery of hematopoiesis after irradiation, and under conditions of stress-induced erythropoiesis, erythrocyte differentiation was accelerated in spleen of

Dhh-deficient mice compared with WT (Lau et al., 2012). Extramedullary hematopoiesis was not studied in Stra13 KO during steady state, ageing or under different genotoxic stress conditions. As a start point, it would be important to know if Stra13 is expressed in the HSC compartment of spleen and whether its expression increases during ageing and stress in a similar fashion as observed in the BM (Figure 3.1.2). We can predict that the expression of Stra13 maybe modulated in the HSC compartment of spleen during stress conditions. Stra13 KO mice under genotoxic stress by radiation or treatment with 5FU may show reduced extramedullary hematopoiesis.

The role of HSCs during infections is not clearly defined. Major reports have focused on the role of committed myeloid and lymphoid progenitors to replenish immune cells after infection. Inflammatory signaling molecules like interferons, toll like receptors and tumor necrosis factor-α (TNF-α) have recently been identified to play important roles in HSC response (Baldridge et al., 2011). Severe infection such as sepsis or lipopolysaccharides treatment leads to T cell apoptosis and CLPs proliferate to compensate for this loss to replenish the immune cells. This homeostatic response is termed as emergency granulopoiesis or emergency lymphopoiesis. Unpublished data from our lab have shown that Stra13 expression is induced in macrophages upon stimulation with LPS. However the effects of LPS in the percentages of HSCs and progenitor fractions in Stra13 KO mice have not been studied. It would be interesting to study the long term effects of LPS in Stra13 KO mice and its impact on HSC function under transplantation settings. Likewise, TNF-α has been known to inhibit colony formation of human BM and

inhibit the in vitro growth of $CD34^+$ cells and their ability to reconstitute NOD-SCID mice (Broxmeyer et al., 1986; Dybedal et al., 2001). TNF-α additionally inhibits the proliferation of murine HSCs (Jacobsen et al., 1994; Zhang et al., 1995). However TNF-α also been demonstrated to promote HSC clonogenecity and increase the proportion of progenitors in vitro and prevent HSC apoptosis in vitro and in vivo. (Rezzoug et al., 2008). Our lab has previously shown that peritoneal macrophages in Stra13 mutants show reduced levels of TNF-α during muscle repair after injury as macrophages are involved in muscle regeneration and clearance of necrotic debris (unpublished data). The expression levels of TNF-α in HSC compartment of Stra13 KO mice has not been investigated and it would be important to explore how TNF-α expression and function is modulated in Stra13 KO mice during infections. Since Stra13 KO mice develop a lymphoproliferative autoimmune disorder resembling systemic lupus erythematosus (SLE), the functional changes resemble to those seen in chronically infected animals. There is massive expansion of the HSC compartment in the bone marrow and abnormal accumulation of HSC in the periphery in lupus mice. Most of the changes in HSCs are induced by proinflammatory cytokines in both lupus and infected mice. Unlike infected mice where the HSCs have a compromised regenerative potential, lupus mice show enhanced self renewal regenerative potential (Niu et al., 2013). Stra13 KO lupus mice show a greatly expanded HSC population in the BM (Appendix-Figure I) and it will be important to analyze the regenerative potential of these HSCs by competitive repopulation assays.

Previous reports have shown that Stra13 physically interacts with Runx1 in Treg cells to synergistically regulate the expression of its target genes (Miyazaki et al., 2010). Runx1 and Stra13 are both induced by oncogenic Ras and the expression of Runx1 is upregulated in aged HSC fraction similar to Stra13 (Chambers et al., 2007; Motoda et al., 2007). We therefore speculate that common target genes could be possibly regulated both by Stra13 and Runx1 during ageing of HSCs. Double knockouts of Stra13 and Runx1 mice could provide valuable insights about the combined role of these factors in ageing of HSCs.

Collectively, our data clearly illustrates Stra13 as a novel gene in hematopoiesis which is dispensable during normal steady state hematopoiesis in adult mouse both *in vitro* and *in vivo* but important for the function of HSCs during ageing and genotoxic stress.

CHAPTER 5

CONCLUSION AND FUTURE IMPLICATIONS

5. CONCLUSION AND FUTURE IMPLICATIONS

Our work has demonstrated for the first time, the role of Stra13 in hematopoiesis. Our detailed study and analysis provides first evidence that Stra13 is expressed in the long term hematopoietic stem cells (LT-HSCs) and its associated progenitors. Using Stra13 knockout mice, we have uncovered its function in hematopoiesis. Our data strongly suggests that Stra13 is dispensable for adult murine hematopoiesis during steady state both *in vitro* and *in vivo*. However, the importance of Stra13 is realized during ageing and stress responses of HSCs where loss of Stra13 leads to reduced hematopoietic stem cell numbers in ageing mice and sensitivity to both forms of genotoxic stress- radiation and chemotherapy. In order to understand the broader role of Stra13 in regulating HSC renewal and numbers during ageing and stress, gene expression profiling revealed a wide array of genes that were deregulated in the absence of Stra13. Some of the genes identified may be direct or indirect targets of Stra13 and are genes previously not known to be involved during ageing or stress hematopoiesis. Future studies should be aimed at dissecting the roles of these genes in HSC self renewal to provide a better insight how hematopoietic stem cell signaling is regulated during ageing and stress or during steady state hematopoiesis. Since Stra13 has been reported to be a target of p53 gene, it will be interesting to explore if Stra13 like p53 is involved in the quiescence of HSCs (Liu et al., 2009; Qian et al., 2008). Stra13 gene is expressed in the suprachiasmatic nucleus (SCN) in a circadian fashion and is a regulator of mammalian clock genes along with

members such as Bmal1, Cry and Per genes (Grechez-Cassiau et al., 2004; Honma et al., 2002; Noshiro et al., 2004). Many reports have confirmed that HSC release, mobilization and trafficking are rhythmically controlled (Giudice et al., 2010; Lucas et al., 2008; Mendez-Ferrer et al., 2009; Mendez-Ferrer et al., 2008; Singh et al., 2011; Tsinkalovsky et al., 2006; Tsinkalovsky et al., 2007). The clock genes mPer1, mPer2, mBmal1 and mClock are active in mouse HSCs (Tsinkalovsky et al., 2005) and it would be interesting to examine the expression pattern of Stra13 in HSCs in mice subjected to different cycles of light and how the rhythmic pattern of HSC is modulated in Stra13 KO mice. Knowing that Stra13 is overexpressed in many solid tumors, it would not be surprising to establish a role for it in leukemia. There have been reports of Stra13 being highly expressed in leukemic cell lines (Ivanova et al., 2001). Preliminary studies from our lab (unpublished data) have indicated that Stra13 is overexpressed in a subset of acute myeloid leukemia patients including those with EVI1 overexpression and CBF-β-MYH11 (inv16) rearrangements. These results may suggest that Stra13 may be also involved in leukemogenesis. Hence, targeting Stra13 may provide a therapeutic approach of eliminating leukemia initiating cells.

APPENDIX

APPENDIX

I. Increased HSCs in Stra13 autoimmune lupus mice.

The pioneer study on Stra13 knockout mice published from our lab has already demonstrated that by 6-8 months 90% of females and 50% of males develop an autoimmune phenotype similar to systemic lupus like disease. Hyper activated T and B cells infiltrate the systemic organs causing severe organ hyperplasia (Sun et al., 2001). A similar study corroborates this autoimmune phenotype although the manifestation of this disease was observed between 12-14 months (Miyazaki et al., 2010). The difference in onset of this lymphoproliferative disease could be due to the different genetic backgrounds used, the former being in a mixed background whereas the latter in C57BL/6 background. In this present study, our Stra13 KO mice have been derived in a pure C57BL/6 background and only about 25-30% of Stra13 KO mice develop the autoimmune disease with ageing by 14-16 months. Hence, we were interested to understand the effect of the lupus like condition in the hematopoietic compartment. To our surprise, we observed a massive expansion of the LSK and $SLAM^{+}$ cell compartments in Stra13 lupus mice (Figure I a&b). This increase in HSCs percentages might be the effect of pro inflammatory cytokines that come into function in lupus mice and hence may play a major role in the upregulation of genes involved in the self renewal of HSCs. Hence, the increase in HSC pool size in Stra13 autoimmune KO mice is an extrinsic or a secondary effect.

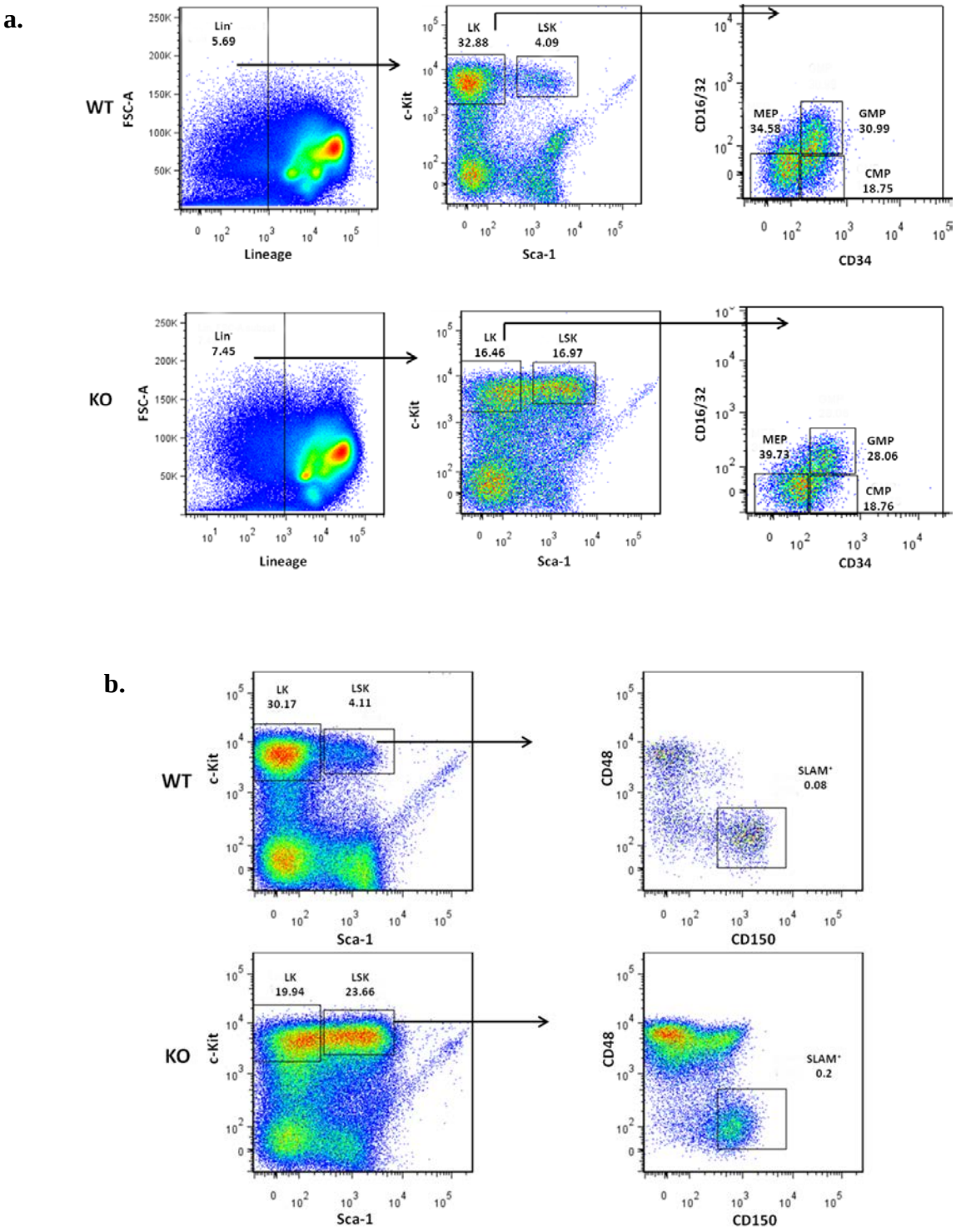


Figure I: Increased HSC pool in Stra13 lupus mice.

a. Ageing Stra13 KO mice 13-16 months that developed the autoimmune phenotype were analyzed for the percentages of LSK and progenitors by flow

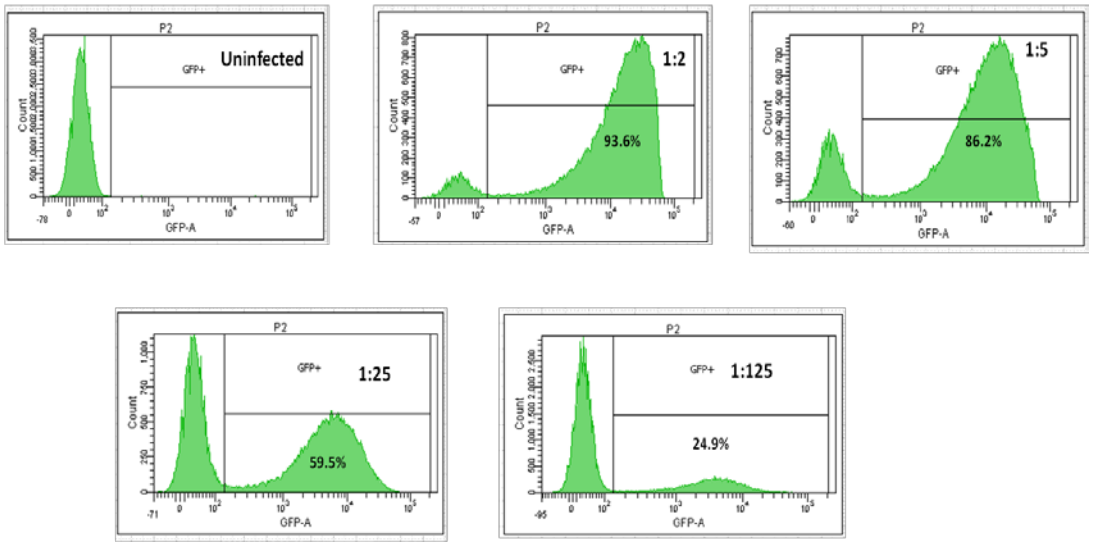
cytometry. LSK and LK cells are gated on Lin^- population. WT littermate ageing mice served as control.
b. $SLAM^+$ fraction of Stra13 KO lupus mice stained with antibodies positive for CD150 and negative for CD48 were gated on LSK fraction. WT littermate ageing mice served as control. Representative images of (n=2) mice analyzed.

II Retrovirus titre determination in NIH 3T3 cells

To determine the titre of the concentrated retrovirus supernatants of pMIG and pMIG-Stra13 that was produced as described in section 2.6 we plated 1 x 10^5 NIH3T3 cell in 6 well plates and the retroviral supernatants were added in the dilutions of 1:2, 1:5, 1:25, 1:125 to the culture medium after 24 hrs. NIH3T3 cells were incubated with the retroviral supernatants for 48 hours in the presence of protamine sulphate (8ug/ml) to increase infection efficiency, after which they were trypsinised and checked for the production of GFP+ cells by flow cytometry. The retrovirus titre was calculated according to the following formula:

$$\text{Titre (TU/ml)} = \frac{(\%\text{GFP positive cells}/100) \times \#\text{ cells transduced})}{\text{Volume of virus (ml)}}$$

a.



b.

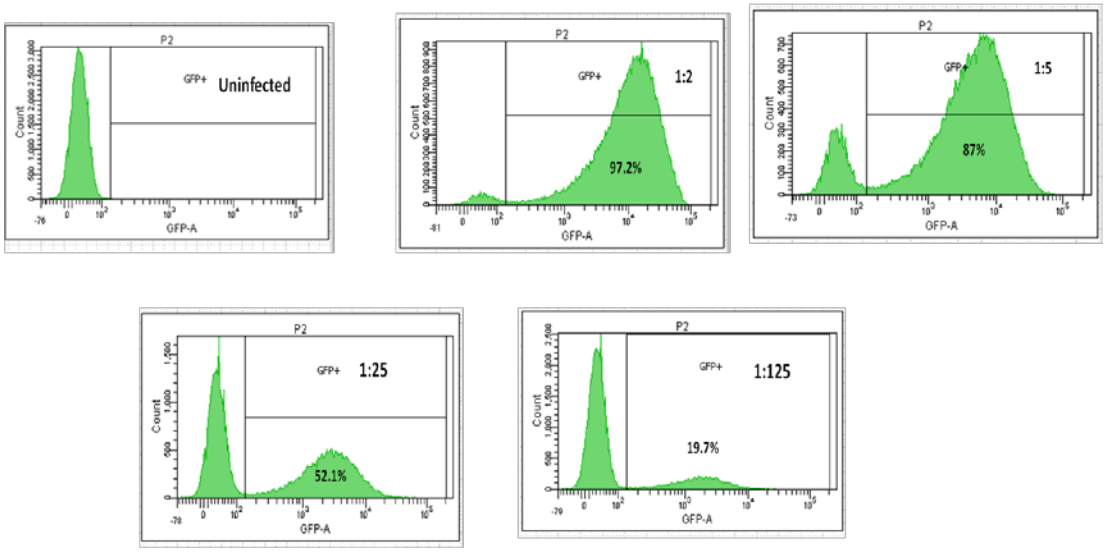


Figure II: Determination of retrovirus titre in NIH 3T3 cells by flow cytometry.

a. Percentage of GFP^+ cells with different dilutions of the pMIG retrovirus. Live cells gated and 20,000 events acquired for each dilution.

b. Percentages of GFP^+ cells with different dilutions of pMIG-Stra13 retrovirus gates similarly and acquired for 20,000 events for each respective dilution.

III Stra13 mice genotyping and agarose gel electrophoresis

Stra13 Primers:

Primer 1 (350rv) 5'-CGTTTTATTCCCCGCCTGGA-3'
Primer-2 (STRA13-2RV) 5'-GGAAGCTCAGGCTAGCTCAT-3'
Primer-3 (STRA13-NEO1) 5'-TCGATTCCACCGCCGCCTTCTATG-3'

Since the wild type and knockout allele PCR bands are approx around 380-400bp, the above PCR is carried out separately for detection of individual bands.

For detection of Wild Type Allele/genotype

Reagents	**X1**
10X Standard Taq Buffer	2.5ul
10mM dNTPs	0.5ul
Primer 1 (10uM)	1ul
Primer 2 (10uM)	1ul
25mM $MgCl_2$	1.5ul
Taq Polymerase	0.2
Water	17.3
Genomic DNA (200ng-300ng)	1ul
Total	25ul

For detection of Knockout Allele/genotype

Reagents	**X1**
10X Standard Taq Buffer	2.5ul
10mM dNTPs	0.5ul
Primer 1 (10uM)	1ul
Primer 3 (10uM)	1ul
25mM $MgCl_2$	1.5ul
Taq Polymerase	0.2
Water	17.3
Genomic DNA (200ng-300ng)	1ul
Total	25ul

PCR conditions

94 deg -5 minutes
94 deg- 30 sec
58 deg- 45 sec
72 deg -1 min
} Total 29 cycles
72 deg- 5 min
4deg - ∞

Both the PCR samples are run simultaneously/in parallel on a 1.5% agarose gel as depicted in figure III.

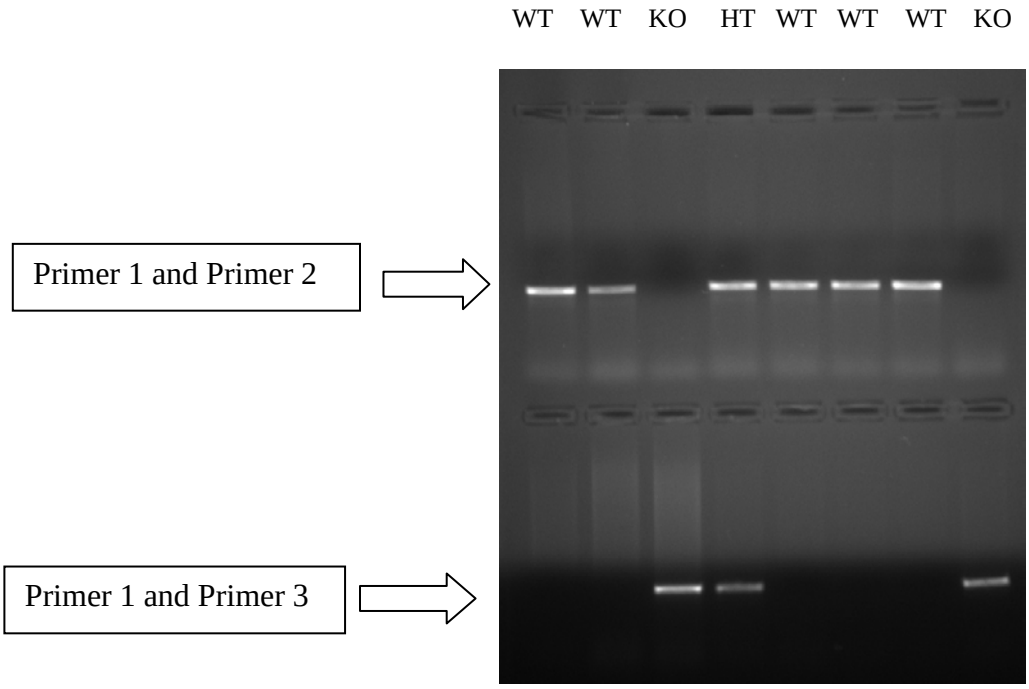


Figure III. Stra13 Genotyping on 1.5% agarose gel electrophoresis
Abbreviation: WT- Wild type; HT- Heterozygous; KO- Knockout
A single band in Primer 1 and Primer 2 indicates wild type genotype. A single band in Primer 1 and Primer 3 indicates knockout genotype. Bands in both (Primer 1 & Primer 2) & (Primer 1 & Primer 3) indicates heterozygous genotype.

Table IV: List of highly differentially expressed genes that were upregulated or downregulated in ageing Stra13 KO LSK vs. ageing WT LSK cells.

Gene Symbol	Gene Name	P value <0.05
	Downregulated Genes	
Ddah1	dimethylarginine dimethylaminohydrolase 1	0.01
Tgm2	transglutaminase 2, C polypeptide	0.03
Fam168b	family with sequence similarity 168, member B	0.001
Trib3	tribbles pseudokinase 3	0.02
Stx12	syntaxin 12	0.01
Ywhab	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta	0.04
Mylk	myosin, light polypeptide kinase	0.02
Bcat1	branched chain aminotransferase 1, cytosolic	0.01
Ptpn14	protein tyrosine phosphatase, non-receptor type 14	0.02
Luc7l	Luc7 homolog (S. cerevisiae)-like	0.04
Cd3eap	CD3e molecule, epsilon associated protein	0.01
Ahi1	Abelson helper integration site 1	0.007
Wdr78	WD repeat domain 78	0.03
Atg9b	autophagy related 9B	0.04
Wfdc2	WAP four-disulfide core domain 2	0.01
Tle2	transducin-like enhancer of split 2, homolog of Drosophila E(spl)	0.02
Dhcr24	24-dehydrocholesterol reductase	0.02
Pdxdc1	pyridoxal-dependent decarboxylase domain containing 1	0.04
Mmrn1	multimerin 1	0.04
Narfl	nuclear prelamin A recognition factor-like	0.02
Trim39	tripartite motif-containing 39	0.04

Creg1	cellular repressor of E1A-stimulated genes 1	0.01
Zfp532	zinc finger protein 532	0.007
Gabarapl1	gamma-aminobutyric acid (GABA) A receptor-associated protein-like 1	0.02
Zfp532	zinc finger protein 532	0.02
Slc35b1	solute carrier family 35, member B1	0.04
Trfr2	transferrin receptor 2	0.01
Acadvl	acyl-Coenzyme A dehydrogenase, very long chain	0.04
Dusp4	dual specificity phosphatase 4	0.02
Ift172	intraflagellar transport 172	0.001
Sqle	squalene epoxidase	0.01
Zfp30	zinc finger protein 30	0.03
Aplp2	amyloid beta (A4) precursor-like protein 2	0.03
Frap1	mechanistic target of rapamycin	0.01
Cryz	crystallin, zeta	0.02
Cish	cytokine inducible SH2-containing protein	0.04
Dlg3	discs, large homolog 3	0.03
Slc16a9	solute carrier family 16 (monocarboxylic acid transporters), member 9	0.04
Ric8b	resistance to inhibitors of cholinesterase 8 homolog B	0.002
Grb7	growth factor receptor bound protein 7	0.04
Otub2	OTU domain, ubiquitin aldehyde binding 2	0.03
Tmem191c	transmembrane protein 191C	0.02
Mat2b	methionine adenosyltransferase II, beta	0.02
Gnptg	N-acetylglucosamine-1-phosphotransferase, gamma subunit	0.02
Evc2	Ellis van Creveld syndrome 2	0.003
Dci/Eci	enoyl-Coenzyme A delta isomerase 1	0.01

Kifap3	kinesin-associated protein 3	0.03
Arnt	aryl hydrocarbon receptor nuclear translocator	0.03
Prr16	proline rich 16	0.03
Prkcq	protein kinase C, theta	0.04
Capns1	calpain, small subunit 1	0.004
Vti1a	vesicle transport through interaction with t-SNAREs 1A	0.04
Slc5a6	olute carrier family 5 (sodium-dependent vitamin transporter), member 6	0.04
Als2cr4	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4	0.05
Smarca2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	0.03
Ptprv	protein tyrosine phosphatase, receptor type, V	0.04
Rrp12	ribosomal RNA processing 12 homolog	0.02
Hdgfl1	hepatoma derived growth factor-like 1	0.02
Eefsec	eukaryotic elongation factor, selenocysteine-tRNA-specific	0.04
Zscan20	zinc finger and SCAN domains 20	0.008
Rnf40	ring finger protein 40	0.04
Ctxn1	cortexin 1	0.04
Mrap	melanocortin 2 receptor accessory protein	0.03
Gm973	predicted gene 973	0.01
Gm773	predicted gene 773	0.03
Smndc1	survival motor neuron domain containing 1	0.04
Chmp4c	charged multivesicular body protein 4C	0.007
Mic2l1/Cd99l2	CD99 antigen-like 2	0.04
Ints5	integrator complex subunit 5	0.03
4930565O14/ Gm16853	predicted gene, 16853	0.03
Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	0.003

Snap23	synaptosomal-associated protein 23	0.03
Rufy1	RUN and FYVE domain containing 1	0.01
Rtn1	reticulon 1	0.04
Mtx1	metaxin 1	0.02
Krtap10-10	keratin associated protein 10-10	0.03
Lrrc41	leucine rich repeat containing 41	0.006
Slc30a7	solute carrier family 30 (zinc transporter), member 7	0.006
Kbtbd4	kelch repeat and BTB (POZ) domain containing 4	0.03
Fgf9	fibroblast growth factor 9	0.01
Pcdhac2	protocadherin alpha subfamily C	0.04
Rtp4	Rtp4 receptor transporter protein 4	0.004
Tada3l	transcriptional adaptor 3	0.02
Aggf1	angiogenic factor with G patch and FHA domains 1	0.02
Gm317/ Ccdc154	Ccdc154 coiled-coil domain containing 154	0.04
Smarcd2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2	0.02
Aifm2	apoptosis-inducing factor, mitochondrion-associated 2	0.02
Upregulated Genes		
Mycbpap	MYCBP associated protein	0.04
Yars2	tyrosyl-tRNA synthetase 2	0.01
Pdhx	pyruvate dehydrogenase complex, component X	0.005
Ift20	intraflagellar transport 20	0.03
Eef1a1	eukaryotic translation elongation factor 1 alpha 1	0.03
Ing5	inhibitor of growth family, member 5	0.04
Rps6	ribosomal protein S6	0.02
Rpl27a	ribosomal protein L27A	0.04
Mfn2	mitofusin 2	0.02

Cul4b	cullin 4B	0.01
Mkrn3	makorin, ring finger protein, 3	0.01
Rrm1	ribonucleotide reductase M1	0.04
D12Ertd647e/ Ifi27	interferon, alpha-inducible protein 27	0.03
Irf2bp1	interferon regulatory factor 2 binding protein 1	0.04
Fam114a2	family with sequence similarity 114, member A2	0.02
Clspn	claspin	0.02
Pml	promyelocytic leukemia	0.03
Cstf3	cleavage stimulation factor, 3' pre-RNA, subunit 3	0.03
Usp3	ubiquitin specific peptidase 3	0.03
Srm	spermidine synthase	0.03
Tstd2	thiosulfate sulfurtransferase (rhodanese)-like domain containing 2	0.03
Bcorl1	BCL6 co-repressor-like 1	0.03
Nedd4l	neural precursor cell expressed, developmentally down-regulated gene 4-like	0.02
Tpst1	protein-tyrosine sulfotransferase 1	0.003
Tctn3	tectonic family member 3	0.03
Igl-V1	immunoglobulin lambda variable 1	0.02

Table V: List of highly differentially expressed genes that were upregulated or downregulated in Stra13 KO LSK vs. ageing WT LSK cells upon 5Gy gamma radiation.

Gene Symbol	Gene Name	P<0.05
	Downregulated Genes	
Upf1	UPF1 regulator of nonsense transcripts homolog	0.02
Tmed7	transmembrane emp24 protein transport domain containing 7	0.03
Nrep	neuronal regeneration related protein	0.03
Zfp930	zinc finger protein 930	0.04
Srxn1	sulfiredoxin 1 homolog	0.02
Pdgfb	platelet derived growth factor, B polypeptide	0.03
Olfr1203	olfactory receptor 1203	0.03
Olfr281	olfactory receptor 281	0.02
Gdpd5	glycerophosphodiester phosphodiesterase domain containing 5	0.02
2900026A02Rik	RIKEN cDNA 2900026A02 gene	0.02
Zdhhc19	zinc finger, DHHC domain containing 19	0.04
Tbc1d10a	TBC1 domain family, member 10a	0.04
LOC243025/ Tmem156	transmembrane protein 156	0.03
Rasal1	RAS protein activator like 1 (GAP1 like	0.02
Tmub1	transmembrane and ubiquitin-like domain containing 1	0.04
Doc2a	double C2-like domains, alpha	0.03
Wdr85	WD repeat domain 85	0.04
Epb4.1l3	erythrocyte protein band 4.1-like 3	0.04
Jarid1a	jumonji, AT rich interactive domain 1A	0.04
Spatc1l	spermatogenesis and centriole associated 1 like	0.03

Gab2	growth factor receptor bound protein 2-associated protein 2	0.04
Tsga8	testis specific gene A8	0.03
Zfp563	zinc finger protein 563	0.04
Polr3h	polymerase (RNA) III (DNA directed) polypeptide H	0.01
Rnf123	ring finger protein 123	0.04
5033413D22Rik/ Ccdc162	coiled-coil domain containing 162	0.03
Zfp532	zinc finger protein 532	0.02
Sfmbt1	Scm-like with four mbt domains 1	0.04
Ugt2b5	UDP glucuronosyltransferase 2 family, polypeptide B5	0.03
Cat	Catalase	0.008
Evi5l	ecotropic viral integration site 5 like	0.03
Yars2	tyrosyl-tRNA synthetase 2 (mitochondrial	0.01
Ano7	anoctamin 7	0.01
Arhgap17	Rho GTPase activating protein 17	0.04
Rnf41	ring finger protein 41	0.004
Gucy1a3	guanylate cyclase 1, soluble, alpha 3	0.02
Rab13	RAB13, member RAS oncogene family	0.04
Zfp87	zinc finger protein 87	0.04
Pcdhac2	protocadherin alpha subfamily C, 2	0.03
Setx	senataxin	0.03
Upregulated Genes		
Traf4	TNF receptor associated factor 4	0.03
Ccdc50	coiled-coil domain containing 50	0.03
Traf4	TNF receptor-associated factor 4	0.04
Ccdc14	coiled-coil domain containing 14	0.01

Sdsl	serine dehydratase-like	0.01
Pign	phosphatidylinositol glycan anchor biosynthesis, class N	0.008
Zfp715	zinc finger protein 715	0.008
Fam118b	family with sequence similarity 118, member B	0.02
Rrbp1	ribosome binding protein 1	0.03
Nanos1	nanos homolog 1	0.02
Snx14	sorting nexin 14	0.04
Sep6	septin 6	0.04
Mt1	metallothionein 1	0.03
Srgap2	SLIT-ROBO Rho GTPase activating protein 2	0.02
F10	coagulation factor X	0.04
Pfkfb4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	0.03
Car12	carbonic anyhydrase 12	0.01
Narfl	nuclear prelamin A recognition factor-like	0.03
Cd59a	CD59a antigen	0.02
2010317E24Rik/ Sapcd2	suppressor APC domain containing 2	0.03
2210408F11Rik	RIKEN cDNA 2210408F11 gene	0.02
Rdh11	retinol dehydrogenase 11	0.02
Slc25a19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	0.01
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	0.03
Zfyve21	zinc finger, FYVE domain containing 21	0.04

REFERENCES

REFERENCES

Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S. E. (2001). Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659-669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O. J., Thoren, L. A., *et al.* (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295-306.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Allsopp, R. C., Morin, G. B., DePinho, R., Harley, C. B., and Weissman, I. L. (2003a). Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. Blood *102*, 517-520.

Allsopp, R. C., Morin, G. B., Horner, J. W., DePinho, R., Harley, C. B., and Weissman, I. L. (2003b). Effect of TERT over-expression on the long-term transplantation capacity of hematopoietic stem cells. Nature Medicine *9*, 369-371.

Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell *109*, 39-45.

Artinger, E. L., Mishra, B. P., Zaffuto, K. M., Li, B. E., Chung, E. K., Moore, A. W., Chen, Y., Cheng, C., and Ernst, P. (2013). An MLL-dependent network sustains hematopoiesis. Proceedings of the National Academy of Sciences of the United States of America *110*, 12000-12005.

Asai, T., Liu, Y., Di Giandomenico, S., Bae, N., Ndiaye-Lobry, D., Deblasio, A., Menendez, S., Antipin, Y., Reva, B., Wevrick, R., and Nimer, S. D. (2012). Necdin, a p53 target gene, regulates the quiescence and response to genotoxic stress of hematopoietic stem/progenitor cells. Blood *120*, 1601-1612.

Attema, J. L., Pronk, C. J., Norddahl, G. L., Nygren, J. M., and Bryder, D. (2009). Hematopoietic stem cell ageing is uncoupled from p16 INK4A-mediated senescence. Oncogene *28*, 2238-2243.

Austin, T. W., Solar, G. P., Ziegler, F. C., Liem, L., and Matthews, W. (1997). A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood *89*, 3624-3635.

Azmi, S., Sun, H., Ozog, A., and Taneja, R. (2003). mSharp-1/DEC2, a basic helix-loop-helix protein functions as a transcriptional repressor of E box activity and Stra13 expression. The Journal of Biological Chemistry *278*, 20098-20109.

Azmi, S., and Taneja, R. (2002). Embryonic expression of mSharp-1/mDEC2, which encodes a basic helix-loop-helix transcription factor. Mechanisms of Development *114*, 181-185.

Azzalin, C. M., and Lingner, J. (2006). The human RNA surveillance factor UPF1 is required for S phase progression and genome stability. Current Biology : CB *16*, 433-439.

Baba, Y., Yokota, T., Spits, H., Garrett, K. P., Hayashi, S., and Kincade, P. W. (2006). Constitutively active beta-catenin promotes expansion of multipotent hematopoietic progenitors in culture. Journal of Immunology (Baltimore, Md : 1950) *177*, 2294-2303.

Baena, E., Ortiz, M., Martinez, A. C., and de Alboran, I. M. (2007). c-Myc is essential for hematopoietic stem cell differentiation and regulates Lin(-)Sca-1(+)c-Kit(-) cell generation through p21. Experimental Hematology *35*, 1333-1343.

Balazs, A. B., Fabian, A. J., Esmon, C. T., and Mulligan, R. C. (2006). Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. Blood *107*, 2317-2321.

Baldridge, M. T., King, K. Y., and Goodell, M. A. (2011). Inflammatory signals regulate hematopoietic stem cells. Trends in Immunology *32*, 57-65.

Ballmaier, M., Germeshausen, M., Krukemeier, S., and Welte, K. (2003). Thrombopoietin is essential for the maintenance of normal hematopoiesis in humans: development of aplastic anemia in patients with congenital amegakaryocytic thrombocytopenia. Annals of the New York Academy of Sciences *996*, 17-25.

Baraibar, M. A., and Friguet, B. (2013). Oxidative proteome modifications target specific cellular pathways during oxidative stress, cellular senescence and aging. Experimental Gerontology *48*, 620-625.

Beadling, C., Cereseto, A., Fan, W., Naramura, M., and Smith, K. A. (2001). Cytokine response gene 8 (CR8) regulates the cell cycle G1-S phase transition and promotes cellular survival. Oncogene *20*, 1771-1783.

Becker, A. J., Mc, C. E., and Till, J. E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature *197*, 452-454.

Beerman, I., Bock, C., Garrison, B. S., Smith, Z. D., Gu, H., Meissner, A., and Rossi, D. J. (2013). Proliferation-dependent alterations of the DNA methylation landscape underlie hematopoietic stem cell aging. Cell Stem Cell *12*, 413-425.

Beerman, I., Maloney, W. J., Weissmann, I. L., and Rossi, D. J. (2010). Stem cells and the aging hematopoietic system. Current Opinion in Immunology *22*, 500-506.

Bellantuono, I., Aldahmash, A., and Kassem, M. (2009). Aging of marrow stromal (skeletal) stem cells and their contribution to age-related bone loss. Biochimica et Biophysica Acta *1792*, 364-370.

Benz, C., Copley, M. R., Kent, D. G., Wohrer, S., Cortes, A., Aghaeepour, N., Ma, E., Mader, H., Rowe, K., Day, C., *et al.* (2012). Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell Stem Cell *10*, 273-283.

Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., *et al.* (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315-326.

Bhardwaj, G., Murdoch, B., Wu, D., Baker, D. P., Williams, K. P., Chadwick, K., Ling, L. E., Karanu, F. N., and Bhatia, M. (2001). Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nature Immunology *2*, 172-180.

Bhawal, U. K., Ito, Y., Tanimoto, K., Sato, F., Fujimoto, K., Kawamoto, T., Sasahira, T., Hamada, N., Kuniyasu, H., Arakawa, H., *et al.* (2012). IL-1beta-mediated up-regulation of DEC1 in human gingiva cells via the Akt pathway. Journal of Cellular Biochemistry *113*, 3246-3253.

Bhawal, U. K., Sato, F., Arakawa, Y., Fujimoto, K., Kawamoto, T., Tanimoto, K., Ito, Y., Sasahira, T., Sakurai, T., Kobayashi, M., *et al.* (2011). Basic helix-loop-

helix transcription factor DEC1 negatively regulates cyclin D1. The Journal of Pathology *224*, 420-429.

Blank, U., Karlsson, G., Moody, J. L., Utsugisawa, T., Magnusson, M., Singbrant, S., Larsson, J., and Karlsson, S. (2006). Smad7 promotes self-renewal of hematopoietic stem cells. Blood *108*, 4246-4254.

Bocker, M. T., Hellwig, I., Breiling, A., Eckstein, V., Ho, A. D., and Lyko, F. (2011). Genome-wide promoter DNA methylation dynamics of human hematopoietic progenitor cells during differentiation and aging. Blood *117*, e182-189.

Boivin, D. B., James, F. O., Wu, A., Cho-Park, P. F., Xiong, H., and Sun, Z. S. (2003). Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood *102*, 4143-4145.

Boudjelal, M., Taneja, R., Matsubara, S., Bouillet, P., Dolle, P., and Chambon, P. (1997). Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes & Development *11*, 2052-2065.

Bowie, M. B., McKnight, K. D., Kent, D. G., McCaffrey, L., Hoodless, P. A., and Eaves, C. J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. The Journal of Clinical Investigation *116*, 2808-2816.

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., and Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science (New York, NY) *317*, 807-810.

Brown, K., Xie, S., Qiu, X., Mohrin, M., Shin, J., Liu, Y., Zhang, D., Scadden, D. T., and Chen, D. (2013). SIRT3 reverses aging-associated degeneration. Cell Reports *3*, 319-327.

Broxmeyer, H. E., Williams, D. E., Lu, L., Cooper, S., Anderson, S. L., Beyer, G. S., Hoffman, R., and Rubin, B. Y. (1986). The suppressive influences of human tumor necrosis factors on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: synergism of tumor necrosis factor and interferon-gamma. Journal of Immunology (Baltimore, Md : 1950) *136*, 4487-4495.

Bryder, D., Rossi, D. J., and Weissman, I. L. (2006). Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. The American Journal of Pathology *169*, 338-346.

Bugeon, L., Taylor, H. B., Progatzky, F., Lin, M. I., Ellis, C. D., Welsh, N., Smith, E., Vargesson, N., Gray, C., Renshaw, S. A., *et al.* (2011). The NOTCH pathway contributes to cell fate decision in myelopoiesis. Haematologica *96*, 1753-1760.

Butler, J. M., Nolan, D. J., Vertes, E. L., Varnum-Finney, B., Kobayashi, H., Hooper, A. T., Seandel, M., Shido, K., White, I. A., Kobayashi, M., *et al.* (2010). Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. Cell Stem Cell *6*, 251-264.

Cancelas, J. A., Koevoet, W. L., de Koning, A. E., Mayen, A. E., Rombouts, E. J., and Ploemacher, R. E. (2000). Connexin-43 gap junctions are involved in multiconnexin-expressing stromal support of hemopoietic progenitors and stem cells. Blood *96*, 498-505.

Capron, C., Lecluse, Y., Kaushik, A. L., Foudi, A., Lacout, C., Sekkai, D., Godin, I., Albagli, O., Poullion, I., Svinartchouk, F., *et al.* (2006). The SCL relative LYL-1 is required for fetal and adult hematopoietic stem cell function and B-cell differentiation. Blood *107*, 4678-4686.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature *335*, 88-89.

Chadburn, A. (2000). The spleen: Anatomy and anatomical function. Seminars in Hematology *37, Supplement 1*, 13-21.

Chakrabarti, J., Turley, H., Campo, L., Han, C., Harris, A. L., Gatter, K. C., and Fox, S. B. (2004). The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. British Journal of Cancer *91*, 954-958.

Chambers, S. M., Shaw, C. A., Gatza, C., Fisk, C. J., Donehower, L. A., and Goodell, M. A. (2007). Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biology *5*, e201.

Chen, J., Ellison, F. M., Keyvanfar, K., Omokaro, S. O., Desierto, M. J., Eckhaus, M. A., and Young, N. S. (2008). Enrichment of hematopoietic stem cells with

SLAM and LSK markers for the detection of hematopoietic stem cell function in normal and Trp53 null mice. Experimental Hematology *36*, 1236-1243.

Cheng, T., Rodrigues, N., Dombkowski, D., Stier, S., and Scadden, D. T. (2000a). Stem cell repopulation efficiency but not pool size is governed by p27(kip1). Nature Medicine *6*, 1235-1240.

Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D. T. (2000b). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science (New York, NY) *287*, 1804-1808.

Cheshier, S. H., Morrison, S. J., Liao, X., and Weissman, I. L. (1999). In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proceedings of the National Academy of Sciences of the United States of America *96*, 3120-3125.

Cho, R. H., Sieburg, H. B., and Muller-Sieburg, C. E. (2008). A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood *111*, 5553-5561.

Cho, Y., Noshiro, M., Choi, M., Morita, K., Kawamoto, T., Fujimoto, K., Kato, Y., and Makishima, M. (2009). The basic helix-loop-helix proteins differentiated embryo chondrocyte (DEC) 1 and DEC2 function as corepressors of retinoid X receptors. Molecular Pharmacology *76*, 1360-1369.

Choi, S. M., Cho, H. J., Cho, H., Kim, K. H., Kim, J. B., and Park, H. (2008). Stra13/DEC1 and DEC2 inhibit sterol regulatory element binding protein-1c in a hypoxia-inducible factor-dependent mechanism. Nucleic Acids Research *36*, 6372-6385.

Choudhury, A. R., Ju, Z., Djojosubroto, M. W., Schienke, A., Lechel, A., Schaetzlein, S., Jiang, H., Stepczynska, A., Wang, C., Buer, J., *et al.* (2007). Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nature Genetics *39*, 99-105.

Christensen, J. L., and Weissman, I. L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proceedings of the National Academy of Sciences of the United States of America *98*, 14541-14546.

Conneally, E., Cashman, J., Petzer, A., and Eaves, C. (1997). Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative

assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proceedings of the National Academy of Sciences of the United States of America *94*, 9836-9841.

Dawson, S. R., Turner, D. L., Weintraub, H., and Parkhurst, S. M. (1995). Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. Molecular and Cellular Biology *15*, 6923-6931.

de Haan, G., Weersing, E., Dontje, B., van Os, R., Bystrykh, L. V., Vellenga, E., and Miller, G. (2003). In vitro generation of long-term repopulating hematopoietic stem cells by fibroblast growth factor-1. Developmental Cell *4*, 241-251.

de Pater, E., Kaimakis, P., Vink, C. S., Yokomizo, T., Yamada-Inagawa, T., van der Linden, R., Kartalaei, P. S., Camper, S. A., Speck, N., and Dzierzak, E. (2013). Gata2 is required for HSC generation and survival. The Journal of Experimental Medicine *210*, 2843-2850.

de Sauvage, F. J., Carver-Moore, K., Luoh, S. M., Ryan, A., Dowd, M., Eaton, D. L., and Moore, M. W. (1996). Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. The Journal of Experimental Medicine *183*, 651-656.

Dear, T. N., Hainzl, T., Follo, M., Nehls, M., Wilmore, H., Matena, K., and Boehm, T. (1997). Identification of interaction partners for the basic-helix-loop-helix protein E47. Oncogene *14*, 891-898.

Denning-Kendall, P., Singha, S., Bradley, B., and Hows, J. (2003). Cobblestone area-forming cells in human cord blood are heterogeneous and differ from long-term culture-initiating cells. Stem Cells (Dayton, Ohio) *21*, 694-701.

Dybedal, I., Bryder, D., Fossum, A., Rusten, L. S., and Jacobsen, S. E. (2001). Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood *98*, 1782-1791.

Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S. J., Brinkman, R., and Eaves, C. (2007). Long-term propagation of distinct hematopoietic differentiation programs in vivo. Cell Stem Cell *1*, 218-229.

Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M., and de Haan, G. (2011). Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. The Journal of Experimental Medicine *208*, 2691-2703.

Dzierzak, E., and Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nature Immunology *9*, 129-136.

Ehata, S., Hanyu, A., Hayashi, M., Aburatani, H., Kato, Y., Fujime, M., Saitoh, M., Miyazawa, K., Imamura, T., and Miyazono, K. (2007). Transforming growth factor-beta promotes survival of mammary carcinoma cells through induction of antiapoptotic transcription factor DEC1. Cancer Research *67*, 9694-9703.

Ema, M., Yokomizo, T., Wakamatsu, A., Terunuma, T., Yamamoto, M., and Takahashi, S. (2006). Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. Blood *108*, 4018-4024.

Fischbach, N. A., Rozenfeld, S., Shen, W., Fong, S., Chrobak, D., Ginzinger, D., Kogan, S. C., Radhakrishnan, A., Le Beau, M. M., Largman, C., and Lawrence, H. J. (2005). HOXB6 overexpression in murine bone marrow immortalizes a myelomonocytic precursor in vitro and causes hematopoietic stem cell expansion and acute myeloid leukemia in vivo. Blood *105*, 1456-1466.

Fisher, A. L., Ohsako, S., and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. Molecular and Cellular Biology *16*, 2670-2677.

Florian, M. C., Dorr, K., Niebel, A., Daria, D., Schrezenmeier, H., Rojewski, M., Filippi, M. D., Hasenberg, A., Gunzer, M., Scharffetter-Kochanek, K., *et al.* (2012). Cdc42 activity regulates hematopoietic stem cell aging and rejuvenation. Cell Stem Cell *10*, 520-530.

Florian, M. C., Nattamai, K. J., Dorr, K., Marka, G., Uberle, B., Vas, V., Eckl, C., Andra, I., Schiemann, M., Oostendorp, R. A., *et al.* (2013). A canonical to non-canonical Wnt signalling switch in haematopoietic stem-cell ageing. Nature *503*, 392-396.

Forsberg, E. C., Prohaska, S. S., Katzman, S., Heffner, G. C., Stuart, J. M., and Weissman, I. L. (2005). Differential expression of novel potential regulators in hematopoietic stem cells. PLoS Genetics *1*, e28.

Fournier, M., Lebert-Ghali, C. E., Krosl, G., and Bijl, J. J. (2012). HOXA4 induces expansion of hematopoietic stem cells in vitro and confers enhancement of pro-B-cells in vivo. Stem Cells and Development *21*, 133-142.

Fox, N., Priestley, G., Papayannopoulou, T., and Kaushansky, K. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. The Journal of Clinical Investigation *110*, 389-394.

Franceschi, C., Bonafe, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., and De Benedictis, G. (2000). Inflamm-aging. An evolutionary perspective on immunosenescence. Annals of the New York Academy of Sciences *908*, 244-254.

Freemont, A. J., and Hoyland, J. A. (2007). Morphology, mechanisms and pathology of musculoskeletal ageing. The Journal of Pathology *211*, 252-259.

Fujimoto, K., Shen, M., Noshiro, M., Matsubara, K., Shingu, S., Honda, K., Yoshida, E., Suardita, K., Matsuda, Y., and Kato, Y. (2001). Molecular cloning and characterization of DEC2, a new member of basic helix-loop-helix proteins. Biochemical and Biophysical Research Communications *280*, 164-171.

Gao, J., Graves, S., Koch, U., Liu, S., Jankovic, V., Buonamici, S., El Andaloussi, A., Nimer, S. D., Kee, B. L., Taichman, R., *et al.* (2009). Hedgehog signaling is dispensable for adult hematopoietic stem cell function. Cell Stem Cell *4*, 548-558.

Geiger, H., de Haan, G., and Florian, M. C. (2013). The ageing haematopoietic stem cell compartment. Nature Reviews Immunology *13*, 376-389.

Geiger, H., Koehler, A., and Gunzer, M. (2007). Stem cells, aging, niche, adhesion and Cdc42: a model for changes in cell-cell interactions and hematopoietic stem cell aging. Cell Cycle (Georgetown, Tex) *6*, 884-887.

Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988). The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell *55*, 185-192.

Gekas, C., Dieterlen-Lievre, F., Orkin, S. H., and Mikkola, H. K. (2005). The placenta is a niche for hematopoietic stem cells. Developmental Cell *8*, 365-375.

Giudice, A., Caraglia, M., Marra, M., Montella, M., Maurea, N., Abbruzzese, A., and Arra, C. (2010). Circadian rhythms, adrenergic hormones and trafficking of hematopoietic stem cells. Expert Opinion on Therapeutic Targets *14*, 567-575.

Gonzalez-Nieto, D., Li, L., Kohler, A., Ghiaur, G., Ishikawa, E., Sengupta, A., Madhu, M., Arnett, J. L., Santho, R. A., Dunn, S. K., *et al.* (2012). Connexin-43

in the osteogenic BM niche regulates its cellular composition and the bidirectional traffic of hematopoietic stem cells and progenitors. Blood *119*, 5144-5154.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. The Journal of Experimental Medicine *183*, 1797-1806.

Grbavec, D., and Stifani, S. (1996). Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. Biochemical and Biophysical Research Communications *223*, 701-705.

Grechez-Cassiau, A., Panda, S., Lacoche, S., Teboul, M., Azmi, S., Laudet, V., Hogenesch, J. B., Taneja, R., and Delaunay, F. (2004). The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. The Journal of Biological Chemistry *279*, 1141-1150.

Growney, J. D., Shigematsu, H., Li, Z., Lee, B. H., Adelsperger, J., Rowan, R., Curley, D. P., Kutok, J. L., Akashi, K., Williams, I. R., *et al.* (2005). Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood *106*, 494-504.

Guiu, J., Shimizu, R., D'Altri, T., Fraser, S. T., Hatakeyama, J., Bresnick, E. H., Kageyama, R., Dzierzak, E., Yamamoto, M., Espinosa, L., and Bigas, A. (2013). Hes repressors are essential regulators of hematopoietic stem cell development downstream of Notch signaling. The Journal of Experimental Medicine *210*, 71-84.

Harrison, D. E., Astle, C. M., and Lerner, C. (1988). Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. Proceedings of the National Academy of Sciences of the United States of America *85*, 822-826.

Harrison, D. E., Jordan, C. T., Zhong, R. K., and Astle, C. M. (1993). Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. Experimental Hematology *21*, 206-219.

Henry, C. J., Marusyk, A., and DeGregori, J. (2011). Aging-associated changes in hematopoiesis and leukemogenesis: what's the connection? Aging *3*, 643-656.

Hock, H., Hamblen, M. J., Rooke, H. M., Schindler, J. W., Saleque, S., Fujiwara, Y., and Orkin, S. H. (2004). Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature *431*, 1002-1007.

Hofmann, I., Stover, E. H., Cullen, D. E., Mao, J., Morgan, K. J., Lee, B. H., Kharas, M. G., Miller, P. G., Cornejo, M. G., Okabe, R., *et al.* (2009). Hedgehog signaling is dispensable for adult murine hematopoietic stem cell function and hematopoiesis. Cell Stem Cell *4*, 559-567.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., and Honma, K. (2002). Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature *419*, 841-844.

Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research *37*, 1-13.

Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols *4*, 44-57.

Ichikawa, M., Goyama, S., Asai, T., Kawazu, M., Nakagawa, M., Takeshita, M., Chiba, S., Ogawa, S., and Kurokawa, M. (2008). AML1/Runx1 negatively regulates quiescent hematopoietic stem cells in adult hematopoiesis. Journal of Immunology (Baltimore, Md : 1950) *180*, 4402-4408.

Ivanova, A. V., Ivanov, S. V., Danilkovitch-Miagkova, A., and Lerman, M. I. (2001). Regulation of STRA13 by the von Hippel-Lindau tumor suppressor protein, hypoxia, and the UBC9/ubiquitin proteasome degradation pathway. The Journal of Biological Chemistry *276*, 15306-15315.

Ivanova, A. V., Ivanov, S. V., and Lerman, M. L. (2005). Association, mutual stabilization, and transcriptional activity of the STRA13 and MSP58 proteins. Cellular and Molecular Life Sciences : CMLS *62*, 471-484.

Ivanova, A. V., Ivanov, S. V., Zhang, X., Ivanov, V. N., Timofeeva, O. A., and Lerman, M. I. (2004). STRA13 interacts with STAT3 and modulates transcription of STAT3-dependent targets. Journal of Molecular Biology *340*, 641-653.

Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Katoh-Fukui, Y., Koseki, H., *et al.* (2004). Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity *21*, 843-851.

Jacobsen, F. W., Rothe, M., Rusten, L., Goeddel, D. V., Smeland, E. B., Veiby, O. P., Slordal, L., and Jacobsen, S. E. (1994). Role of the 75-kDa tumor necrosis

factor receptor: inhibition of early hematopoiesis. Proceedings of the National Academy of Sciences of the United States of America *91*, 10695-10699.

Jang, Y. Y., and Sharkis, S. J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood *110*, 3056-3063.

Janzen, V., Forkert, R., Fleming, H. E., Saito, Y., Waring, M. T., Dombkowski, D. M., Cheng, T., DePinho, R. A., Sharpless, N. E., and Scadden, D. T. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature *443*, 421-426.

Jeannet, G., Scheller, M., Scarpellino, L., Duboux, S., Gardiol, N., Back, J., Kuttler, F., Malanchi, I., Birchmeier, W., Leutz, A., *et al.* (2008). Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. Blood *111*, 142-149.

Jia, Y. F., Xiao, D. J., Ma, X. L., Song, Y. Y., Hu, R., Kong, Y., Zheng, Y., Han, S. Y., Hong, R. L., and Wang, Y. S. (2013). Differentiated embryonic chondrocyte-expressed gene 1 is associated with hypoxia-inducible factor 1alpha and Ki67 in human gastric cancer. Diagnostic Pathology *8*, 37.

Jude, C. D., Climer, L., Xu, D., Artinger, E., Fisher, J. K., and Ernst, P. (2007). Unique and independent roles for MLL in adult hematopoietic stem cells and progenitors. Cell Stem Cell *1*, 324-337.

Jude, C. D., Gaudet, J. J., Speck, N. A., and Ernst, P. (2008). Leukemia and hematopoietic stem cells: balancing proliferation and quiescence. Cell Cycle (Georgetown, Tex) *7*, 586-591.

Karlsson, G., Blank, U., Moody, J. L., Ehinger, M., Singbrant, S., Deng, C. X., and Karlsson, S. (2007). Smad4 is critical for self-renewal of hematopoietic stem cells. The Journal of Experimental Medicine *204*, 467-474.

Katayama, Y., Battista, M., Kao, W. M., Hidalgo, A., Peired, A. J., Thomas, S. A., and Frenette, P. S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell *124*, 407-421.

Kaushansky, K. (2005). The molecular mechanisms that control thrombopoiesis. The Journal of Clinical Investigation *115*, 3339-3347.

Kerber, R. A., O'Brien, E., and Cawthon, R. M. (2009). Gene expression profiles associated with aging and mortality in humans. Aging Cell *8*, 239-250.

Kiel, M. J., He, S., Ashkenazi, R., Gentry, S. N., Teta, M., Kushner, J. A., Jackson, T. L., and Morrison, S. J. (2007). Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. Nature *449*, 238-242.

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., and Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kimura, S., Roberts, A. W., Metcalf, D., and Alexander, W. S. (1998). Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. Proceedings of the National Academy of Sciences of the United States of America *95*, 1195-1200.

Kinzler, K. W., and Vogelstein, B. (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature *386*, 761, 763.

Klauke, K., and de Haan, G. (2011). Polycomb group proteins in hematopoietic stem cell aging and malignancies. International Journal of Hematology *94*, 11-23.

Kobune, M., Ito, Y., Kawano, Y., Sasaki, K., Uchida, H., Nakamura, K., Dehari, H., Chiba, H., Takimoto, R., Matsunaga, T., *et al.* (2004). Indian hedgehog gene transfer augments hematopoietic support of human stromal cells including NOD/SCID-beta2m-/- repopulating cells. Blood *104*, 1002-1009.

Koch, U., Wilson, A., Cobas, M., Kemler, R., Macdonald, H. R., and Radtke, F. (2008). Simultaneous loss of beta- and gamma-catenin does not perturb hematopoiesis or lymphopoiesis. Blood *111*, 160-164.

Kohler, A., Schmithorst, V., Filippi, M. D., Ryan, M. A., Daria, D., Gunzer, M., and Geiger, H. (2009). Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. Blood *114*, 290-298.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Krishnamurthy, J., Ramsey, M. R., Ligon, K. L., Torrice, C., Koh, A., Bonner-Weir, S., and Sharpless, N. E. (2006). p16INK4a induces an age-dependent decline in islet regenerative potential. Nature *443*, 453-457.

Krishnamurthy, J., Torrice, C., Ramsey, M. R., Kovalev, G. I., Al-Regaiey, K., Su, L., and Sharpless, N. E. (2004). Ink4a/Arf expression is a biomarker of aging. The Journal of Clinical Investigation *114*, 1299-1307.

Ku, H., Yonemura, Y., Kaushansky, K., and Ogawa, M. (1996). Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. Blood *87*, 4544-4551.

Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T., *et al.* (2003). Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity *18*, 699-711.

Kuranda, K., Vargaftig, J., de la Rochere, P., Dosquet, C., Charron, D., Bardin, F., Tonnelle, C., Bonnet, D., and Goodhardt, M. (2011). Age-related changes in human hematopoietic stem/progenitor cells. Aging Cell *10*, 542-546.

Lacorazza, H. D., Yamada, T., Liu, Y., Miyata, Y., Sivina, M., Nunes, J., and Nimer, S. D. (2006). The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. Cancer Cell *9*, 175-187.

Larsson, J., Blank, U., Helgadottir, H., Bjornsson, J. M., Ehinger, M., Goumans, M. J., Fan, X., Leveen, P., and Karlsson, S. (2003). TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood *102*, 3129-3135.

Lau, C. I., Outram, S. V., Saldana, J. I., Furmanski, A. L., Dessens, J. T., and Crompton, T. (2012). Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. Blood *119*, 4741-4751.

Lawrence, H. J., Christensen, J., Fong, S., Hu, Y. L., Weissman, I., Sauvageau, G., Humphries, R. K., and Largman, C. (2005). Loss of expression of the Hoxa-9 homeobox gene impairs the proliferation and repopulating ability of hematopoietic stem cells. Blood *106*, 3988-3994.

Lemischka, I. R., Raulet, D. H., and Mulligan, R. C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. Cell *45*, 917-927.

Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature *423*, 255-260.

Li, C. L., and Johnson, G. R. (1992). Rhodamine123 reveals heterogeneity within murine Lin-, Sca-1+ hemopoietic stem cells. The Journal of Experimental Medicine *175*, 1443-1447.

Li, Y., Song, X., Ma, Y., Liu, J., Yang, D., and Yan, B. (2004). DNA binding, but not interaction with Bmal1, is responsible for DEC1-mediated transcription regulation of the circadian gene mPer1. The Biochemical Journal *382*, 895-904.

Li, Y., Xie, M., Song, X., Gragen, S., Sachdeva, K., Wan, Y., and Yan, B. (2003). DEC1 negatively regulates the expression of DEC2 through binding to the E-box in the proximal promoter. The Journal of Biological Chemistry *278*, 16899-16907.

Li, Y., Xie, M., Yang, J., Yang, D., Deng, R., Wan, Y., and Yan, B. (2006). The expression of antiapoptotic protein survivin is transcriptionally upregulated by DEC1 primarily through multiple sp1 binding sites in the proximal promoter. Oncogene *25*, 3296-3306.

Li, Y., Zhang, H., Xie, M., Hu, M., Ge, S., Yang, D., Wan, Y., and Yan, B. (2002). Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation. The Biochemical Journal *367*, 413-422.

Liang, Y., Van Zant, G., and Szilvassy, S. J. (2005). Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood *106*, 1479-1487.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709-715.

Liu, Y., Elf, S. E., Miyata, Y., Sashida, G., Liu, Y., Huang, G., Di Giandomenico, S., Lee, J. M., Deblasio, A., Menendez, S., *et al.* (2009). p53 regulates hematopoietic stem cell quiescence. Cell Stem Cell *4*, 37-48.

Lo Celso, C., and Scadden, D. T. (2011). The haematopoietic stem cell niche at a glance. Journal of Cell Science *124*, 3529-3535.

Lotem, J., and Sachs, L. (1993). Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. Blood *82*, 1092-1096.

Lucas, D., Battista, M., Shi, P. A., Isola, L., and Frenette, P. S. (2008). Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell Stem Cell *3*, 364-366.

Luis, T. C., Naber, B. A., Roozen, P. P., Brugman, M. H., de Haas, E. F., Ghazvini, M., Fibbe, W. E., van Dongen, J. J., Fodde, R., and Staal, F. J. (2011). Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell Stem Cell *9*, 345-356.

Lyman, S. D., and Jacobsen, S. E. (1998). c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. Blood *91*, 1101-1134.

Malhotra, S., and Kincade, P. W. (2009). Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. Cell Stem Cell *4*, 27-36.

Mancini, S. J., Mantei, N., Dumortier, A., Suter, U., MacDonald, H. R., and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood *105*, 2340-2342.

Mandal, P. K., and Rossi, D. J. (2012). DNA-damage-induced differentiation in hematopoietic stem cells. Cell *148*, 847-848.

Mansson, R., Hultquist, A., Luc, S., Yang, L., Anderson, K., Kharazi, S., Al-Hashmi, S., Liuba, K., Thoren, L., Adolfsson, J., *et al.* (2007). Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity *26*, 407-419.

Martinez-Llordella, M., Esensten, J. H., Bailey-Bucktrout, S. L., Lipsky, R. H., Marini, A., Chen, J., Mughal, M., Mattson, M. P., Taub, D. D., and Bluestone, J. A. (2013). CD28-inducible transcription factor DEC1 is required for efficient autoreactive CD4+ T cell response. The Journal of Experimental Medicine *210*, 1603-1619.

Matsumoto, A., Takeishi, S., Kanie, T., Susaki, E., Onoyama, I., Tateishi, Y., Nakayama, K., and Nakayama, K. I. (2011). p57 is required for quiescence and maintenance of adult hematopoietic stem cells. Cell Stem Cell *9*, 262-271.

McCulloch, E. A., and Till, J. E. (1960). The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiation Research *13*, 115-125.

McMahon, K. A., Hiew, S. Y., Hadjur, S., Veiga-Fernandes, H., Menzel, U., Price, A. J., Kioussis, D., Williams, O., and Brady, H. J. (2007). Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell *1*, 338-345.

McNiece, I. K., Bertoncello, I., Kriegler, A. B., and Quesenberry, P. J. (1990). Colony-forming cells with high proliferative potential (HPP-CFC). International Journal of Cell Cloning *8*, 146-160.

Mendez-Ferrer, S., Chow, A., Merad, M., and Frenette, P. S. (2009). Circadian rhythms influence hematopoietic stem cells. Current Opinion in Hematology *16*, 235-242.

Mendez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P. S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. Nature *452*, 442-447.

Merchant, A., Joseph, G., Wang, Q., Brennan, S., and Matsui, W. (2010). Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors. Blood *115*, 2391-2396.

Mercier, F. E., Ragu, C., and Scadden, D. T. (2012). The bone marrow at the crossroads of blood and immunity. Nature Reviews Immunology *12*, 49-60.

Meyers, S., and Hiebert, S. W. (1995). Indirect and direct disruption of transcriptional regulation in cancer: E2F and AML-1. Critical Reviews in Eukaryotic Gene Expression *5*, 365-383.

Miller, J. P., and Allman, D. (2005). Linking age-related defects in B lymphopoiesis to the aging of hematopoietic stem cells. Seminars in Immunology *17*, 321-329.

Minegishi, N., Ohta, J., Yamagiwa, H., Suzuki, N., Kawauchi, S., Zhou, Y., Takahashi, S., Hayashi, N., Engel, J. D., and Yamamoto, M. (1999). The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. Blood *93*, 4196-4207.

Minegishi, N., Suzuki, N., Yokomizo, T., Pan, X., Fujimoto, T., Takahashi, S., Hara, T., Miyajima, A., Nishikawa, S., and Yamamoto, M. (2003). Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. Blood *102*, 896-905.

Miyamoto, K., Araki, K. Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., *et al.* (2007). Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell *1*, 101-112.

Miyazaki, K., Miyazaki, M., Guo, Y., Yamasaki, N., Kanno, M., Honda, Z., Oda, H., Kawamoto, H., and Honda, H. (2010). The role of the basic helix-loop-helix transcription factor Dec1 in the regulatory T cells. Journal of Immunology (Baltimore, Md : 1950) *185*, 7330-7339.

Mohrin, M., Bourke, E., Alexander, D., Warr, M. R., Barry-Holson, K., Le Beau, M. M., Morrison, C. G., and Passegue, E. (2010). Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. Cell Stem Cell *7*, 174-185.

Morita, Y., Ema, H., and Nakauchi, H. (2010). Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. The Journal of Experimental Medicine *207*, 1173-1182.

Motoda, L., Osato, M., Yamashita, N., Jacob, B., Chen, L. Q., Yanagida, M., Ida, H., Wee, H. J., Sun, A. X., Taniuchi, I., *et al.* (2007). Runx1 protects hematopoietic stem/progenitor cells from oncogenic insult. Stem Cells (Dayton, Ohio) *25*, 2976-2986.

Muller-Sieburg, C. E., Cho, R. H., Karlsson, L., Huang, J. F., and Sieburg, H. B. (2004). Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood *103*, 4111-4118.

Muller-Sieburg, C. E., Cho, R. H., Thoman, M., Adkins, B., and Sieburg, H. B. (2002). Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. Blood *100*, 1302-1309.

Murdoch, B., Chadwick, K., Martin, M., Shojaei, F., Shah, K. V., Gallacher, L., Moon, R. T., and Bhatia, M. (2003). Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proceedings of the National Academy of Sciences of the United States of America *100*, 3422-3427.

Nakamura, H., Tanimoto, K., Hiyama, K., Yunokawa, M., Kawamoto, T., Kato, Y., Yoshiga, K., Poellinger, L., Hiyama, E., and Nishiyama, M. (2008). Human mismatch repair gene, MLH1, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2. Oncogene *27*, 4200-4209.

Navarro, S., Meza, N. W., Quintana-Bustamante, O., Casado, J. A., Jacome, A., McAllister, K., Puerto, S., Surralles, J., Segovia, J. C., and Bueren, J. A. (2006). Hematopoietic dysfunction in a mouse model for Fanconi anemia group D1. Molecular Therapy: The Journal of the American Society of Gene Therapy *14*, 525-535.

Nemeth, M. J., Topol, L., Anderson, S. M., Yang, Y., and Bodine, D. M. (2007). Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. Proceedings of the National Academy of Sciences of the United States of America *104*, 15436-15441.

Nijnik, A., Woodbine, L., Marchetti, C., Dawson, S., Lambe, T., Liu, C., Rodrigues, N. P., Crockford, T. L., Cabuy, E., Vindigni, A., *et al.* (2007). DNA repair is limiting for haematopoietic stem cells during ageing. Nature *447*, 686-690.

Nishino, J., Kim, I., Chada, K., and Morrison, S. J. (2008). Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression. Cell *135*, 227-239.

Niu, H., Fang, G., Tang, Y., Xie, L., Yang, H., Morel, L., Diamond, B., and Zou, Y. R. (2013). The function of hematopoietic stem cells is altered by both genetic and inflammatory factors in lupus mice. Blood *121*, 1986-1994.

Norddahl, G. L., Pronk, C. J., Wahlestedt, M., Sten, G., Nygren, J. M., Ugale, A., Sigvardsson, M., and Bryder, D. (2011). Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. Cell Stem Cell *8*, 499-510.

Noshiro, M., Kawamoto, T., Furukawa, M., Fujimoto, K., Yoshida, Y., Sasabe, E., Tsutsumi, S., Hamada, T., Honma, S., Honma, K., and Kato, Y. (2004). Rhythmic expression of DEC1 and DEC2 in peripheral tissues: DEC2 is a potent suppressor for hepatic cytochrome P450s opposing DBP. Genes to Cells : Devoted to Molecular & Cellular Mechanisms *9*, 317-329.

Nygren, J. M., Bryder, D., and Jacobsen, S. E. (2006). Prolonged cell cycle transit is a defining and developmentally conserved hemopoietic stem cell property. Journal of Immunology (Baltimore, Md : 1950) *177*, 201-208.

Oguro, H., Ding, L., and Morrison, S. J. (2013). SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell Stem Cell *13*, 102-116.

Oguro, H., Yuan, J., Ichikawa, H., Ikawa, T., Yamazaki, S., Kawamoto, H., Nakauchi, H., and Iwama, A. (2010). Poised lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb protein Bmi1. Cell Stem Cell *6*, 279-286.

Ohta, H., Sawada, A., Kim, J. Y., Tokimasa, S., Nishiguchi, S., Humphries, R. K., Hara, J., and Takihara, Y. (2002). Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. The Journal of Experimental Medicine *195*, 759-770.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044-3050.

Orkin, S. H., and Zon, L. I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science (New York, NY) *273*, 242-245.

Palis, J., and Yoder, M. C. (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. Experimental Hematology *29*, 927-936.

Park, I. K., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., Morrison, S. J., and Clarke, M. F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature *423*, 302-305.

Park, Y., and Gerson, S. L. (2005). DNA repair defects in stem cell function and aging. Annual Review of Medicine *56*, 495-508.

Passegue, E., Wagers, A. J., Giuriato, S., Anderson, W. C., and Weissman, I. L. (2005). Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. The Journal of Experimental Medicine *202*, 1599-1611.

Peng, Y., Liu, W., Xiong, J., Gui, H. Y., Feng, X. M., Chen, R. N., Hu, G., and Yang, J. (2012). Down regulation of differentiated embryonic chondrocytes 1 (DEC1) is involved in 8-methoxypsoralen-induced apoptosis in HepG2 cells. Toxicology *301*, 58-65.

Petit-Cocault, L., Volle-Challier, C., Fleury, M., Peault, B., and Souyri, M. (2007). Dual role of Mpl receptor during the establishment of definitive hematopoiesis. Development (Cambridge, England) *134*, 3031-3040.

Plowden, J., Renshaw-Hoelscher, M., Engleman, C., Katz, J., and Sambhara, S. (2004). Innate immunity in aging: impact on macrophage function. Aging Cell *3*, 161-167.

Pollina, E. A., and Brunet, A. (2011). Epigenetic regulation of aging stem cells. Oncogene *30*, 3105-3126.

Qian, H., Buza-Vidas, N., Hyland, C. D., Jensen, C. T., Antonchuk, J., Mansson, R., Thoren, L. A., Ekblom, M., Alexander, W. S., and Jacobsen, S. E. (2007). Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell *1*, 671-684.

Qian, Y., Jung, Y. S., and Chen, X. (2011). DeltaNp63, a target of DEC1 and histone deacetylase 2, modulates the efficacy of histone deacetylase inhibitors in growth suppression and keratinocyte differentiation. The Journal of Biological Chemistry *286*, 12033-12041.

Qian, Y., Zhang, J., Yan, B., and Chen, X. (2008). DEC1, a basic helix-loop-helix transcription factor and a novel target gene of the p53 family, mediates p53-dependent premature senescence. The Journal of Biological Chemistry *283*, 2896-2905.

Rando, T. A., and Chang, H. Y. (2012). Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. Cell *148*, 46-57.

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Rezzoug, F., Huang, Y., Tanner, M. K., Wysoczynski, M., Schanie, C. L., Chilton, P. M., Ratajczak, M. Z., Fugier-Vivier, I. J., and Ildstad, S. T. (2008). TNF-alpha is critical to facilitate hemopoietic stem cell engraftment and function. Journal of Immunology (Baltimore, Md : 1950) *180*, 49-57.

Rizo, A., Vellenga, E., de Haan, G., and Schuringa, J. J. (2006). Signaling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche? Human Molecular Genetics *15 Spec No 2*, R210-219.

Robert-Moreno, A., Espinosa, L., de la Pompa, J. L., and Bigas, A. (2005). RBPjkappa-dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. Development (Cambridge, England) *132*, 1117-1126.

Robert-Moreno, A., Guiu, J., Ruiz-Herguido, C., Lopez, M. E., Ingles-Esteve, J., Riera, L., Tipping, A., Enver, T., Dzierzak, E., Gridley, T., *et al.* (2008). Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. The EMBO Journal *27*, 1886-1895.

Ronnstrand, L. (2004). Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci *61*, 2535-2548.

Ross, K., Sedello, A. K., Todd, G. P., Paszkowski-Rogacz, M., Bird, A. W., Ding, L., Grinenko, T., Behrens, K., Hubner, N., Mann, M., *et al.* (2012). Polycomb group ring finger 1 cooperates with Runx1 in regulating differentiation and self-renewal of hematopoietic cells. Blood *119*, 4152-4161.

Rossi, D. J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I. L. (2007a). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature *447*, 725-729.

Rossi, D. J., Bryder, D., Zahn, J. M., Ahlenius, H., Sonu, R., Wagers, A. J., and Weissman, I. L. (2005). Cell intrinsic alterations underlie hematopoietic stem cell aging. Proceedings of the National Academy of Sciences of the United States of America *102*, 9194-9199.

Rossi, D. J., Seita, J., Czechowicz, A., Bhattacharya, D., Bryder, D., and Weissman, I. L. (2007b). Hematopoietic stem cell quiescence attenuates DNA damage response and permits DNA damage accumulation during aging. Cell Cycle (Georgetown, Tex) *6*, 2371-2376.

Rossner, M. J., Dorr, J., Gass, P., Schwab, M. H., and Nave, K. A. (1997). SHARPs: mammalian enhancer-of-split- and hairy-related proteins coupled to neuronal stimulation. Molecular and Cellular Neurosciences *10*, 460-475.

Rowlinson, J. M., and Gering, M. (2010). Hey2 acts upstream of Notch in hematopoietic stem cell specification in zebrafish embryos. Blood *116*, 2046-2056.

Rube, C. E., Fricke, A., Widmann, T. A., Furst, T., Madry, H., Pfreundschuh, M., and Rube, C. (2011). Accumulation of DNA damage in hematopoietic stem and progenitor cells during human aging. PloS One *6*, e17487.

Sablina, A. A., Budanov, A. V., Ilyinskaya, G. V., Agapova, L. S., Kravchenko, J. E., and Chumakov, P. M. (2005). The antioxidant function of the p53 tumor suppressor. Nature Medicine *11*, 1306-1313.

Sahin, E., Colla, S., Liesa, M., Moslehi, J., Muller, F. L., Guo, M., Cooper, M., Kotton, D., Fabian, A. J., Walkey, C., *et al.* (2011). Telomere dysfunction induces metabolic and mitochondrial compromise. Nature *470*, 359-365.

Sato, F., Kawamoto, T., Fujimoto, K., Noshiro, M., Honda, K. K., Honma, S., Honma, K., and Kato, Y. (2004). Functional analysis of the basic helix-loop-helix transcription factor DEC1 in circadian regulation. Interaction with BMAL1. European Journal of Biochemistry / FEBS *271*, 4409-4419.

Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., and Humphries, R. K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes & Development *9*, 1753-1765.

Scandura, J. M., Boccuni, P., Massague, J., and Nimer, S. D. (2004). Transforming growth factor beta-induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. Proceedings of the National Academy of Sciences of the United States of America *101*, 15231-15236.

Schwanbeck, R., Schroeder, T., Henning, K., Kohlhof, H., Rieber, N., Erfurth, M. L., and Just, U. (2008). Notch signaling in embryonic and adult myelopoiesis. Cells, Tissues, Organs *188*, 91-102.

Seimiya, M., Bahar, R., Wang, Y., Kawamura, K., Tada, Y., Okada, S., Hatano, M., Tokuhisa, T., Saisho, H., Watanabe, T., *et al.* (2002). Clast5/Stra13 is a negative regulator of B lymphocyte activation. Biochem Biophys Res Commun *292*, 121-127.

Seimiya, M., Wada, A., Kawamura, K., Sakamoto, A., Ohkubo, Y., Okada, S., Hatano, M., Tokuhisa, T., Watanabe, T., Saisho, H., *et al.* (2004). Impaired lymphocyte development and function in Clast5/Stra13/DEC1-transgenic mice. European Journal of Immunology *34*, 1322-1332.

Shen, M., Yoshida, E., Yan, W., Kawamoto, T., Suardita, K., Koyano, Y., Fujimoto, K., Noshiro, M., and Kato, Y. (2002). Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. J Biol Chem *277*, 50112-50120.

Shi, X. H., Zheng, Y., Sun, Q., Cui, J., Liu, Q. H., Qu, F., and Wang, Y. S. (2011). DEC1 nuclear expression: a marker of differentiation grade in hepatocellular carcinoma. World Journal of Gastroenterology : WJG *17*, 2037-2043.

Signer, R. A., and Morrison, S. J. (2013). Mechanisms that regulate stem cell aging and life span. Cell Stem Cell *12*, 152-165.

Singh, K. P., Garrett, R. W., Casado, F. L., and Gasiewicz, T. A. (2011). Aryl hydrocarbon receptor-null allele mice have hematopoietic stem/progenitor cells with abnormal characteristics and functions. Stem Cells and Development *20*, 769-784.

Sitnicka, E., Bryder, D., Theilgaard-Monch, K., Buza-Vidas, N., Adolfsson, J., and Jacobsen, S. E. (2002). Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. Immunity *17*, 463-472.

Solar, G. P., Kerr, W. G., Zeigler, F. C., Hess, D., Donahue, C., de Sauvage, F. J., and Eaton, D. L. (1998). Role of c-mpl in early hematopoiesis. Blood *92*, 4-10.

Spangrude, G. J., Heimfeld, S., and Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science (New York, NY) *241*, 58-62.

St-Pierre, B., Flock, G., Zacksenhaus, E., and Egan, S. E. (2002). Stra13 homodimers repress transcription through class B E-box elements. The Journal of Biological Chemistry *277*, 46544-46551.

Staal, F. J., and Luis, T. C. (2010). Wnt signaling in hematopoiesis: crucial factors for self-renewal, proliferation, and cell fate decisions. Journal of Cellular Biochemistry *109*, 844-849.

Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D. T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood *99*, 2369-2378.

Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. The Journal of Experimental Medicine *192*, 1273-1280.

Sun, H., Ghaffari, S., and Taneja, R. (2007). bHLH-Orange Transcription Factors in Development and Cancer. Translational Oncogenomics *2*, 107-120.

Sun, H., Lu, B., Li, R. Q., Flavell, R. A., and Taneja, R. (2001). Defective T cell activation and autoimmune disorder in Stra13-deficient mice. Nature Immunology *2*, 1040-1047.

Sun, H., and Taneja, R. (2000). Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. Proceedings of the National Academy of Sciences of the United States of America *97*, 4058-4063.

Szilvassy, S. J., Humphries, R. K., Lansdorp, P. M., Eaves, A. C., and Eaves, C. J. (1990). Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proceedings of the National Academy of Sciences of the United States of America *87*, 8736-8740.

Tajima, Y., Moore, M. A., Soares, V., Ono, M., Kissel, H., and Besmer, P. (1998). Consequences of exclusive expression in vivo of Kit-ligand lacking the major proteolytic cleavage site. Proceedings of the National Academy of Sciences of the United States of America *95*, 11903-11908.

Taswell, C. (1981). Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. Journal of Immunology (Baltimore, Md : 1950) *126*, 1614-1619.

TeKippe, M., Harrison, D. E., and Chen, J. (2003). Expansion of hematopoietic stem cell phenotype and activity in Trp53-null mice. Experimental Hematology *31*, 521-527.

Thin, T. H., Li, L., Chung, T. K., Sun, H., and Taneja, R. (2007). Stra13 is induced by genotoxic stress and regulates ionizing-radiation-induced apoptosis. EMBO Reports *8*, 401-407.

Thomas, S., Legendre, M., Saunier, S., Bessières, B., Alby, C., Bonnière, M., Toutain, A., Loeuillet, L., Szymanska, K., Jossic, F., *et al.* (2012). TCTN3 Mutations Cause Mohr-Majewski Syndrome. The American Journal of Human Genetics *91*, 372-378.

Thorsteinsdottir, U., Mamo, A., Kroon, E., Jerome, L., Bijl, J., Lawrence, H. J., Humphries, K., and Sauvageau, G. (2002). Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood *99*, 121-129.

Till, J. E., and Mc, C. E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiation Research *14*, 213-222.

Tipping, A. J., Pina, C., Castor, A., Hong, D., Rodrigues, N. P., Lazzari, L., May, G. E., Jacobsen, S. E., and Enver, T. (2009). High GATA-2 expression inhibits human hematopoietic stem and progenitor cell function by effects on cell cycle. Blood *113*, 2661-2672.

Tothova, Z., Kollipara, R., Huntly, B. J., Lee, B. H., Castrillon, D. H., Cullen, D. E., McDowell, E. P., Lazo-Kallanian, S., Williams, I. R., Sears, C., *et al.* (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Trowbridge, J. J., Scott, M. P., and Bhatia, M. (2006). Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. Proceedings of the National Academy of Sciences of the United States of America *103*, 14134-14139.

Tsinkalovsky, O., Filipski, E., Rosenlund, B., Sothern, R. B., Eiken, H. G., Wu, M. W., Claustrat, B., Bayer, J., Levi, F., and Laerum, O. D. (2006). Circadian expression of clock genes in purified hematopoietic stem cells is developmentally regulated in mouse bone marrow. Experimental Hematology *34*, 1249-1261.

Tsinkalovsky, O., Rosenlund, B., Laerum, O. D., and Eiken, H. G. (2005). Clock gene expression in purified mouse hematopoietic stem cells. Experimental Hematology *33*, 100-107.

Tsinkalovsky, O., Smaaland, R., Rosenlund, B., Sothern, R. B., Hirt, A., Steine, S., Badiee, A., Abrahamsen, J. F., Eiken, H. G., and Laerum, O. D. (2007). Circadian variations in clock gene expression of human bone marrow CD34+ cells. Journal of Biological Rhythms *22*, 140-150.

Tuljapurkar, S. R., McGuire, T. R., Brusnahan, S. K., Jackson, J. D., Garvin, K. L., Kessinger, M. A., Lane, J. T., BJ, O. K., and Sharp, J. G. (2011). Changes in human bone marrow fat content associated with changes in hematopoietic stem cell numbers and cytokine levels with aging. Journal of Anatomy *219*, 574-581.

Uchida, N., Dykstra, B., Lyons, K. J., Leung, F. Y., and Eaves, C. J. (2003). Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. Experimental Hematology *31*, 1338-1347.

van Os, R. P., Dethmers-Ausema, B., and de Haan, G. (2008). In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C. Methods in Molecular Biology (Clifton, NJ) *430*, 143-157.

Vas, V., Senger, K., Dorr, K., Niebel, A., and Geiger, H. (2012a). Aging of the microenvironment influences clonality in hematopoiesis. PloS One *7*, e42080.

Vas, V., Wandhoff, C., Dorr, K., Niebel, A., and Geiger, H. (2012b). Contribution of an aged microenvironment to aging-associated myeloproliferative disease. PloS One *7*, e31523.

Venezia, T. A., Merchant, A. A., Ramos, C. A., Whitehouse, N. L., Young, A. S., Shaw, C. A., and Goodell, M. A. (2004). Molecular signatures of proliferation and quiescence in hematopoietic stem cells. PLoS Biology *2*, e301.

Wagner, W., Horn, P., Bork, S., and Ho, A. D. (2008). Aging of hematopoietic stem cells is regulated by the stem cell niche. Experimental Gerontology *43*, 974-980.

Wang, C. Q., Jacob, B., Nah, G. S. S., and Osato, M. (2010). Runx family genes, niche, and stem cell quiescence. Blood Cells, Molecules, and Diseases *44*, 275-286.

Wang, J., Sun, Q., Morita, Y., Jiang, H., Gross, A., Lechel, A., Hildner, K., Guachalla, L. M., Gompf, A., Hartmann, D., *et al.* (2012a). A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. Cell *148*, 1001-1014.

Wang, K., Saito, M., Bisikirska, B. C., Alvarez, M. J., Lim, W. K., Rajbhandari, P., Shen, Q., Nemenman, I., Basso, K., Margolin, A. A., *et al.* (2009). Genome-wide identification of post-translational modulators of transcription factor activity in human B cells. Nature Biotechnology *27*, 829-839.

Wang, N., Morra, M., Wu, C., Gullo, C., Howie, D., Coyle, T., Engel, P., and Terhorst, C. (2001). CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells. Immunogenetics *53*, 382-394.

Wang, Y., Rao, V. K., Kok, W. K., Roy, D. N., Sethi, S., Ling, B. M., Lee, M. B., and Taneja, R. (2012b). SUMO modification of Stra13 is required for repression of cyclin D1 expression and cellular growth arrest. PloS One *7*, e43137.

Warr, M. R., Pietras, E. M., and Passegue, E. (2011). Mechanisms controlling hematopoietic stem cell functions during normal hematopoiesis and hematological malignancies. Wiley interdisciplinary reviews Systems Biology and Medicine *3*, 681-701.

Weber, J. M., and Calvi, L. M. (2010). Notch signaling and the bone marrow hematopoietic stem cell niche. Bone *46*, 281-285.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448-452.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C. F., Eshkind, L., Bockamp, E., *et al.* (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. Cell *135*, 1118-1129.

Wilson, A., Murphy, M. J., Oskarsson, T., Kaloulis, K., Bettess, M. D., Oser, G. M., Pasche, A. C., Knabenhans, C., Macdonald, H. R., and Trumpp, A. (2004). c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes & Development *18*, 2747-2763.

Wilson, N. K., Foster, S. D., Wang, X., Knezevic, K., Schutte, J., Kaimakis, P., Chilarska, P. M., Kinston, S., Ouwehand, W. H., Dzierzak, E., *et al.* (2010). Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. Cell Stem Cell *7*, 532-544.

Wu, Y., Sato, F., Bhawal, U. K., Kawamoto, T., Fujimoto, K., Noshiro, M., Morohashi, S., Kato, Y., and Kijima, H. (2011). Basic helix-loop-helix transcription factors DEC1 and DEC2 regulate the paclitaxel-induced apoptotic pathway of MCF-7 human breast cancer cells. International Journal of Molecular Medicine *27*, 491-495.

Xing, Z., Ryan, M. A., Daria, D., Nattamai, K. J., Van Zant, G., Wang, L., Zheng, Y., and Geiger, H. (2006). Increased hematopoietic stem cell mobilization in aged mice. Blood *108*, 2190-2197.

Yahata, T., Takanashi, T., Muguruma, Y., Ibrahim, A. A., Matsuzawa, H., Uno, T., Sheng, Y., Onizuka, M., Ito, M., Kato, S., and Ando, K. (2011). Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood *118*, 2941-2950.

Yalcin, S., Zhang, X., Luciano, J. P., Mungamuri, S. K., Marinkovic, D., Vercherat, C., Sarkar, A., Grisotto, M., Taneja, R., and Ghaffari, S. (2008). Foxo3 is essential for the regulation of ataxia telangiectasia mutated and oxidative stress-mediated homeostasis of hematopoietic stem cells. The Journal of Biological Chemistry *283*, 25692-25705.

Yamada, K., Kawata, H., Mizutani, T., Arima, T., Yazawa, T., Matsuura, K., Shou, Z., Sekiguchi, T., Yoshino, M., Kajitani, T., and Miyamoto, K. (2004). Gene expression of basic helix-loop-helix transcription factor, SHARP-2, is regulated by gonadotropins in the rat ovary and MA-10 cells. Biology of Reproduction *70*, 76-82.

Yamada, K., Kawata, H., Shou, Z., Mizutani, T., Noguchi, T., and Miyamoto, K. (2003). Insulin induces the expression of the SHARP-2/Stra13/DEC1 gene via a phosphoinositide 3-kinase pathway. The Journal of Biological Chemistry *278*, 30719-30724.

Yamada, K., and Miyamoto, K. (2005). Basic helix-loop-helix transcription factors, BHLHB2 and BHLHB3; their gene expressions are regulated by multiple extracellular stimuli. Frontiers in Bioscience : a journal and virtual library *10*, 3151-3171.

Yeoh, J. S., van Os, R., Weersing, E., Ausema, A., Dontje, B., Vellenga, E., and de Haan, G. (2006). Fibroblast growth factor-1 and -2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures. Stem Cells (Dayton, Ohio) *24*, 1564-1572.

Yilmaz, O. H., Kiel, M. J., and Morrison, S. J. (2006). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood *107*, 924-930.

Yu, H., Yuan, Y., Shen, H., and Cheng, T. (2006a). Hematopoietic stem cell exhaustion impacted by p18 INK4C and p21 Cip1/Waf1 in opposite manners. Blood *107*, 1200-1206.

Yu, X., Alder, J. K., Chun, J. H., Friedman, A. D., Heimfeld, S., Cheng, L., and Civin, C. I. (2006b). HES1 inhibits cycling of hematopoietic progenitor cells via DNA binding. Stem Cells (Dayton, Ohio) *24*, 876-888.

Yuan, Y., Shen, H., Franklin, D. S., Scadden, D. T., and Cheng, T. (2004). In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. Nature Cell Biology *6*, 436-442.

Yun, Z., Maecker, H. L., Johnson, R. S., and Giaccia, A. J. (2002). Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Developmental Cell *2*, 331-341.

Zawel, L., Yu, J., Torrance, C. J., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Zhou, S. (2002). DEC1 is a downstream target of TGF-beta with sequence-specific transcriptional repressor activities. Proceedings of the National Academy of Sciences of the United States of America *99*, 2848-2853.

Zelenock, J. A., Welling, T. H., Sarkar, R., Gordon, D. G., and Messina, L. M. (1997). Improved retroviral transduction efficiency of vascular cells in vitro and in vivo during clinically relevant incubation periods using centrifugation to increase viral titers. Journal of Vascular Surgery *26*, 119-127.

Zeng, H., Yucel, R., Kosan, C., Klein-Hitpass, L., and Moroy, T. (2004). Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. The EMBO Journal *23*, 4116-4125.

Zhang, J., Grindley, J. C., Yin, T., Jayasinghe, S., He, X. C., Ross, J. T., Haug, J. S., Rupp, D., Porter-Westpfahl, K. S., Wiedemann, L. M., *et al.* (2006). PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature *441*, 518-522.

Zhang, Y., Harada, A., Bluethmann, H., Wang, J. B., Nakao, S., Mukaida, N., and Matsushima, K. (1995). Tumor necrosis factor (TNF) is a physiologic regulator of hematopoietic progenitor cells: increase of early hematopoietic progenitor cells in TNF receptor p55-deficient mice in vivo and potent inhibition of progenitor cell proliferation by TNF alpha in vitro. Blood *86*, 2930-2937.

Zhao, C., Chen, A., Jamieson, C. H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H. Y., Kim, J., Chute, J. P., Rizzieri, D., *et al.* (2009). Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature *458*, 776-779.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., and Sorrentino, B. P. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature Medicine *7*, 1028-1034.