UNIVERSITY OF CALIFORNIA, SAN DIEGO

Deciphering a Kit-Shp2-*Kit* Axis in Regulation of Adult Hematopoietic Stem and Progenitor Cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

He Zhu

Committee in charge:

Professor Gen-Sheng Feng, Chair
Professor Mark Kamps, Co-Chair
Professor Michael David
Professor Robert Rickert
Professor Dong-Er Zhang

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The dissertation of He Zhu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

DEDICATION

To my dear husband Michael Chengjun Qian ,

parents Yongzhong Zhu and Xianglan Yi,

and in laws Jianguo Qian and Ping Cheng.

⋆⋆⋆⋆ *"To raise new questions, new possibilities,* ⋆⋆⋆⋆

to regard old questions from a new angle, requires creative imagination and marks real advances in science."

--Albert Einstein

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LIST OF ABBREVIATIONS

34^-LSK	$lin^-Sca1^+Kit^+CD34^-$
IGF-1	insulin-like growth factor-1
5-FU	5-fluorouracil
AGM	aorta-gonad-mesonephros
AKT	protein kinase B
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANG1	Angiopoietin 1
BCR	anti-B cell antigen receptor
BMNC	bone marrow nucleated cell
BMP	bone morphogenetic protein
BMPRIA	bone morphogenetic protein (BMP) receptor 1A
Brdu	5-bromo-2'-deoxy-uridine
CBC	complete blood cell
CBF	core-binding factor
Cbfα1	core binding factor α1
CFU	Colony Forming Unit
CHIP	Chromatin Immunoprecipitation
CLP	common lymphoid progenitors
CMP	common myeloid progenitor
Cola1	type 1 collagen α1

csw	Corkscrew
C-terminal	carboxy terminal
CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	C-X-C chemokine receptor type 4
DMEM	dulbecco's modified eagle medium
DN	double-negative
DP	double-positive
E	embryonic day
EBs	embryonic bodies
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EML	Erythroid myeloid lymphoid
MEP	megakaryocytic-erythroid progenitor
Epo	Erythropoietin
Erk	extracellular signal regulated kinase
ES cells	embryonic stem cells
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
floxed	loxP-flanked
FLT3	FMS-like tyrosine kinase 3
FRS-2	growth factor receptor substrate-2

GAB	Grb2-associated binder
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GM-CSFR	granulocyte macrophage colony-stimulating factor receptor
GMP	granulocyte-monocyte progenitors
Hcd2	human CD2
HSC	hematopoietic stem cell
IFN	interferon
IL-3	interleukin 3
IMDM	iscove's modified dulbecco's medium
IRES	internal ribosomal entry site
IRS-1	insulin receptor substrate-1
JM	juxtamembrane
JMML	juvenile myelomonocytic leukemia
JNK	c-Jun NH2-terminal kinase
LIF	leukemia inhibitory factor
LMO2	Lim-only 2
LS	LEOPARD syndrome
LSK	lineage^{-} Sca-1^{+} c-Kit^{+}
LT-HSC	long-term repopulating HSC
MAPK	mitogen-activated protein kinase
M-CSFR	macrophage colony-stimulating factor receptor

me	*Motheaten*
me v	*motheaten viable*
MMP	matrix metalloproteinase
MPP	multi-potential progenitor
MPN	myeloproliferative neoplasm
NF1	neurofibromatosis type 1
NS	Noonan Syndrome
N-terminal	amino-terminal
P.I.	post final injection
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
poly-I:C	polyinosinic: polycytidylic acid
PPR	PTH-related protein receptor
pSp	para-aortic splanchnopleura
PTH	parathyroid hormone
PTP	protein tyrosine phosphatase
PTPN11	none-receptor protein tyrosine phosphatase number 11
pTyr	phosphotyrosine
PVDF	Polyvinylidene fluoride
RARγ	Retinoic acid receptor γ
RB	retinoblastoma protein
RTK	receptor tyrosine kinase

SCF	stem cell factor
SDF-1alpha	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	sinusoidal endothelial cell
SH2	Src homology-2
SHP	Src homology-2 domain-containing phosphatase
Sl/Sl mice	steel-Dickie mice
SLAM	signaling lymphocytic activation molecule
SNO	spindle-shaped N-cadherin^{+}CD45^{-} osteoblastic cell
STAT	signal transducers and activators of transcription
ST-HSC	short-term repopulating HSC
TCR	T cell antigen receptor
TNF	tumor necrosis factor
Tpo	thrombopoietin
Tunel	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGFR2	Vascular endothelial growth factor receptor 2
W	*white spotting*

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The author of this dissertation was the major investigator and first author of both papers.

VITA

2002-2006 B.S., College of Life Science, Fudan University, P.R. China

2006-2011 Ph.D., Molecular Pathology Graduate Program, University of California, San Diego

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

Deciphering a Kit-Shp2-*Kit* Axis in Regulation of Adult Hematopoietic Stem and Progenitor Cells

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It is now widely accepted that the fine balance between hematopoietic stem cell (HSC) self-renewal and differentiation is required for blood cell homeostasis and is evidently orchestrated by the dynamic interplay between environmental cues and intrinsic genetic setup. Nevertheless, how the niche signal-initiated intracellular

signaling cascades are regulated in HSCs is less understood. The Stem cell factor (SCF)/Kit system has served as a classical model in deciphering molecular signaling events in the hematopoietic compartment. It has long been known that the microenvironment, or the so-called niche, of *Sl/Sl* mouse (steel-Dickie mice) that harbors a mutation in membrane-bound SCF or Kit-ligand, is not capable of supporting normal HSC functions. Study of various *white spotting* (*W*) mutant mice revealed an essential role of SCF-Kit signaling in HSC survival and quiescence maintenance. Kit is now a most frequently used marker for HSCs and progenitors. However, it remains to be elucidated how Kit expression is regulated in HSCs. In this dissertation, we report that a cytoplasmic tyrosine phosphatase Shp2, acting downstream of Kit, promotes *Kit* gene expression, constituting a Kit-Shp2-*Kit* signaling loop. Inducible ablation of none-receptor protein tyrosine phosphatase number 11 (*PTPN11/Shp2*) in adult hematopoietic compartment led to severe cytopenia in bone marrow, spleen and peripheral blood in mice. Shp2 removal resulted in elevated HSC death and loss of HSC quiescence, causing suppression of the functional HSCs/progenitors pool. Shp2-deficient HSCs failed to reconstitute lethally irradiated recipients in a cell autonomous manner due to combined defects in homing, self-renewal and survival. We show that Shp2 regulates coordinately multiple signals, including AKT, MAPK and STAT3 pathways, to promote *Kit* expression via the transcriptional factor Gata2. Therefore, this study reveals a novel signaling mechanism of kinase-phosphatase-*kinase* cascade in HSCs/progenitors in adult mammals.

I. Introduction

HSCs constitute a tiny portion of the bone marrow compartment but are maintained throughout life for sustained production of all lineages of mature blood cells (Weissman and Shizuru, 2008). The hypothesis of ancestors for mature hematopoietic cells was first proposed by Ray Owen in 1945 (Owen, 1945). Since then a wealth of knowledge has been established for the basis of HSC research, including the development of quantitative analysis of hematopoietic progenitors, identification of HSC markers and enrichment of phenotypic HSCs, understanding of HSC ontogeny, and characterization of HSC maintaining microenvironments, etc. It is evidently demonstrated that the maintenance of functional HSCs depends on the dynamic interplay between environmental cues and intrinsic genetic programming (Martinez-Agosto et al., 2007). We have known for long that the bone marrow niche of *Sl/Sl* mouse with a mutation in membrane-bound SCF is not capable of supporting normal HSC functions (Barker, 1997; McCulloch et al., 1965). A number of other niche cell components, cell membrane-bound or secreted growth factors, cytokines, or small molecules were subsequently identified to maintain HSC quiescence, self-renewal and survival (Yin and Li, 2006). Nonetheless, how the niche signal-initiated intracellular signaling cascades are regulated in HSCs remains obscure. Elucidation of the signaling mechanisms governing HSC activities is pivotal to the understanding of physiological hematopoiesis and also the pathogenesis of blood disorders.

1. Ontogeny of HSCs.

During mammalian development, hematopoiesis takes place in a sequential order from distinct embryonic sites. Hematopoietic and endothelial lineages, which emerge in almost the same stage at physically close spots during embryogenesis, share a common precursor, the hemangioblast. Hemangioblasts are originated from a subset of mesoderm cells. *In vitro* stimulation of murine embryonic stem cells (ES cells)-derived embryonic bodies (EBs) by vascular endothelial growth factor (VEGF) led to the production of hematopoietic and vascular bi-potent hemangioblasts expressing both brachyury and flk-1 (Choi et al., 1998; Fehling et al., 2003). *In vivo* dissecting of mice embryos with an enhanced green fluorescent protein (EGFP) reporter targeted to the *brachyury* locus suggested hemangioblasts first appear in the posterior region of the primitive streak, then migrate to the yolk sac and proceed to hematopoietic or endothelial fate specifications (Huber et al., 2004). Blood islands are formed in the mouse yolk sac later on, from which primitive erythrocytes are generated on embryonic day 7.5 (E7.5) (Ferkowicz and Yoder, 2005; Ueno and Weissman, 2006). Definite hematopoiesis takes place in at least four different anatomic sites: the yolk sac, allantois, aorta-gonad-mesonephros (AGM), and placenta. Erythroid-myeloid progenitors or pro-definitive hematopoietic progenitors can be detected in the yolk sac, allantoises, and para-aortic splanchnopleura (pSp) shortly after primitive hematopoiesis between E7.5 and E8.5 when the mouse embryonic circulation is established (Corbel et al., 2007; Cumano et al., 1996; Ferkowicz et al., 2003; Palis et al., 1999; Zeigler et al., 2006). After E9, myeloid progenitors are abundantly generated

in AGM region (Medvinsky and Dzierzak, 1996). Another type of progenitors, meso-definitive progenitors with both lymphoid and myeloid potential and low long-term multi-lineage reconstitution capacity, can be detected on E8 in the pSp region (Cumano et al., 1996). By E9, a third class of progenitors or the meta-definitive progenitors, which possess multi-potent repopulating potential in newborns but not adult mice, are generated in yolk sac and AGM (Yoder et al., 1997). Definitive HSCs with full reconstitution capacity appear firstly in AGM on E10.5 then migrate to and expand in the fetal liver (de Bruijn et al., 2000; Johnson and Moore, 1975; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Adult definitive HSCs begin to seed in the bone marrow from E15 and thereafter reside in the marrow throughout the rest of life.

2. Phenotypic and functional quantification of HSCs.

Three important characteristics in defining HSCs are multi-potency, self-renewal, and niche-dependence. To replenish the short-lived blood cells, HSCs harbor extensive differentiation capacity by an intricately regulated hierarchy. When studied in experimental transplantation models, HSCs can be divided into two subgroups: the long-term HSCs (LT-HSCs) and the short-term HSCs (ST-HSCs). LT-HSCs with long term repopulating potential give rise to more actively cycling ST-HSCs and multi-potential progenitors (MPPs) (Morrison et al., 1997; Morrison and Weissman, 1994). MPPs then differentiate into committed common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) (Akashi et al., 2000; Kondo et al., 1997). CLPs give rise to all the lymphocyte progenitors which ultimately differentiate into T-

lymphocytes, B-lymphocytes and NK cells. CMPs are the progenitors of megakaryocytic-erythroid progenitors (MEPs) as well as granulocyte-monocyte progenitors (GMPs), which will finally differentiate into mature erythrocytes, platelets, granulocytes and macrophages.

Specific bone marrow subpopulations can be identified by a combination of cell surface markers. Mouse HSCs are enriched in the bone marrow cells that do not express mature cell surface lineage markers, including CD3, CD45R/B220, CD11b, TER-119, Ly-6G, CD4 and CD8 et al, but express high level of c-Kit and stem cell antigen Sca-1. This subset of cells represents around 0.15% to 0.2% of total bone marrow and is named as lineage$^-$ Sca-1$^+$ c-Kit$^+$ (LSK) cells. Flk2, Thy and signaling lymphocytic activation molecule (SLAM) family receptors CD150 and CD48 were further adopted to enrich highly purified HSC (Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48 CD150$^+$Thy^{low})(Christensen and Weissman, 2001; Kiel et al., 2005; Kim et al., 2006; Morrison et al., 1995; Osawa et al., 1996). The more committed progenitors CMPs are found to be most enriched in IL-7Rα$^-$Lin$^-$c-Kit$^+$Sca-1 FcγR^{low}CD34$^+$ cells, while CLPs are exclusively found in Lin$^-$IL-7R$^+$Thy-1$^-$Sca-1^{lo}c-Kit^{lo} bone marrow subset. GMPs are in IL-7Rα$^-$Lin$^-$c-Kit$^+$Sca-1$^-$FcγR^{hi}CD34$^+$ bone marrow fraction and MEPs are in IL-7Rα$^-$Lin$^-$c-Kit$^+$Sca-1$^-$FcγR^{lo}CD34$^-$ subpopulation (Akashi et al., 2000; Barker, 1997).

In addition to phenotypic identification, a few functional assays for the quantification of HSC and primitive progenitors have also been developed, such as the *in vitro* Colony Forming Unit (CFU) assay and the *in vivo* CFU-spleen assay. In a

CFU assay, hematopoietic cells from bone marrow or other hemogenic organs are seeded in a methylcellulose semi-solid medium containing a combination of different cytokines such as erythropoietin, thrombopoietin, insulin, interleukins and so on for optimal growth and differentiation of erythroid progenitors (BFU-E), granulocyte progenitors (CFU-G), macrophage progenitors (CFU-M) or granulocyte-macrophage progenitors (CFU-GM) and multi-potential granulocyte, erythroid, macrophage, megakaryocyte progenitors (CFU-GEMM). Colony number will be enumerated after a certain period of incubation time and can be used to quantify a specific type of progenitors. *In vivo* CFU-Spleen assay in lethally irradiated mice is used as a short-term quantitative assay for pluripotent progenitor cells. Committed myeloid progenitors form discrete nodules in recipients' spleens on day 8 after transplantation to irradiated mice. Colonies in host animals on day 12 are generated by more primitive multipotent progenitors (Till and Mc, 1961). Besides all the methods described above, the most reliable way to quantify fully functional HSCs is the long term transplantation assay. Fractionated or whole hematopoietic cells are transplanted into lethally irradiated host mice with or without competitive donor cells. Multi-lineage reconstitution of the hematopoietic system in recipients is monitored at different times for up to 4 to 6 months. Secondary and even tertiary transplantation are sometimes adopted to determine self-renewal and multi-lineage differentiation capacity of HSCs (Weissman and Shizuru, 2008).

3. HSC niches

Life-long preservation of the HSC pool is enabled by the self-renewing property, a biological process allowing for HSC to reproduce itself with the same differentiation potential. The balance between HSC self-renewal and differentiation is required for homeostasis in the blood system. Under homeostatic conditions, maintenance of HSCs in a relatively dormant status is required for long-term preservation of the stem cell reservoir. In response to bone marrow injury, dormant HSCs enter a self-renewal cycling that leads to quick expansion of the stem cell pool and differentiation into mature cells. Once homeostasis is re-established, HSCs will switch back to quiescence (Wilson et al., 2008). Therefore precise cell cycle control is essential for normal HSC function. This hypothesis is substantiated by a recent study that deletion of *PTEN* in bone marrow caused short-term HSC hyper-proliferation, resulting in reduced stem cell activity and exhaustion of LT-HSCs (Zhang et al., 2006). The quiescence and entry into cycling of HSCs are tightly controlled and maintained in postnatal hematopoiesis by cytokines/growth factors and other signals in the microenvironment (Mendez-Ferrer et al., 2010; Xie and Li, 2007). HSCs are proposed to reside in specific bone marrow microenvironments, or the so called niche, constituted by different supporting cells and their secreted factors. Although debates still exist, current literature supports two types of niches, the endosteal niche and peri-vascular niche (Kiel and Morrison, 2008; Li and Xie, 2005; Wilson and Trumpp, 2006). Recently developed *ex vivo* or *in vivo* real time images of individual hematopoietic stem/progenitor cells by the combination usage of high-resolution

confocal microscopy and two-photon imaging confirmed the physical adjacency between HSCs and the endosteal niche (Lo Celso et al., 2009; Xie et al., 2009). Different genetically engineered mice models suggested that osteoblasts and osteoclasts, which build or remodel bone structure, critically influence HSC functions and are therefore considered active components of the HSC endosteal niche. Knock out mice of core binding factor α1 (*Cbfα1*), a key transcriptional factor in osteogenesis, showed maturational arrest of osteoblasts and consequent loss of bone marrow and extramedullary hematopoiesis in the liver and spleen (Deguchi et al., 1999). Constitutive expression of active parathyroid hormone (PTH) or PTH-related protein receptor (PPR) in osteoblasts under the type 1 collagen α1 (*Cola1*) promoter led to an increased number of osteoblasts accompanied with augmented HSC compartment in transgenic mice (Calvi et al., 2003). A similar phenotype was observed in bone morphogenetic protein (BMP) receptor 1A (*BMPR1A*) deficient mice (Zhang et al., 2003). In this study, the long-term HSCs were found in close association with spindle-shaped N-cadherin+CD45- osteoblastic cells (SNO) through two adherens junction molecules, N-cadherin and β-catenin. The importance of osteoblasts in supporting HSC activities was further substantiated by the decreased HSC number in bone marrow and enhanced extramedullary hematopoiesis in *Col1a1* promoter driven–thymidine-kinase transgenic mice in which osteoblasts were conditionally ablated by treatment of ganciclovir (Visnjic et al., 2004). SNO cells express membrane proteins such as SCF, N-cadherin or secrete factors such as angiopoietin 1 (ANG1) and thrombopoietin (Tpo), all of which have been proved to enhance HSC survival, self-

renewal, and quiescence (Arai et al., 2004; Barker, 1994; Yoshihara et al., 2007; Zhang et al., 2003). Apart from the endosteum, the peri-vascular site is suggested to be another stem cell niche. $CD150^{+}CD48^{-}CD41^{-}Lineage^{-}$ enriched HSCs were found to be in contact with sinusoidal endothelial cells (SECs) in mouse bone marrow (Kiel et al., 2005). Vascular endothelial growth factor receptor 2 (VEGF2) is exclusively expressed in SECs within the bone marrow and is essential in SECs regeneration. Inducible deletion of *Vegf2* or inhibition of VEGFR2 activation by neutralizing antibody prevents regeneration of SECs, impairs engraftment of HSCs and reconstitution of the hematopoietic system after severe myelosuppression (Hooper et al., 2009; Yao et al., 2005). Although a great effort has been made in the exploration of HSC niche, a complete picture is still lacking. How the niches maintain a functional HSC pool, if there are other niches or other components in the two niches, whether the two niches function distinctly or interactively, etc., remains unanswered.

Under homeostatic conditions, a small fraction of HSCs circulates in the peripheral blood and may become accessible for immediate repopulation upon bone marrow injury (Wright et al., 2001). Enhanced HSC mobilization from the bone marrow niche to peripheral blood can be induced by myelosuppression or treatment of cytokines such as granulocyte colony-stimulating factor (G-CSF) (Wilson and Trumpp, 2006). Meanwhile, circulating HSCs are also able to migrate back to the bone marrow vasculature and lodge into the bone marrow niche, a biological process termed homing (Nilsson and Simmons, 2004). Homing of HSCs is a complex process that involves a series of sequential steps including chemotactic mobilization, transendothelial

migration, adhesion and retention to the endosteal niche. The chemokine stromal cell-derived factor (SDF-1alpha), or chemokine (C-X-C motif) ligand 12 (CXCL12), is the only known potent chemotactic reagent for HSCs so far (Wright et al., 2002). CXCL12 is expressed in osteoblasts, SECs, and some other bone marrow stromal cells, while its ligand C-X-C chemokine receptor type 4 (CXCR-4) is highly presented on the surfaces of HSCs (Ara et al., 2003; Ponomaryov et al., 2000). Some cytoskeleton regulation molecules, such as RAC1 and RAC2, cell-surface adhesion molecules, such as selectins and integrins, and matrix metalloproteinases (MMPs) as well as angiogenetic factor VEGF are also found to be essential for homing (Gu et al., 2003; Lapidot et al., 2005; Potocnik et al., 2000). Gene deletion of those factors led to failed engraftment and homing of HSCs in mouse transplantation models.

Bone marrow niches, under normal physiological conditions, provide suitable environments for intricate control of HSC self-renewal division and differentiation. However, disruption of the niches or aberrant niche signals may lead to hematopoietic pathogenesis. Retinoic acid receptor γ (RARγ) knock out mice develop a myeloproliferative neoplasm (MPN) in a non-autonomous manner. Wild type bone marrow cells, when introduced to RARγ deficient microenvironment, rapidly develop MPS. Myeloproliferation caused by deregulated environmental cues was also seen in retinoblastoma protein (RB) deficient animals (Walkley et al., 2007a; Walkley et al., 2007b).

4. Shp2 mediated signal pathways

Shp2, comprised by two amino-terminal (N-terminal) Src homology-2 (SH2) domains, a central phosphatase domain, carboxy terminal (C-terminal) tail, and a proline-rich motif, is a widely expressed cytoplasmic protein tyrosine phosphatase (PTP) acting downstream of various growth factors, cytokines, hormones, antigens and extracellular matrixes. The enzymatic activity of Shp2 is controlled by an auto-inhibitory loop (Feng et al., 1993). Without stimulation, Shp2 remains in an inactive conformation by intra-molecular interactions between the N-terminal SH2 domain and the phosphatase domain. Crystal structure reveals that hydrogen bonds between D61, E76 (in N-terminal SH2 domain) and C459, S502 (in phosphatase domain), respectively, occupy critical sites in the phosphatase catalytic pocket, therefore blocking substrate access and inhibiting enzyme activity (Hof et al., 1998). In response to growth factor or cytokine stimulation, the N-terminal SH2 domain docks on phosphorylated tyrosyl residues of activated receptor tyrosine kinases (RTKs) or cytoplasmic tyrosine kinases via direct interaction or indirect binding to scaffold proteins such as fibroblast growth factor receptor substrate-2 (FRS-2), insulin receptor substrate-1 (IRS-1), or Grb2-associated binders (GABs), resulting in release of the PTP domain and transient activation of the phosphatase activity (Feng et al., 1993; Hof et al., 1998). Although the binding between the C-terminal SH2 domain and phosphatase domain is barely detectable, association of the C-terminal SH2 domain to a phosphotyrosine (pTyr) protein could possibly increase local ligand concentration and enhance substrate specificity (O'Reilly and Neel, 1998). The tyrosine residues in

the C-terminal tail of Shp-2 could be phosphorylated and bind to Grb2, leading to the initiation of downstream signal relay emanating from platelet-derived growth factor (PDGF), interleukin 3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Bennett et al., 1994; Songyang et al., 1994; Welham et al., 1994; Yin et al., 1997)

Biochemical and genetic evidence from different animal models, including *Caenorhabditis Elegans, Drosophila, Xenophous* and *Mus musculus,* suggested that Shp2 transduces and enhances signal strength from RTKs and cytoplasmic tyrosine kinases (Feng, 1999). Corkscrew (csw), an ortholog of Shp2 in *Drosophila,* operates positively in concert with D-Raf in the terminal signal transduction from the RTK Torso (Freeman et al., 1992; Perkins et al., 1992). Moreover, Corkscrew acts downstream of or in parallel with Ras1 in the RTK sevenless signaling during Drosophila eye development (Allard et al., 1996). Deficiency of Ptp-2, Shp2 homolog in *Caenorhabditis Elegans,* causes defective oogenesis. This phenotype can be rescued by gain-of-function let-60 Ras expression (Gutch et al., 1998). Shp2 is required for the FGF induced mitogen-activated protein kinase (MAPK) activation in normal Xenopus mesoderm induction and gastrulation (O'Reilly and Neel, 1998; Tang et al., 1995). Both the N-terminal SH2 domain and phosphatase domain of Shp2 are needed in the signal relay from FGFR (O'Reilly and Neel, 1998). In mammalian tissues, Shp2 was shown by different studies to promote sustained activation of extracellular signal regulated kinase (Erk) by epidermal growth factor (EGF), FGF, insulin-like growth factor-1 (IGF-1), insulin or SCF (Chan et al., 2003; Milarski and Saltiel, 1994;

Noguchi et al., 1994; Qu et al., 1999; Wu et al., 2001). On the other hand, c-Jun NH2-terminal kinase (JNK) activation by heat shock was markedly augmented in Shp2 mutant (loss of function) fibroblast cell lines, suggesting opposite effects of Shp2 on different MAPK pathways (Shi et al., 1998). Shp2 is described to regulate strength of the phosphoinositide-3 kinase (PI3K)/ protein kinase B (AKT) signaling pathway in a negative or positive way depending on different cell lines and growth factor stimulations (Wu et al., 2001; Zhang et al., 2002). Shp2 also participates in various cytokine receptor signalings during inflammatory and immune responses. Mutation in the Shp2 binding site of IL-6 receptor gp130 leads to hyperactive immune response and autoimmune disease due to elevated signal transducers and activators of transcription 3 (STAT3) activity (Atsumi et al., 2002). Consistently, Shp2 was shown to suppress the cytotoxic effects of the signal transducers and activators of transcription (STATs) activation by interferon α or γ (IFNα or γ), indicating a negative role in JAK/STAT signal pathway (You et al., 1999). Shp2 is also required for NF-kappa B activation for IL-6 production upon IL-1 alpha or tumor necrosis factor (TNF)-alpha stimulation, independent of MAPKs, Erk, Jnk or p38 pathways (You et al., 2001). In addition, by operating downstream of integrin signal through activation of Src family kinases, Shp2 functions in the regulation of cell-cell contact and cell spreading (Oh et al., 1999). Shp2 is further shown to modify focal adhesion kinase (FAK) signal strength and therefore plays an important role in cell mobility, migration and focal adhesion (Yu et al., 1998).

Shp1 (encoded by *PTPN6*), or PTP1C, is the other member of the Src homology-2 domain-containing phosphatases (SHPs) family besides Shp2 (Feng and Pawson, 1994; Neel and Tonks, 1997). Distinctly from the wide expression pattern of Shp2, Shp1 is predominantly expressed in hematopoietic cells. Shp1 is a negative regulator of most proliferative and growth-promoting signals, which again stands in contrast to the bidirectional roles of Shp2 in various signal pathways (Kozlowski et al., 1998). *Motheaten (me)* and *motheaten viable (me v)* mutant mice, which express Shp1 with non or significantly reduced catalytic activity, suffer from inflammatory disease caused by excessive function of myeloid cells, leukocyte hypersensitivity and abnormal mast cell function(Yu et al., 1996). In agreement with the enhanced myelomonocytic growth in *me* mice , Shp1 has been shown to downregulate signals emanating from receptors for IL-3, CSF1, and GM-CSF (Neel, 1997). Interaction of Shp1 with PIR-B and SHPS-1 was later suggested to be responsible for the negative regulation of macrophage activities (Siminovitch and Neel, 1998). Shp1 deficiency also leads to hypersensitivity of T lymphocytes to T cell antigen receptor (TCR) activation and enhanced proliferation of splenic B lymphocytes to anti-B cell antigen receptor (BCR) stimulation, suggesting inhibitory roles of Shp1 in modification of signal strength from TCR or BCR (Carter et al., 1999; Cyster and Goodnow, 1995; Pani et al., 1995). Moreover, Shp1 was also found to negatively regulate cell adhesion and migration of hematopoietic cells in response to chemokine SDF-1 (Kim et al., 1999)

5. Shp2 in embryonic hematopoiesis.

Great effort from other research groups and our laboratory has been devoted to the study of Shp2 in embryonic hematopoiesis. Homozygous Shp2 exon3 knock out mice ($Shp2^{\Delta 46\text{-}110}$) died between E10.5 to E11.5 due to impaired gastrulation and mesoderm patterning (Saxton et al., 1997). Although primitive erythrocytes and vasculature was presented, the yolk sac of the Shp2 $^{\Delta 46\text{-}110}$ mutant embryos on E9.5 was abnormally wrinkled and thin. *Flk1-LacZ* staining of mutant yolk sac sections displayed a defective vascularized network formation and lack of thick wall blood vessels. Functional analysis of disaggregated yolk sac in CFU assay indicated a severe loss of myeloid progenitors in Shp2 $^{\Delta 46\text{-}110}$ embryos (Qu et al., 1998). The homozygous $Shp2^{\Delta 46\text{-}110}$ ES cell line gave rise to significantly less EBs with visible erythrocytes in semisolid medium in the absence of leukemia inhibitory factor (LIF) (Qu et al., 1997a). EBs from $Shp2^{\Delta 46\text{-}110}$ ES cells, when dissociated and plated for *in vitro* primitive or definitive differentiation, produced a dramatically lower number of erythroid colonies. CFU-GM, CFU-Mix and CFU-mast were equally suppressed in $Shp2^{\Delta 46\text{-}110}$ EBs, suggesting a requirement of wild type Shp2 in both erythroid and myeloid differentiation during embryogenesis. Consistently, transcription of critical transcriptional factors in hematopoiesis such as Gata1, PU.1, and cytokine receptor AIC-2B, c-fms, and IL-7R, as well as β-H1 and β major globins was markedly reduced in mutant EBs (Qu et al., 1997a). $Shp2^{\Delta 46\text{-}110}$ /wild type chimeric animals were used to further examine the *in vivo* function of Shp2 in early hematopoiesis. Definitive hematopoiesis from $Shp2^{\Delta 46\text{-}110}$ origin is almost abolished within fetal liver and bone

marrow in chimeric mice, despite significant contribution of $Shp2^{\Delta 46\text{-}110}$ cells in various other tissues (Qu et al., 1998). Careful analysis of early stages in embryogenesis revealed that Shp2 functions as early as the formation of mesoderm and hemangioblasts. Mechanistically, Shp2 negatively modifies STAT3 and promotes Erk signaling strength elicited by LIF in the regulation of ES self-renewal and differentiation (Chan et al., 2003). A requirement for Shp2 in early lymphopoiesis was supported by the Rag2–deficient blastocyst complementation assay, as lymphoid cell development in $Shp2^{-/-}$/Rag-$2^{-/-}$ chimeric mice was blocked at the precursor stage (Qu et al., 2001). T cell-specific deletion of Shp2 in the Lck-cre shp2 lox-P flanked (floxed) mice model resulted in differentiation blockage from double-negative (DN) cells to double-positive (DP) cells and reduced proliferation stimulated by the pre-TCR signals (Nguyen et al., 2006). By using a B cell specific Shp2 knock-out model (CD19-cre Shp2 flox), Dr Robert Rickert's group suggested a negative role of Shp2 in regulating germinal center B cell differentiation and CD40 signaling (their unpublished data).

Of note, recent studies indicate that different mechanisms are involved in embryonic and adult hematopoiesis (Orkin and Zon, 2008). For example, although it is required for specification of HSCs from mesoderm during embryogenesis, SCL/tal-1 is dispensable for HSC self-renewal and differentiation into myeloid and lymphoid cells in the adult (Mikkola et al., 2003; Porcher et al., 1996). A more recent study suggested a redundant role between *SCL* and *Lyl* in adult hematopoiesis, as compound deletion of both genes resulted in severe apoptosis of adult HSCs (Souroullas et al., 2009). A similar observation was made for transcription factor Runx1, although a

number of hematopoietic abnormalities, such as lymphoid development blockage, were detected in conditional Runx1 knockout mice (Chen et al., 2009; Growney et al., 2005; Ichikawa et al., 2004).

6. Association of Shp2 mutations with human genetic diseases and hematological disorders.

Shp2 is the first identified *bona fide* proto-oncogene that encodes a PTP, but its importance in human disease was first identified in Noonan Syndrome (NS), a genetic disease with incidence of 1:1000 to 1: 2500 in newborns characterized by dementia, cardiac defect and short stature (Tartaglia et al., 2001). Germline active mutations in *PTPN11/Shp2* gene are responsible for approximately 50% of NS patients. Based on screening data, the most common mutations found in NS patients are Asparagine 308, tyrosine 63 and glutamine 79 (Lee et al., 2005; Takahashi et al., 2005; Tartaglia and Gelb, 2005). Germline missense mutations in *PTPN11* have also been detected in another autosomal dominant disease LEOPARD syndrome (LS). Patients with LS suffer from multiple lentigines, growth retardation, deafness, electrocardiographic conduction abnormalities, ocular hypertelorism, abnormal genitalia and pulmonary stenosis, some of which are interestingly overlapping with NS. Nevertheless, the most prevalent mutations of PTPN11 in LS are tyrosine 279 and threonine 468, distinctively from those in NS (Conti et al., 2003; Keren et al., 2004; Sarkozy et al., 2003). Noonnanlike/multiple giant cell lesion syndrome is also associates with *PTPN11* mutations.

NS patients have high risk of hepatosplenomegaly and juvenile myelomonocytic leukemia (JMML) (Bader-Meunier et al., 1997; Choong et al., 1999; Tartaglia et al., 2001). The most common mutation of NS associated myeloproliferative disorders is threonine 73 isoleucine substitution (Kratz et al., 2005). Somatic gain-of-function mutations in *PTPN11* have also been detected in around 30% *de novo* nonsyndromic JMML, 4% pediatric acute myeloid leukemia (AML), 7% B-cell precursor acute lymphoblastic leukemia (ALL) and 10% myelodysplastic syndromes (Loh et al., 2004; Tartaglia et al., 2004; Tartaglia et al., 2003). Frequent mutations sites of *PTPN11* locus in the childhood leukemias are identified in the N-terminal SH2 domain, including glycine 60, aspartic acid 61, glutamic acid 69, alanine 72, and glutamic acid 76，most of which are not detectable in NS (Kratz et al., 2005; Loh et al., 2004; Niihori et al., 2005; Tartaglia et al., 2004; Tartaglia et al., 2005). In hematopoietic progenitor cells, Shp2 was previously found to physically associate with ligand-activated cytokine receptors such as c-Kit, IL3 receptor, granulocyte macrophage colony-stimulating factor receptor (GM-CSFR), macrophage colony-stimulating factor receptor (M-CSFR), FMS-like tyrosine kinase 3 (FLT3), fibroblast growth factor receptor (FGFR) by docking on its autophosphorylated tyrosyl residues for signal relay to downstream molecules. The majority of leukemia-associated *PTPN11* mutations disrupt the auto-inhibitory interactions between its N-terminal SH2 and the phosphatase domain, resulting in elevated catalytic activity. Moreover, higher Shp2 activity correlates with increased leukemic cell proliferation and disease severity at a certain level, because high basal and unregulated enzyme

activity of mutations at codon 60, 61, 72 or 76 is associated with leukemia, while high basal but regulated enzyme activity of mutation D61G or T73I is found in NS associated MPD, and mutation N308D with only moderate elevated phosphatase activity are only detected in NS patients without MPD (Keilhack et al., 2005; Niihori et al., 2005). However, later studies in a larger number of samples from NS or leukemia patients suggested disease development may also be attributable to the binding affinity between SH2 domain and phosphotyrosine peptide and also to the substrate specificity (Keilhack et al., 2005).

Retroviral expression of Shp2 mutants E76K, D61V or D61Y in bone marrow cells induced hyperproliferation and hypersensitivity of myeloid progenitors to GM-CSF and IL-3 (Chan et al., 2005; Mohi et al., 2005; Schubbert et al., 2005). E76K mutation conquers fetal livers BFU-E forming ability in the absence of EPO, GM-CSF and IL3. Consistently, bone marrow nucleated cells transduced with Shp2 active mutants display elevated basal and sustained activation of Erk and AKT following IL3 stimulation. Shp2 mutants expressing macrophage progenitors show constitutively increased and sustained high phospho-Erk in response to GM-CSF. Enhance STAT5 activation was also detected in E76K transformed bone marrow nucleated cells. Shp2 phosphatase activity and the pTyr residue binding site on SH2 domains are both required for the transforming capacity of E76K, since C463S catalytic dead mutation, or E76K/R32K mutations at N-SH2 domain or E76K/R138K mutations at C-terminal SH2 domain with ablated pTyr peptide binding ability, abrogated the hypersensitive growth of myeloid progenitors to GM-CSF. Transplantation of $Shp2^{E76K}$ or $Shp2^{D61Y}$

expressing bone marrow cells elicited fatal MPD in recipient mice (Mohi et al., 2005), and knock-in mice bearing an inducible $Shp2^{D61Y}$ allele displayed a similar phenotype (Chan et al., 2009). Expression of NS/MPD associated germ line mutation $Ptpn11^{D61G}$ in mice led to transplantable MPD in primary and secondary recipients (Xu et al., 2010). An experiment involving carefully fractionated bone marrow transplantation revealed that the MPD initiating cells resides in the LSK compartment. D61G mutation causes accelerated HSC proliferation and expansion of the stem cell pool. Genetic study suggested the transforming ability of D61G mutation is dependent on GAB2 protein.

Although mutations of Shp2 have not been detected in adulthood hematopoietic disorders, over-expression of Shp2 was documented in different types of adult human leukemia (Xu et al., 2005). Limited knowledge of Shp2 function in normal adult hematopoiesis prevents us from fully understanding the molecular mechanism by which activated Shp2 mutants induce malignant blood cell hyper-proliferation.

7. Stem cell factor and Kit interaction in the regulation of HSCs

SCF/Kit signaling serves as a classical model for mammalian hematopoiesis and possesses historic importance in deciphering signal cascades for HSC regulation. Within adult bone marrow, SCF (also known as Kit ligand, mast cell factor or Steel factor) is expressed by stromal cells, fibroblasts and endothelial cells. Due to alternative splicing of RNA transcript, SCF can be translated into transmembrane or

soluble form, both of which can bind and activate the Kit receptor. Kit (also called c-Kit, CD117) is presented on the surface of HSCs, MMPs, CMPs, CLPs, committed myeloid progenitors and mast cells (Lyman and Jacobsen, 1998). Kit is composed by five immunoglobulin-like domains, a single transmembrane helix, a cytoplasmic juxtamembrane (JM) domain, and a kinase domain. The binding of SCF to Kit results in dimerization of two receptor monomers followed by autophosphorylation of the tyrosines in the JM domain and stabilization of the active conformation of the receptor (Mol et al., 2003). Shp2, Shp1, Src family kinase and scaffold proteins dock to phosphorylated tyrosyl residue 567 or 569 of Kit, while p85 subunit of the PI3K interacts with tyrosyl residue 721 for downstream signal transduction. Kit can also activate STATs via JAK or Src family kinases (Masson and Ronnstrand, 2009). SCF is encoded by the mouse *Steel* locus, and Kit is encoded by the mouse *White* locus (Chabot et al., 1988; Chui et al., 1976). The microenvironment of *Sl/Sl* mice which harbor a mutation in membrane bound SCF was found to be defective in supporting normal HSC functions (McCulloch et al., 1965). In addition, transplantation of bone marrow or spleen cells from newborn but not 16 week old *Sl/Sl* mice was able to restore the hematopoietic system in irradiated wild type mice (Barker, 1994, 1997). The biological significance of Kit in hematopoiesis was first revealed in the *white spotting* (*W*) mutant mice (Russell, 1979). A number of *W* mutations with different levels of Kit kinase deficiency were found to correlate with the phenotype severity (Reith et al., 1990). The W^{37} mutation in the conserved tyrosine kinase domain of Kit leads to complete loss of kinase activity, and W^{37} homozygous mutant mice are

embryonic lethal due to severe macrocytic anemia. W^{41}/W^{41} and W^{55}/W^{55} mutant mice with partially compromised kinase activity are viable but develop severe anemia and mast cell defects. In contrast, W^{44}/W^{44} and $W^{57}/^{57}$ mutants which do not affect the kinase domain but express lower level of c-kit protein are viable and only mild anemia is detectable. Heterozygous or viable mild *W* mutants have been extensively investigated recently, identifying a pivotal role of Kit in development of multiple hematopoietic lineages (Waskow et al., 2002). Kit is highly expressed in HSCs and is currently used as a phenotypic marker for HSCs (Ikuta and Weissman, 1992). Studies in the viable primary *W* mutant mice or post-transplantation mice indicated the importance of Kit signaling in maintaining adult HSC quiescence and survival (Li and Johnson, 1994; Sharma et al., 2007; Thoren et al., 2008). Despite the wealth of knowledge on SCF/Kit signaling, it is poorly understood how Kit expression is regulated in HSCs and progenitors.

Wild-type Kit is presented in most cases of AML. SCF stimulation enhances proliferation of AML cells (Pietsch et al., 1992). Expression of SCF is also found in some AML blasts (Tohda et al., 1993). Dominantly active *Kit* mutations have been detected in core-binding factor (CBF) subtypes of AML and mast cell leukemia (Paschka et al., 2006). The most common mutation of Kit is the gain-of-function mutation D816V in the activation loop of its kinase domains. Expression of constitutively active Kit induces MPD, but is not sufficient to initiate AML, in mouse bone marrow transduction and transplantation models (Xiang et al., 2007). *Kit* mutations were frequently found to be associated with oncogenic fusion genes

such as *Pml/Rarα* and *Aml/Eto*. Co-expression of D816V Kit and Aml/Eto in bone marrow rapidly induces AML in mouse models, a consequence not observed with Aml/Eto expression alone (Wang et al.; Wang et al., 2005). Interestingly, dominant Shp2 activation in leukemic cells leads to prolonged and elevated Erk activation in response to SCF stimulation, suggesting a possible role of aberrant Shp2 activation in the pathogenesis of AML patients with Kit mutations (Niimi et al., 2006).

8. Objectives of this dissertation.

Although the cellular basis of hematopoiesis has been extensively investigated, the important question of how the intracellular signaling pathways elicited by extracellular cues regulate HSC activities remains largely unexploited. *In vivo* study of signaling molecules using animal models will provide fundamental insights into the underlying molecular mechanisms and facilitate therapeutic use of HSCs. On this thesis project, we have focused on dissecting the biological and biochemical functions of Shp2 mediated signals in the regulation and coordination of HSC activities, including HSC self-renewal, differentiation and niche interaction. We believe the study of physiological Shp2 functions in the regulation of adult HSCs and progenitors will shed light on the understanding of related hematopoietic malignancies induced by active *Shp2/Ptpn11* mutations.

Based on our previous knowledge that Shp2 serves as a pivotal signal molecule downstream of many RTKs and cytoplasmic kinases, and its importance in embryonic hematopoiesis, we hypothesized that it might have a critical role in adult HSC

regulation by orchestrating different signaling pathways. To test the hypothesis, we asked the following specific questions:

1) Is Shp2 required for maintenance of adult HSC and progenitor cell pool?
2) Is Shp2 involved in HSC-Niche interaction, HSC homing, mobilization and engraftment efficiency?
3) What is the molecular mechanism for Shp2 regulation of HSC functions?

Different methods, described extensively in Chapter 2, were set up to tackle these questions. We generated an inducible gene knock-out mouse model *Shp2*$^{flox/flox}$*:Mx1-Cre*$^{+}$, in which *Shp2* can be acutely ablated in the hematopoietic system. Hematological examination, flow cytometric analysis, *in vitro* functional assays and *in vivo* transplantation experiments were employed for the first two questions. Biochemical analysis of both primary hematopoietic progenitor cells and cell lines was used for the study of the third specific question. We describe the generation and conditional gene knockout strategy of *Shp2*$^{flox/flox}$*:Mx1-Cre*$^{+}$ mice in Chapter 3.1. Chapter 3.2 focuses on experimental results for specific question one, from which we unrevealed an important role of Shp2 in maintaining adult HSC self-renewal, survival and quiescence. In Chapter 3.3, we demonstrate that Shp2 is dispensable for the HSC supporting bone marrow microenvironment by reciprocal transplantation, but is indeed essential for homing of engrafted HSCs. Chapter 2.4 was devoted to specific question three. We show that Shp2 not only operates downstream

of the RTK Kit but also regulates *Kit* gene transcription and surface expression, which in turn positively amply the HSC supporting signals from Kit.

II. Methods and Materials

1. Mice and polyinosinic: polycytidylic acid (Poly-I:C) treatment

Experimental mice were housed in Sanford/Burnham Medical Research Institute or University of California San Diego Hopkins Research Center in a pathogen-free environment with a controlled temperature, humidity, and 12-hour light cycle. All animal handling protocols were approved by Sanford/Burnham Medical Research Institute or University of California, San Diego animal care program. The generation strategy of Shp2 floxed animals was reported previously (Zhang et al., 2004). *Shp2*$^{flox/flox}$*:Mx1-Cre*$^{-}$ mice were bred with *Shp2*$^{flox/flox}$*:Mx1-Cre*$^{+}$ mice to produce control and knockout littermates. *Pten* $^{flox/flox}$ mice were purchased from Jackson Laboratory and those mice were originally deposited by Dr. Hong Wu (Groszer et al., 2001). *Pten* $^{flox/flox}$ mice were bred with *Shp2*$^{flox/flox}$*: Mx1-Cre*$^{+}$ to produce *Shp2*$^{flox/flox}$ *Pten* $^{flox/flox}$*:Mx1-Cre*$^{+}$ double knock out animals. CD45.1^{+} mice were kindly donated by Dr. Robert Rickert. Poly-I:C (GE Amersham) was reconstituted in autoclaved phosphate buffered saline (PBS) buffer (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.46mM KH_2PO_4) to form a concentration of 0.8mg/ml solution. Five doses of Poly-I:C (10 mg/kg) were given to control and experimental groups by intra-peritoneal injection every other day.

2. Bone marrow transplantation

Bone marrow cells were harvested from mice femurs and tibiae. Red blood

cells were lysed by the NH_4Cl red blood cell lysis buffer (15.5mM NH4Cl, 1mM KHCO3, 0.01mM EDTA) at room temperature for 5 minutes and neutralized by PBS buffer. In the competitive reconstitution experiment, 5×10^5 bone marrow nucleated cells (BMNCs) from control or knock out animals were mixed with 5×10^5 or 2×10^6 BMNCs from CD45.1 mice and transplanted into lethally irradiated recipient mice by tail vein injection. In the reciprocal transplantation experiment, 5×10^5 wild type BMNCs were transplanted into lethally irradiated control or Shp2 knock out recipients. For lethal irradiation of recipients, we used two doses of 500rad/time with a four-hour interval.

3. Flow cytometric analysis and sorting

Lineage staining was performed using the mouse hematopoietic lineage flow panel from eBioscience (biotin-conjugated antibodies against CD3 (145-2C11), CD45R/B220 (RA3-6B2), CD11b (M1/70), TER-119, Ly-6G (RB6-8C5)) supplemented with biotin-conjugated antibodies against mouse CD4 (L3T4), mouseCD8 (CD8b, Ly-3) (eBioscience). Streptavidin conjugated with APC-CY7 was used as secondary staining. For further staining of HSCs, antibody against CD117 (cKit)-PE, FITC (2B8); Sca1-FITC, APC (D7); Flk2-PE-CY5 (A2F10) (all above from eBioscience); CD34-APC, PE-CY5 (MEC14.7) (Biolegend) were used. In the competitive reconstitution experiment, we used anti-mouse CD45.1-FITC (A20) and anti- mouseCD45.2-FITC (104) antibodies (eBioscience). $Lineage^-$ cells were enriched by lineage depletion kit (Miltenyi) according to the manufacturer's instructions. For

the myeloid and lymphoid progenitor cell staining, additional antibodies against mouse IL-7Ra-PE-CY5 (A7R34), anti-mouse CD16/32-PE (93) (eBioscience) were used. CMPs were gated as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}FcgR^{lo}CD34^{+}$**.** CLPs were gated as $Lin^{-}IL\text{-}7R^{+}$ $Sca\text{-}1^{lo}c\text{-}Kit^{lo}$**.** GMPs were gated as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}FcgR^{hi}CD34^{+}$**.** MEPs were gated as $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}FcgR^{lo}CD34^{-}$. Correspondent isotype controls (eBioscience or Santa Cruz) were used for proper gating. All flow cytometry experiments were performed on BD FACSCANTO or BD FACSARIA machines (BD bioscience) and the data was analyzed by flowjo software (Tree Star, Inc).

4. Cell lines and plasmids

Erythroid myeloid lymphoid (EML) cell line is a multipotent bone marrow progenitor cell line and was cultured in iscove's modified dulbecco's medium (IMDM from Mediatech) with 20% horse serum (Gibco) and 10% BHK-KL conditioned medium. We received the BHK-KL cell line which expresses and secretes SCF to the supernatant from Dr Schickwann Tsai. The BHK-KL and HEK-293 cell line were maintained in dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). Scramble siRNA and Shp2 specific siRNA were purchased from Qiagen. siRNA Sequences used for human Shp2 are: forward 5'-UUAAUUGCCCGUGAUGUUCUU-3', reverse 5'-GAACAUCACGGGCAAUUAAUU-3'. siRNA Sequences used for mouse Shp2 are: forward 5'-GGA CUA UGA CCU CUA UTT-3' reverse 5'-AUA GAG GUC AUA GUA GUC CTT-3'. Jetprime (polyplus transfection) transfection reagent was used for

siRNA and plasmid transfection to the HEK293 cell line, and the nucleofector kit (Lonza) was used to introduce siRNA and plasmids into the EML cell line. For nucleofection, 2.5 million EML cells were pelleted and resuspended in 100μl Amaxa nucleofector buffer V for each reaction. Program X-001 was used in the electroporation. The GFP reporter plasmid p13kit and p70kit were generously provided by Dr. Susanna Dolci. The pBABE-gata2 plasmid was a gift from Dr John Crispino.

5. Analysis of cell cycle and apoptosis

To assess the cell cycle, 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay was performed as described before (Ficara et al., 2008). For 34^-LSK or LSK cell cycle analysis, 100 mg BrdU was injected intraperitoneally to the mice 19 hours or 2 hours before euthanasia respectively. Pyronin Y/ Hoechest33342 co-staining was used to distinguish the G0/G1 phase of LSK cells. Around 50,000 LSK cells were sorted into ice-cold 100% ethanol and incubated for at least 16 hours. We developed a new method of carrier cells to avoid the loss of LSK cells during the sequential staining process. EML cells were stained with 1 μM DDAO-SE (Invitrogen) and then fixed in ice-cold 100% ethanol for at least 16 hours. EML cells were mixed with LSK cells and stained in 0.5 μg/ml Pyronin Y and 2 μg/ml Hoechest33342 45 minutes before FACS analysis. We gated on DDAO-SE^- cells for additional G_0/G_1 gating of LSK cells. For the 5-fluorouracil (5-FU) challenge assay, one week after the final injection of poly-I:C, *fl/fl:cre*$^+$ or *fl/fl:cre*$^-$ mice were injected with three sequential doses of 5-FU

(150mg/kg, Fluka) intraperitoneally every 7 days. Mice were monitored carefully every morning for survival for consecutive 28 days. To assess the cell apoptosis, AnnexinV-EGFP (Biovision) or terminal deoxynucleotidyl transferase dUTP nick end labeling (Tunel) staining (Roche) was performed according to the manufacturer's instructions.

6. PCR and Immunoblotting

DNA from peripheral blood was extracted using DNeasy Blood & Tissue Kit (Qiagen). Primers for genotyping are 5'AAGAGGAAATAGGAAGCATTGAGGA3' and 5'TAGGGAATGTGACAAGAAAGCAGTC3'. PCR products for floxed allele and knockout allele are 1000 bp and 400 bp, respectively. For immunoblotting, cells were lysed in RIPA buffer (Tris 50mM, NaCl 150 mM, SDS 0.1 %, Sodium Deoxycholate 0.5 %, Triton X 100 pH 8.0) with protease and phosphatase inhibitor cocktail (Roche). Protein extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Polyvinylidene fluoride (PVDF) membrane. Home-made anti-Shp2 antibody and anti-Erk antibodies were used to detect Shp2 and Erk expression. Antibody against GFP (Santa Cruz), Flag (Cell Signaling), Gata2 (Santa Cruz) were used to detect respective protein. For the detection of phosphorylated protein, anti-phospho AKT (Ser 473), anti-phospho Erk (Thr202/Tyr204), anti-phospho STAT3(Tyr727), anti-phospho JNK (Thr183/Tyr185), anti-phospho p38 (Thr180/Tyr182) (all from Cell Signaling) were

used. Antibody against Tubulin (Sigma), AKT, STAT3, p38 (all from Cell Signaling) and JNK (Santa Cruz) were used to detect whole protein.

7. CBC count

Peripheral blood was collected into EDTA pretreated tubes (BD bioscience) trough intra orbital bleeding. Complete Blood Cell (CBC) counts were performed with the VetScan HMII Hematology System (Abaxis).

8. *In vitro* CFU assays and *in vivo* CFU-S assays

For the *in vitro* colony forming unit assay, 50,000 bone marrow cells were seeded in MethoCult GF M3434 media (Stemcell Technologies) according to the manufacturer's recommendations. Colonies were visualized and counted under the microscope on day 14. The experiment was performed in duplicate for each sample. To assess the CFU forming capacity of LSK cells, 250 LSK cells were sorted (as described above) and seeded into MethoCult GF M3434 media (Stemcell Technologies). For the *in vivo* colony forming unit assay, 10^5 bone marrow cells were transplanted to lethally irradiated recipient mice. Spleens were harvested from recipients 12 days post transplantation and fixed in Tellesniczky's fixative solution (87% ethanol, 4% acetic acid, 9% formalin) for colony numeration.

9. Homing and migration assay

For the *in vivo* homing assay, 2×10^6 lineage-depleted bone marrow cells were stained with 5μM CFDA-SE (invitrogen) for 5 minutes and injected through the tail vein into lethally irradiated recipient mice. Bone marrow from recipients was harvested and analyzed 16 hours later. CDFA-SE^+ cells were considered as homed cells. To access the homed progenitor cells, we adopted a previously published method (Katayama et al., 2003). 5×10^6 bone marrow nucleated cells from donors were transplanted to lethally irradiated recipient mice. Recipients were euthanized 24 hours later to harvest the femur. $0.9x10^5$ to $2.5x10^5$ nucleated cells were plated in MethoCult 3434 medium for a CFU assay (Stem Cell Technologies). Colonies were numerated on day 8 and normalized against the seeding cell number. Bone marrow cells from lethally irradiated mice without transplantation were also plated as negative control and no colonies were detectable. For the *in vitro* migration assay, 10^6 lineage-depleted bone marrow cells were seeded to the top chamber of 5 μm pore size 24-well transwell plate (Corning) and migrated against 5 nm SCF. Migrated cells were numerated after 3-hour incubation at 37°C, 5%CO_2.

10. Chromatin Immunoprecipitation (CHIP) assay

The CHIP assay was conducted as described before with minor modification (Zhang et al., 2009). EML cells with different treatments were cross-linked with formaldehyde (1%, v/v) (sigma) for 10min at room temperature. Cross-link was stopped by adding glycine to a final concentration of 125 mM. Cells were then

collected and washed with PBS buffer three times, followed by re-suspension in lysis buffer (1% SDS, 0.01M EDTA, 0.05M Tris.Cl pH 7.8) containing protease inhibitor cocktail (Simga) for 20 min on ice. Sonication was then performed on ice to shear the chromation to about 500bp to 1000bp DNA fragments, which were verified by electrophoresis. Debris was cleared by centrifugation at 14000rpm for 10 min at 4 °C. The supernatant containing chromation was further diluted 5 folds in ChIP dilution buffer (1% Triton X-100, 2mM EDTA, 150 mM NaCl, 20 mM Tris/HCl, pH 7.8) with protease inhibitors. 20% of the supernatant was saved for input DNA and the rest was divided into two tubes: one for normal rabbit IgG (Santa Cruz Biotechnology) antibody control and the other was incubated with 5 µg of anti-Gata2 antibody (sc-8334 Santa Cruz) overnight at 4 °C with rotation. Protein A slurry (20 µl) (Sigma) was added with agitation at 4 °C for 4 h. The agarose was pelleted by centrifugation at 4 °C for 1 min at 1000 *g* and the supernatant was discarded. The beads were washed consecutively with the following sequence for 5 minutes on a rotation platform: low salt washing buffer (0.1% SDS, 1% Triton X-100, 4 mM EDTA, 20 mM Tris/HCl, pH 8.0, and 150 mM NaCl), high salt washing buffer (0.1% SDS, 1%Triton X-100, 4 mM EDTA, 20 mM Tris/HCl, pH 8.0, and 500 mM NaCl) and twice in TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). Elution buffer (1% SDS in TE buffer) was added to elute immunoprecipitated DNA-protein complexes from pelleted beads. The DNA was then purified by QIAquick Spin Columns (QIAGEN) according to manufacture's protocol. Q-PCR was then performed on Mx3000P machine (Stratagene) as described below. PCR conditions were: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95

°C for 20 s, 55 °C for 20 s, 72 °C for 30 s, and finally 1 cycle at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. The primers used for Gata2 binding to *c-kit* promoter ChIP assay were designed by invitrogen online tool OligoPerfect Designer. (http://tools.invitrogen.com/content.cfm?pageid=9716). The primers used for Gata2 binding to *c-kit* -114kb and +5kb region ChIP assays were adopted from former publication (Jing et al., 2008) (table1). Kit promoter sequence is listed as follows:

CACACTTAAACACTATTAAGTGTACAATCTTGGGACTTAAAGAACC
CCCAAAACAAACGTTTATCTTCTCTACTTCATAACGTTAACTGTAGACCCG
GATACTATAAAAACCCAGATTGTTACTGTCCACCCCCGGATAGCCACAGTG
ACTGTGAAATGTCCCTAGGTAGAAATTGTCTTAAGAGAATTACTTTAGGAG
TTGGAGAGTTGGGTCAATTAGTTAAGAACAAGAAAGTTGGCGCATGACTT
TAATCCCAGCACTTGGGAGGCAGAGGCAGGCGAATTTCTGAGTTCGAGGC
AGCCTGGTCTACAAAGTGAGTTCCAGGACACCAGTAACCCTGTCTCGAAA
CAAACAAACAAACAAACAAAGAAACAAACAAAGAACAAGAGTAGTTGGG
CTCCAGGCACCCACAAGATGATACACAAGCATCCGTAACTCTAGTTTCAG
GAGATCTGACATCCTTCTGACCTCCCTGGGCACTAGTCACACACGTGGCAC
ACACACATATTCATGTAAGTAAAACACTCATACACATTAAAAAAAAACTT
TAAAAGGAAATTATTTTGCTTTTATCTTGCTCCTAAGGCAGACCACAGTCG
GATCGAGTTGGGGAAAGGGGGACTCAGAGGGGATGGGGCGGGGGGGGGG
TGTTGAGGGTGAGATTAAAAAAATAATTAACACAAGAGAAAAGGCACCCA
AATGCAAATAGGTTTTAAAAACCGAGTTTCAAAGCTAACTCCTTCACAGG
ATGCCTCTGTAGTCCACTGGCCCAGTTTCCACAAGTCTCAGCAGGAGCTTG

GCTGGGTGGAGGTCCACGCTGGCCAATAGAAATCAGCTGTATTCTTACAG
GTTTCGCAGCAGGTGGAGAAACTGAGCATGAAAAATTACTTAAACGTGGG
CTCGGTCTTTTACTGAGGTCAGGGGTGCCACGATCCGTCCTCCTCTACCAA
CAGGAACAGAAATAAATGTTGGGGACCCAGTTTCCTAATCCCTTCGCCCG
GCAATCCCGACTAGTAACACCTCCACCATAAGCCGAATATATTCTCCCCGC
TCCGGAGCTTGCTGGAGGGACCGGTGGTTGTCCTTTATTGTCTAGGGAGCA
CCTGCCAGGTGGCTGGCCCGTACCTAATGCGCTCGTGGCGCTGGGCTTCAC
AAAGCGCGGGCAGCACCTGCGTGGCCAGCCAGCCGCCTGGACTGAAGGAC
CACCGATGGAGGGAGAGTGCTAGGAGGAAGAGGATCCAGGGTGGAGGGC
CTGTGGGGGCTCCTGGTCTTAGAGGGCACAGCGCCCCCGGGATCAGCTTAT
TGCAGCCCGAGAGCCCCGGGCACTAGGCAGCGGGAGGGAGTGCGACCCG
GGCGGGAGAAGGGAGGGGCGTGGCCACGAGCTGGGAGGAGGGCTGGAGG
AGGGGCTGTCGCGCGCCGCTAGTGGCTCTGGGGGCTCGGCTTTGCCGCGCT
CGGTGCACTTGGGCGAGAGCTGTAGCAGAGAGAGGAGCTCAGAGTCTAGC
GCAGCACCGCG

11. Q-PCR primer and gene expression analysis

SYBR green qPCR master mix (stratagene) was used for the quantitative-PCR assay. Target gene expression was calculated by ΔCT method. q-PCR was conducted on a Mx3000P machine (Stratagene). q-PCR conditions were: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 22 s, 72 °C for 30 s, and finally 1 cycle at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. Most q-PCR primers in this

study were adopted from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). Others were designed using invitrogen online tool OligoPerfect Designer. (http://tools.invitrogen.com/content.cfm?pageid=9716).

12. Retroviral infection of bone marrow cells

Retroviral construct MSCV-Cre- IRES (internal ribosomal entry site) -hCD2 that co-expresses Cre recombinase and human CD2 (hCD2) marker and control construct MSCV-hCD2 was a generous gift from Robert Rickert. The retrovirus was prepared using packaging cell line Plat-E following manufacture's protocol (Cell Biolabs). 2-3 months old +/+ or *fl/fl* mice were injected with 500 µl (10 mg/ml) 5-FU and bone marrow was harvested 5 days after the injection. Bone marrow cells were pre-stimulated with DMEM medium containing 15% FBS and 10ng/µl recombinant mouse IL-6, 6 ng/µl recombinant mouse IL-3 (PropTech), 100 ng/µl recombinant mouse SCF (R&D) and 10% WEHI conditional medium (BD bioscience) for 24 hours. Retroviral infection was performed by spin inoculation at 3000rpm for 90 minutes. Bone marrow cells were stained with PE-anti-hCD2 antibody (Biolegend) 48 hours later to detect positively infected cells.

13. Cytokine stimulation and small molecule inhibitor treatment

For SCF stimulation, EML cells were starved 6 hours in UltraCULTURE serum free medium (Lonza) supplemented with 2mM Glutamine and then stimulated with 100 ng/ml recombinant mouse SCF (R&D) for 5 minutes. Cells were quickly

harvested by centrifugation for immunoblotting. For pharmacological inhibition of PI3K, MAPK and JAK pathway, LY294004, U0126 and AG490 (Cell Signaling) was added to culture medium at indicated concentration for 12 or 24 hours before immunostaining.

14. Statistical analysis

Statistical analysis was performed by unpaired two tailed student's *t* test using GraphPad Prism 5 software. Differences were considered significant when p value < 0.05.

Acknowledgement

Chapter two, in part, is a reprint of the paper Zhu, H.H., Ji, K., Alderson, N., He, Z., Li, S., Liu, W., Zhang, D.E., Li, L., and Feng, G.S. (2011). Kit-Shp2-Kit signaling acts to maintain a functional hematopoietic stem and progenitor cell pool.

The author of this dissertation was the major investigator and first author of the paper.

Table 1. List of q-PCR primers used in CHIP assay

Primer name	location	sequence
1F	promoter	5’GTGGTTGTCCTTTATTGTCTAGGG3’
1R	promoter	5’GCTGTGCCCTCTAAGACCAGGAG3’
2F	+5kb	5’GGCTGGAAACCACTGCCTTA3’
2R	+5kb	5’AGCCTTGCCTGTGCTTAAAGC3’
3F	-114kb	5’GCACACAGGACCTGACTCCA3’
3R	-114kb	5’GTTCTGAGATGCGGTTGCTG3’

Table 2. List of q-PCR primers used in gene expression analysis

shp2 forward	5' TCCATGGTCACTTGTCTGGA 3'
shp2 reverse	5' GACGTGGGTCACTTTGGACT 3'
c-kit forward	5' GCCACGTCTCAGCCATCTG 3'
c-kit reverse	5' GTCGCCAGCTTCAACTATTAACT 3'
gata1 forward	5' TGGGGACCTCAGAACCCTTG 3'
gata1 reverse	5' GGCTGCATTTGGGGAAGTG 3'
gata 2 forward	5' CACCCCGCCGTATTGAATG 3'
gata 2 reverse	5' CCTGCGAGTCGAGATGGTTG 3'
sp1 forward	5' GCCGCCTTTTCTCAGACTC 3'
sp11 reverse	5' TTGGGTGACTCAATTCTGCTG 3'
scl forward	5' CACTAGGCAGTGGGTTCTTTG 3'
scl reverse	5' GGTGTGAGGACCATCAGAAATCT 3'

III. Results

1. Generation of *Shp2$^{flox/flox}$: Mx1-cre* mice and gene deletion strategy.

Shp2$^{flox/flox}$ mice were generated from our laboratory previously using the Cre-loxP system (Zhang et al., 2004). To conditionally delete *Shp2* in the hematopoietic system, *Shp2$^{flox/flox}$* animals were crossed with Mx1-Cre transgenic mice (obtained from Jackson's laboratory and deposited by Dr Roberta Pelanda), in which the transcription of Cre recombinase is under the regulation of *Mx-1* gene promoter. *Shp2$^{flox/flox}$: Mx1-cre* mice were then back crossed to C57BL/6 background strain. *Mx-1* gene is involved in immune response against viruses and remains silent in healthy animals. High level of Mx-1 gene transcription can be transiently induced by IFNα, IFNβ, or synthetic double-stranded RNA stimulation (Kuhn et al., 1995). Therefore, administration of IFN or virus mimic poly-I:C to *Shp2$^{flox/flox}$:Mx1-cre* mice will rapidly induce Cre recombinase expression and then cause excision of floxed *Shp2* allele. As shown in Figure 1, five doses of poly-I:C were injected intraperitoneally every other day to *fl/fl:cre^{+}* and their wild type littermates *fl/fl:cre^{-}*. Two weeks post final injection (P.I.), genomic DNA was extracted from peripheral blood cells, and PCR analysis indicated clear excision of the floxed allele in *fl/fl:cre^{+}* animals (Figure 2A). Quantitative PCR and immunoblotting showed approximately 90% Shp2 deletion in poly-I:C-treated *fl/fl:cre^{+}* bone marrow cells at RNA and protein levels, respectively (Figure 2B, 2C).

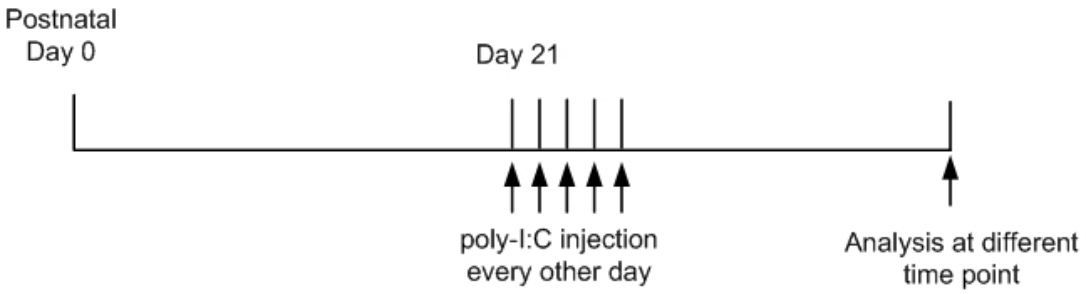

Figure 1. poly-I:C injection strategy.
Five doses of Poly-I:C (10 mg/kg) were given to *fl/fl:cre*$^{+}$ mice and their wild type littermates *fl/fl:cre*$^{-}$ by intra-peritoneal injection every other day from the weaning dates. Animals were sacrificed at different time points after the final injection for tissue collection and analysis.

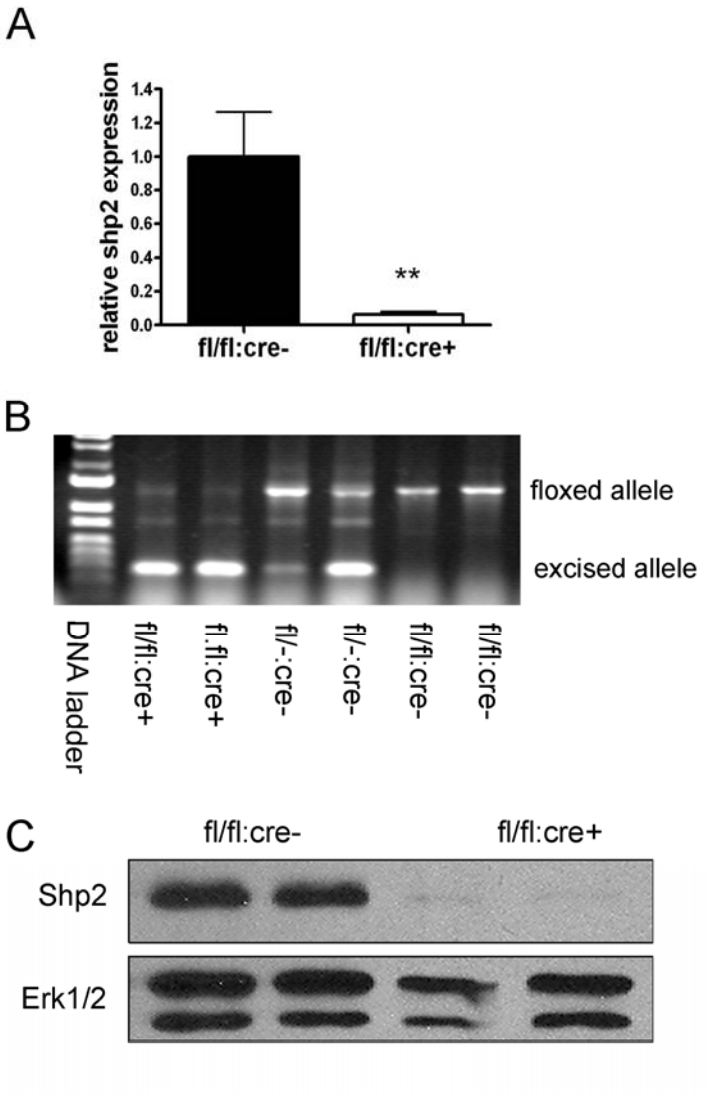

Figure 2. Inducible deletion of Shp2 *in vivo*.
(A) Deletion of the floxed region was verified by PCR. Peripheral blood PCR from mice at 2 weeks post final Poly (I: C) injection (P.I.). (B,C) Deletion of Shp2 in bone marrow was verified by q-PCR and immunoblotting at 2 weeks P.I

2. Investigation of Shp2 functions in HSC self-renewal, differentiation and survival.

2.1. Shp2 ablation leads to bone marrow failure and peripheral blood cytopenia.

No significant differences in different hematopoietic parameters were observed among these groups of mice: *+/+:cre$^-$, fl/+:cre$^-$, +/+:cre$^+$, fl/+:cre$^-$ and fl/fl:cre$^-$*, excluding an effect of Cre-mediated toxicity (Figure 3A-C). Therefore, *fl/fl:cre$^-$* mice were used as controls throughout the study. After poly-I:C treatment, all parameters except platelets in a complete blood cell (CBC) count were significantly lower in *fl/fl:cre$^+$* mice than that in *fl/fl:cre$^-$* mice (Figure 4A). H&E staining of the trabecular bone showed cytopenia in the bone marrow of mutant animals (Figure 4B). Further quantification assays indicated a nearly 2/3rd loss of total bone marrow cellularity starting from one week post final injection in mutant mice, compared to controls (Figure 4C). Other hematopoietic organs, such as the spleen, also displayed significant loss of cellularity in mutant mice (Figure 4D), suggesting a severe defect in the hematopoietic compartment following acute deletion of *Ptpn11/Shp2*.

2.2. Removal of Shp2 suppresses the phenotypic and functional HSCs in adults.

Flow cytometric analysis showed dramatic decrease in the frequency and absolute numbers of HSC-enriched lin$^-$Sca1$^+$Kit$^+$CD34$^-$ (34$^-$LSK) cells in *Shp2$^{\Delta/\Delta}$* bone marrow (Figures 5A-C). We also carefully checked the LSK cell pool in the peripheral blood and spleen. A reduction of splenic LSK cell frequency was observed one week post final poly-I:C injection, and the LSK frequency in peripheral blood remained

indistinguishable between control and mutant animals (Figures 6A, B). Those results suggest that the decrease in the LSK cell pool in the bone marrow is not due to enhanced mobilization to peripheral blood or spleen. The frequency of CLPs in the bone marrow was elevated while the myeloid progenitors were significantly decreased in the knockout animals (Figure 7A). When calculated against the total bone marrow number, the CLPs, CMPs, GMPs and EMPs were significantly lower in mutant animals, with the most dramatic reduction seen in myeloid progenitors, indicating different roles of Shp2 in lymphoid and myeloid lineage commitment/differentiation (Figure 7B).

We performed a colony forming unit (CFU) assay to examine HSC/progenitor functions in bone marrow and spleen. As shown in Figure 8A, Shp2 removal severely suppressed the total colony numbers. An unbiased suppression was detected in all colony types including BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. Similar results were obtained in assays using sorted LSK cells (Figure 8B), suggesting impaired differentiation capacity of mutant LSK cells. Therefore, Shp2 ablation resulted in a decrease in the size and differentiation potential of the LSK pool, as revealed by the CFU assay *in vitro*. Consistently, *in vivo* CFU-spleen assay performed on day 12 following bone marrow transplantation demonstrated a significant decrease of HSCs and MPPs derived from mutant animals (Figure 8C).

The HSC function was further evaluated using a bone marrow competitive reconstitution assay. *fl/fl:cre*$^{-}$ or *fl/fl:cre*$^{+}$ donor mice were treated with poly-I:C, and bone marrow cells (CD45.2^{+}) harvested 2 weeks post final injection were transplanted

into lethally irradiated recipients in competition with CD45.1^{+} competitor bone marrow cells at a ratio of 1:1 or 4:1 (Figure 9). *Shp2*$^{\Delta/\Delta}$ bone marrow gave rise to few, if any, blood cells in the 1:1 ratio transplantation (Figure 10A,B) and contributed a significantly lower percentage in the 4:1 ratio set-up (Figure 10E), compared to the expected chimerism in the controls. In the 1:1 ratio experiment, specific lineage markers were used to evaluate donor-derived cells in different cell lineages after 16 weeks of transplantation. *Shp2*$^{\Delta/\Delta}$ bone marrow produced less than 5% of T, B lymphocytes or myeloid cells (Figure 10C). Consistently, very few (less than 1%) bone marrow LSK cells were derived from *Shp2*$^{\Delta/\Delta}$ bone marrow donors (Figure 10D), indicating a requirement for Shp2 in self-renewal of HSCs *in vivo*.

2.3. Aberrant cell cycle entry and enhanced apoptosis of Shp2-deficient HSCs/progenitors

The majority of adult HSCs remain in a dormant/quiescent status for hematopoietic homeostasis (Wilson et al., 2008). We assessed the ratio of actively cycling cells by a BrdU incorporation assay. Two weeks after the final poly-I:C treatment, *fl/fl:cre*$^{+}$ or *fl/fl:cre*$^{-}$ mice received intraperitoneal injections of BrdU before euthanasia. Bone marrow cells were then collected and stained for surface CD34 LSK markers. BrdU and DNA staining were performed sequentially. As shown in Figure 11 and Figure 12, significantly more LSK (Figure 11A, B) and 34^{-}LSK (Figure 12A) cells in S phase were observed in *Shp2*$^{\Delta/\Delta}$ bone marrow than in control, while fewer mutant 34^{-}LSK cells or LSK cells resided in G_0/G_1 quiescent phase. Pyronnin Y and

Hoechst 33342 co-staining were used to distinguish the G_0 phase from G_1 phase, and Shp2 ablation reduced the number of LSK cells in the quiescent G_0 phase (Figure 11C, D). The enhanced cell cycle entry of *Shp2*$^{\Delta/\Delta}$ HSCs is confirmed by 5-FU challenge experiment. 5-FU is a cell cycle specific myeloablative agent and causes depletion of cycling HSCs. Three sequential sublethal doses of 5-FU was injected to poly-I:C treated *fl/fl:cre*$^{+}$ or *fl/fl:cre*$^{-}$ mice every 7 days. A remarkably higher mortality rate was observed in *fl/fl:cre*$^{+}$ animals than controls (Figure 12B).

We performed Annexin V staining to assess HSC/progenitor cell apoptosis. No difference in cell apoptosis was detected in lineage^{-} bone marrow cell pool. *Shp2*$^{\Delta/\Delta}$ lin^{-} ckit^{+}Sca1^{-} or LSK cells contained higher percent of Annexin V^{+} cells than the control (Figure 13A, B). Significantly increased apoptosis rate was also seen in CD34^{-}LSK cells (Figure 14A, B). These results indicate a more stringent requirement for Shp2 in promoting cell survival in the bone marrow HSCs/progenitors than in more developed/committed precursor cells. We also performed a TUNEL assay in parallel and detected more apoptotic cells in LSK and flk2^{-}LSK pools (Figure 14C), thus confirming a critical role of Shp2 in HSC/progenitor survival. Collectively, the data shown here indicate that disruption of quiescence and increased apoptosis contribute to the severe loss of HSCs in the conditional Shp2 knockout animals.

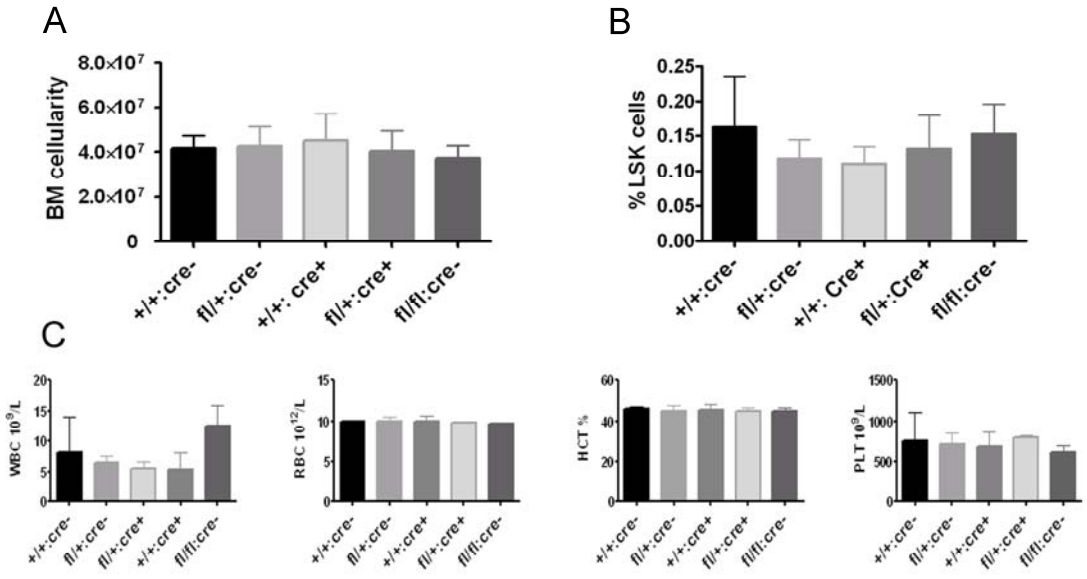

Figure 3. No toxicity of Cre recombinase expression on hematopoietic system. (A, B, C) Comparable bone marrow cellularity (A), bone marrow LSK frequency (B) and CBC counts among different control genotypes as shown (C).

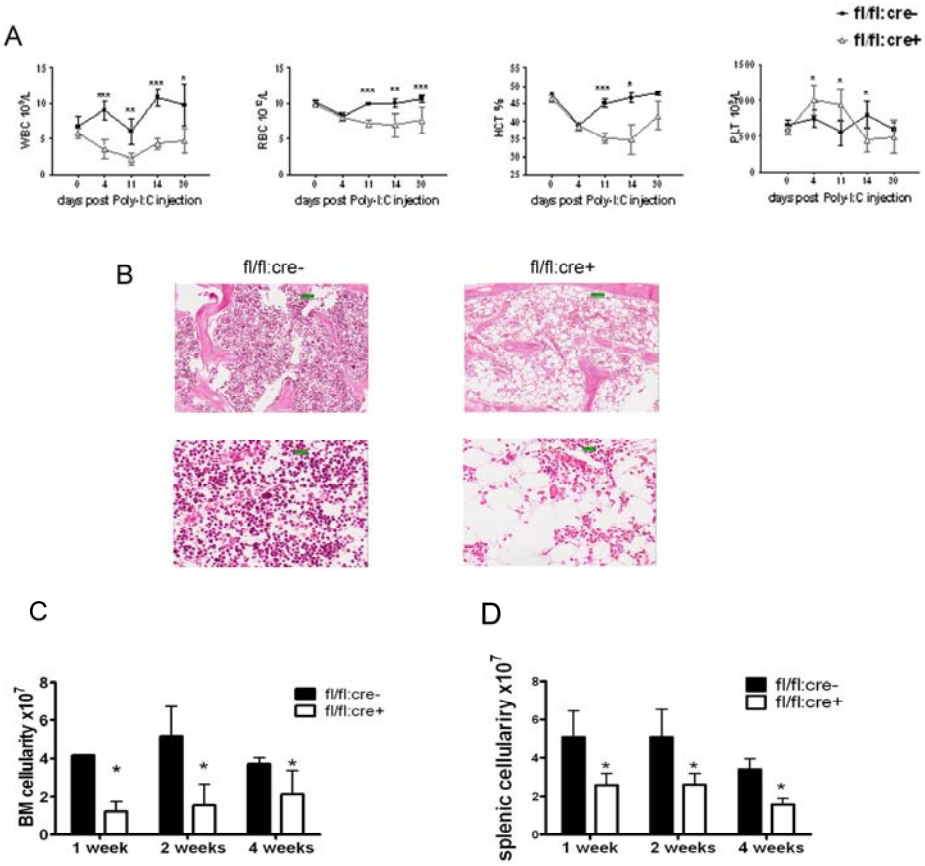

Figure 4. Inducible deletion of Shp2 *in vivo* leads to decreased cellularity in peripheral blood, bone marrow and spleen.

(A) Complete blood cell counting (CBC). Peripheral blood was collected at time points post final Poly-I:C injection (n=5-9). WBC, white blood cells; RBC, red blood cells; HCT, hematocrit; PLT, platelet.

(B) Representative hematoxlin & eosin (H&E) staining of trabecular femur 2 weeks post final Poly-I:C injection indicates a severe hypocellular phenotype in *fl/fl:cre*$^{+}$ bone marrow. Upper panel shows images scanned by ScanScope Digital Slide Scanners with 100x magnification. Lower panel shows images scanned with 400x magnification.

(C) Bone marrow cellularity decreased significantly in *fl/fl:cre*$^{+}$ mice starting from 1 week post final Poly-I:C injection (n=3-15).

(D) Splenic cellularity in the mutant animal was significantly reduced. Time points were after final Poly-I:C injection (n=3-4).

(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

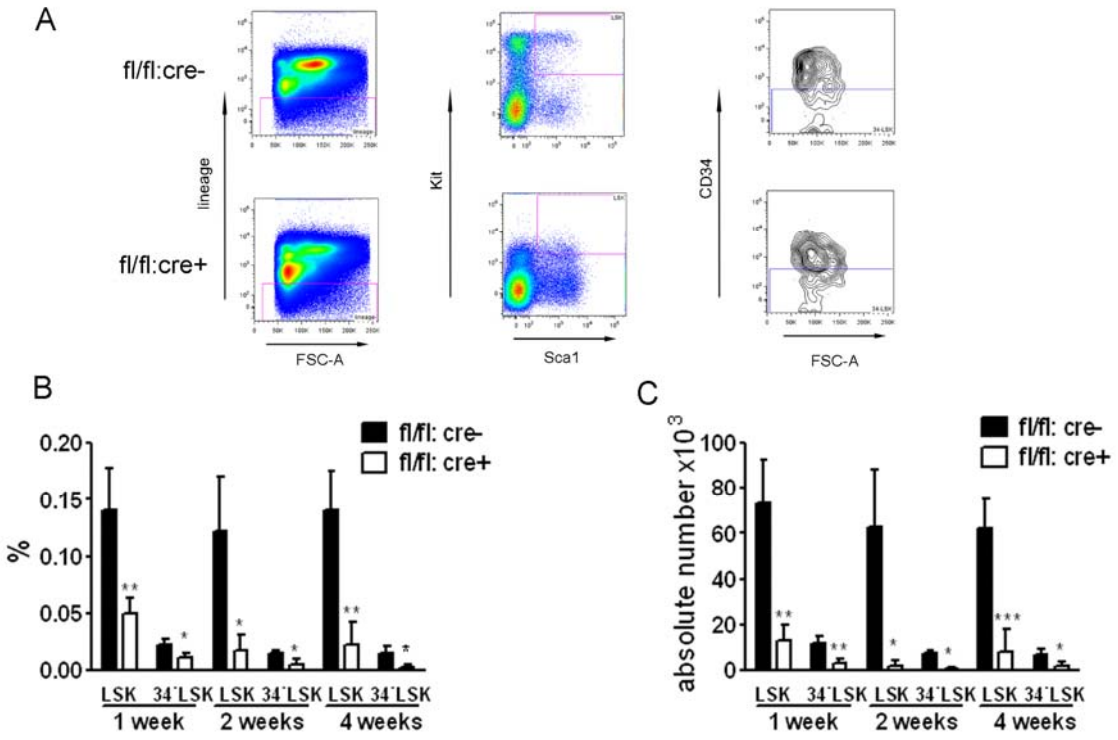

Figure 5. Loss of phenotypic HSCs and myeloid progenitors in Shp2 knock out animals.

(A) Representative FACS plots for lin$^-$Kit$^+$Sca1$^+$ CD34$^-$ (34$^-$LSK) staining 2 weeks post final Poly-I:C injection.

(B) Quantification of CD34$^-$LSK cell frequency per two hindlimbs at indicated time points post final Poly-I:C injection (*n*=3-4).

(C) Quantification of CD34$^-$LSK cell number per two hindlimbs at indicated time points post final Poly-I:C injection (*n*=3-4).

(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, data are presented as mean +/- S.D.)

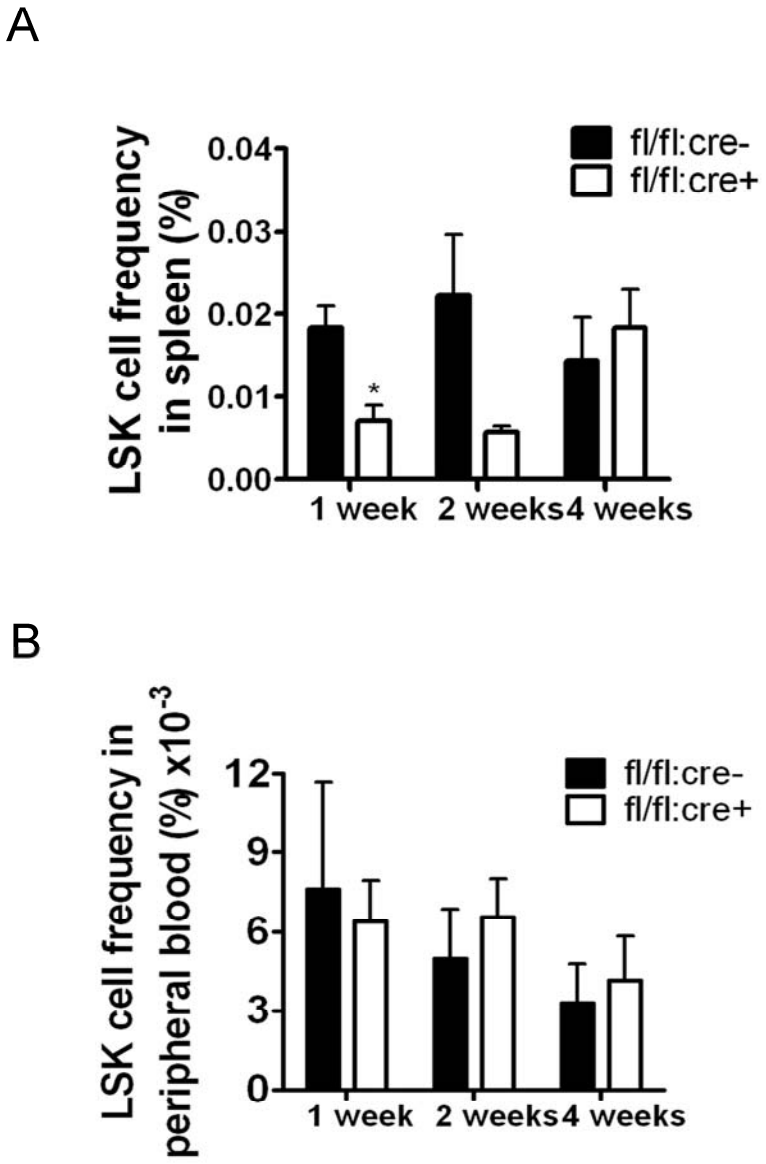

Figure 6. LSK cell mobilization is not affected by Shp2 knock out.
(A) LSK cell frequency in the spleen at indicated time points post final Poly-I:C injection (*n*=3-4).
(B) LSK cell frequency in the peripheral blood was not changed in mutant animal. Time points were after final Poly-I:C injection (*n*=3-4).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, data are presented as mean +/- S.D.)

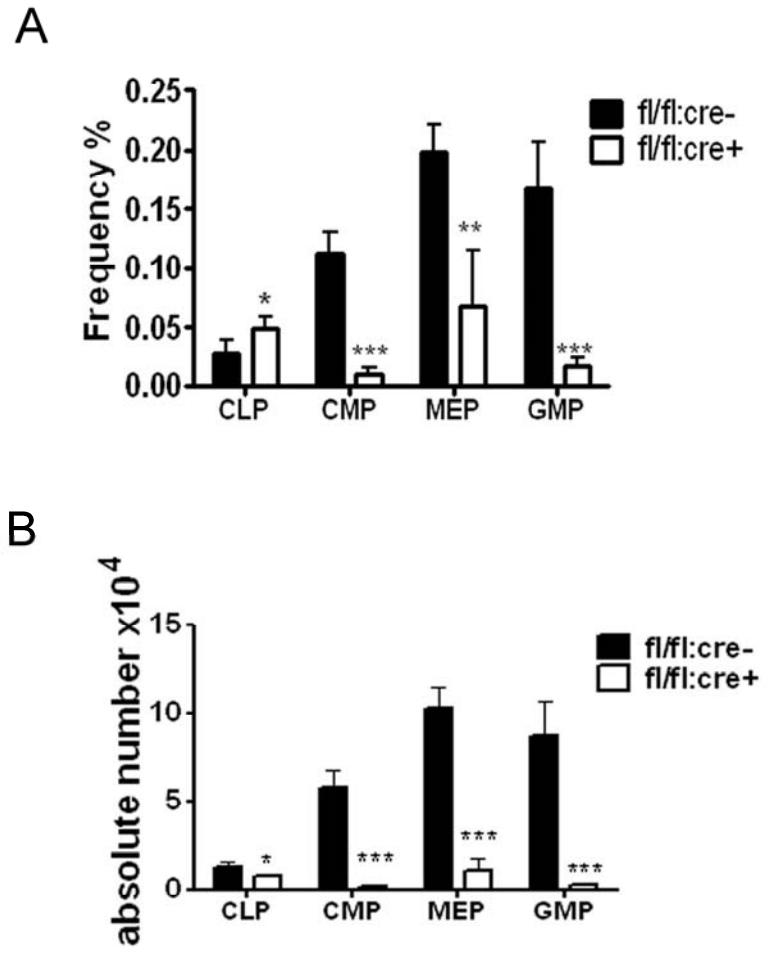

Figure 7. Effect of Shp2 deficiency on committed hematopoietic progenitors.
(A) CMPs, GMPs, EMPs and CLPs frequency in the bone marrow at 2 weeks post final Poly-I:C injection. CMPs were gated as $Lin^{-}Sca\text{-}1^{-}Kit^{+}FcgR^{lo}CD34^{+}$**.** CLPs were gated as $Lin^{-}IL\text{-}7R^{-}$ $Sca\text{-}1^{lo}Kit^{lo}$**.** GMPs were gated as $Lin^{-}Sca\text{-}1^{-}Kit^{+}FcgR^{hi}CD34^{+}$**.** MEPs were gated as $Lin^{-}Sca\text{-}1^{-}Kit^{+}FcgR^{lo}CD34^{-}$.
(B) Absolute numbers of CMPs, GMPs, EMPs and CLPs, 2 weeks post final Poly-I:C injection (n=4).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, data are presented as mean +/- S.D.)

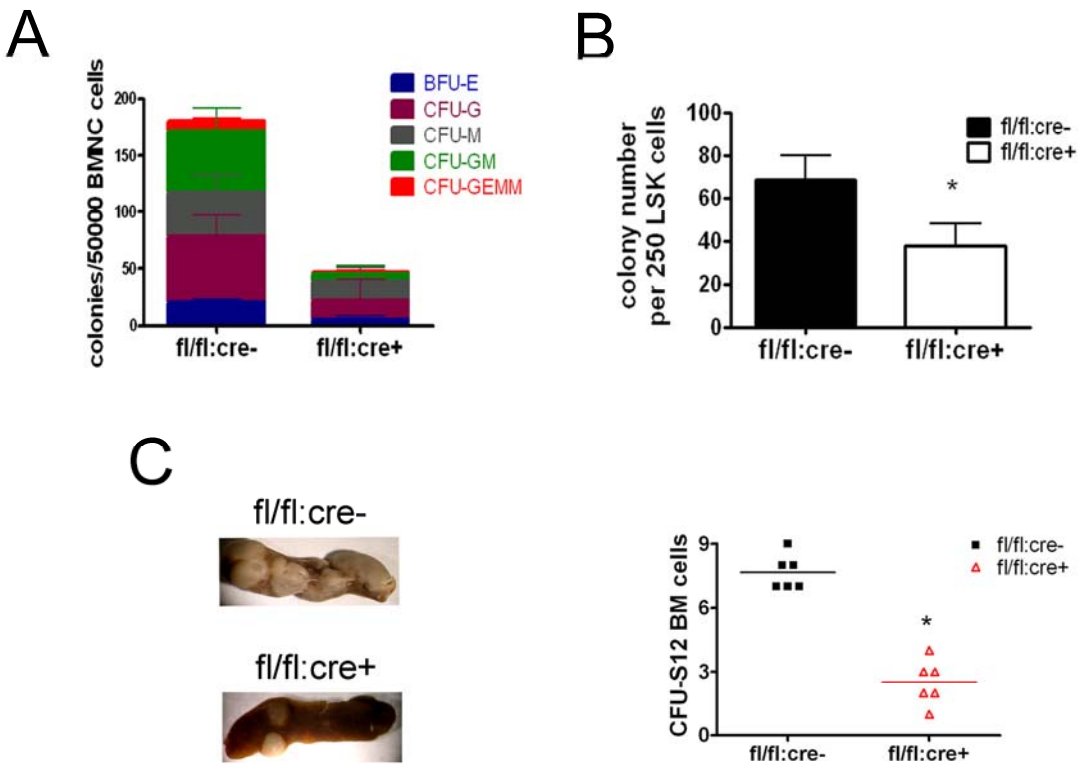

Figure 8. Defective myeloid colony formation of Shp2 knock-out bone marrow
(A, B) *In vitro* CFU assays for whole bone marrow cells or purified LSK cells. 50,000 bone marrow (A) or 250 sorted LSK (B) cells were seeded on methylcellulose medium Methocult 3434 (StemCell Technologies Inc.) with cytokines for 14 days before colony numeration. (*n*=3-6).
(C) Representative spleen colonies from CFU-S day 12 assay. Quantification of CFU-S_{12} suggests a decrease of HSCs and multi-potent progenitors (MMPs) (*n*=5-6).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars were S.D

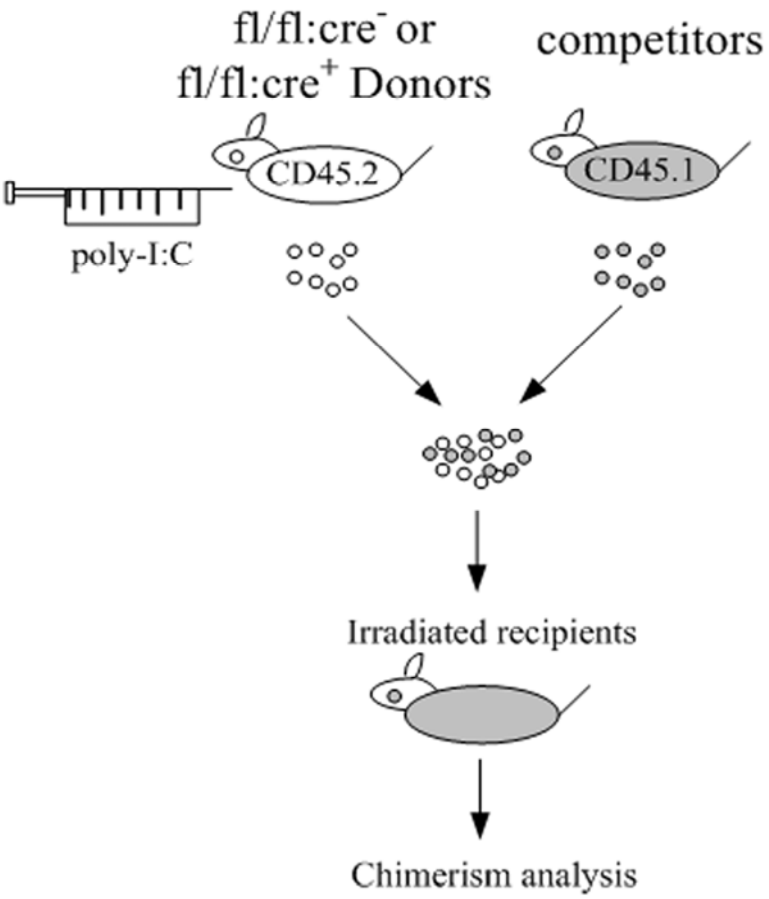

Figure 9. Competitive reconstitution strategy 1: injection first/transplantation later.
Donor mice were treated with poly-I:C before bone marrow transplantation.

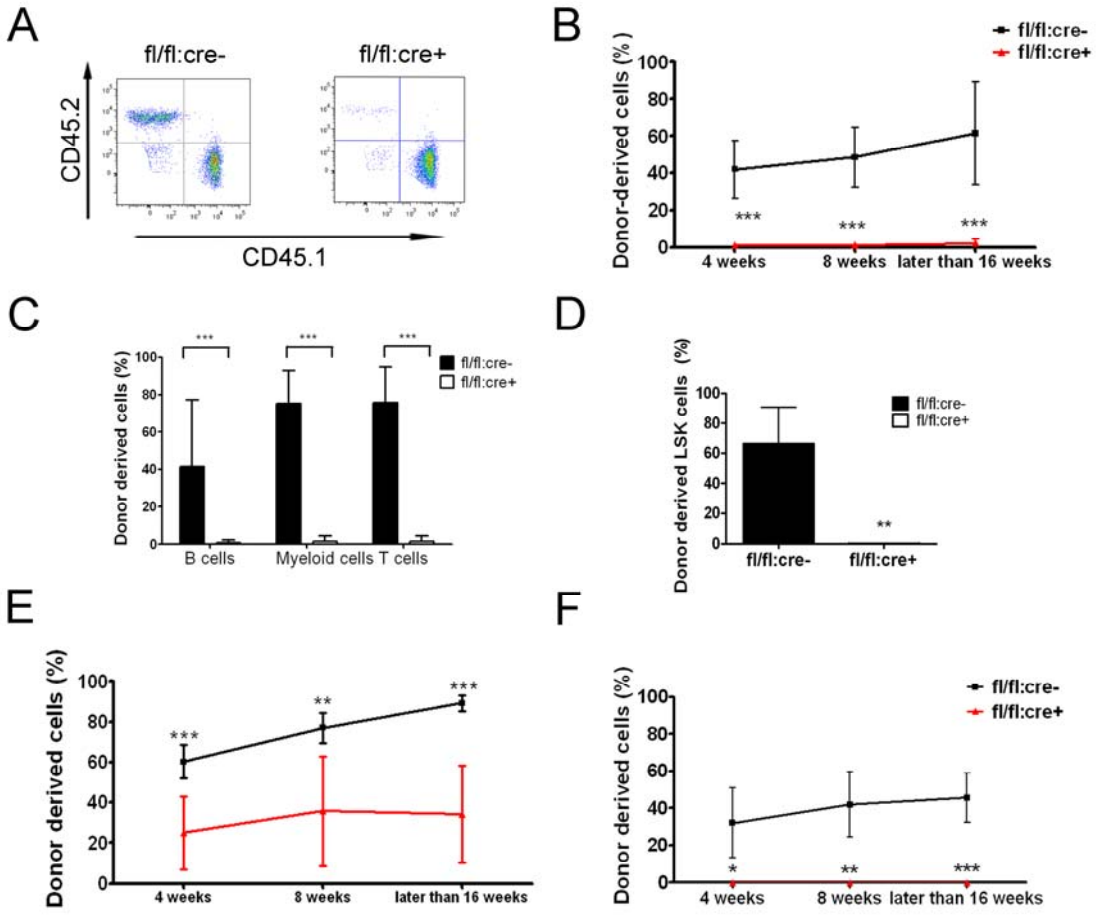

Figure 10. Shp2 ablation results in bone marrow reconstitution failure.
(A) Representative FACS plots illustrate bone marrow chimerism in 1:1 donor versus competitor reconstitution assay.
(B) Percentage of CD45.2 donor derived cells using 1:1 donor to competitor ratio (*n*=6-10).
(C) Percentage of CD45.2 donor-derived cells within different lineages in 1:1 ratio competitive reconstitution (*n*=4-5).
(D) *Shp2*$^{\Delta/\Delta}$ bone marrow contributes to less than 1% of LSK chimerism in 1:1 ratio competitive reconstitution (*n*=4).
(E) Competitive reconstitution assay with donor to competitor ratio at 4:1 in the *injection first/transplantation later* setting (n=4-9)
(F) 500 sorted LSK cells were transplanted into lethally irradiated recipients in competition with 5x10^5 CD45.1 cells in the *injection first/transplantation later* setting. Peripheral chimerism was analyzed at indicated time points. *fl/fl:cre*$^-$ LSK cells barely gave rise to any peripheral blood cells (*n*=4-5).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars were S.D.)

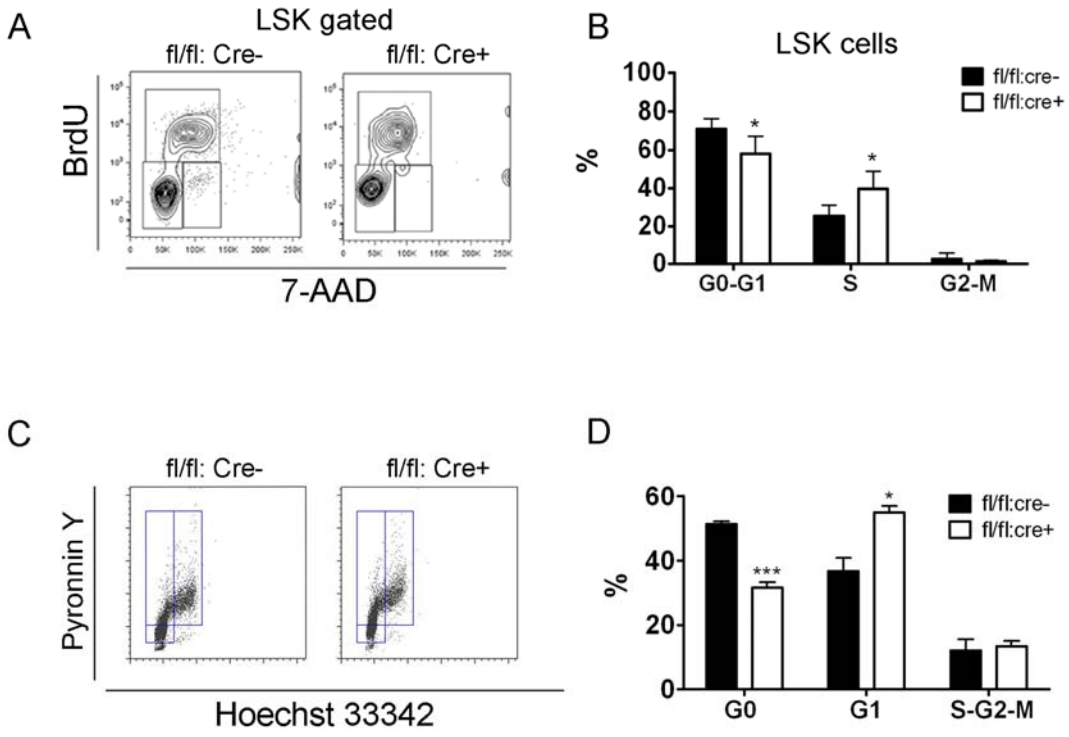

Figure 11. Disturbed quiescence in LSK cells derived from Shp2$^{\Delta/\Delta}$ bone marrow
(A) Representative FACS plots illustrate BrdU/7AAD staining in LSK cells.
(B) Quantification of S phase or G_0/G_1 phase cell percentage in LSK subpopulation indicating more actively proliferating LSK cells in *Shp2* animals. Results were obtained from 3 independent experiments.
(C) Representative FACS plots illustrate Pyronin Y/Hoechest33342 staining in LSK cells.
(D) Quantification of G_0, G_1 and S-G_2-M phase cell percentage in LSK subpopulation. More knockout LSK cells entered G_1 phase, with reduced number in the quiescent G_0 phase. Results were obtained from 3 independent experiments.
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

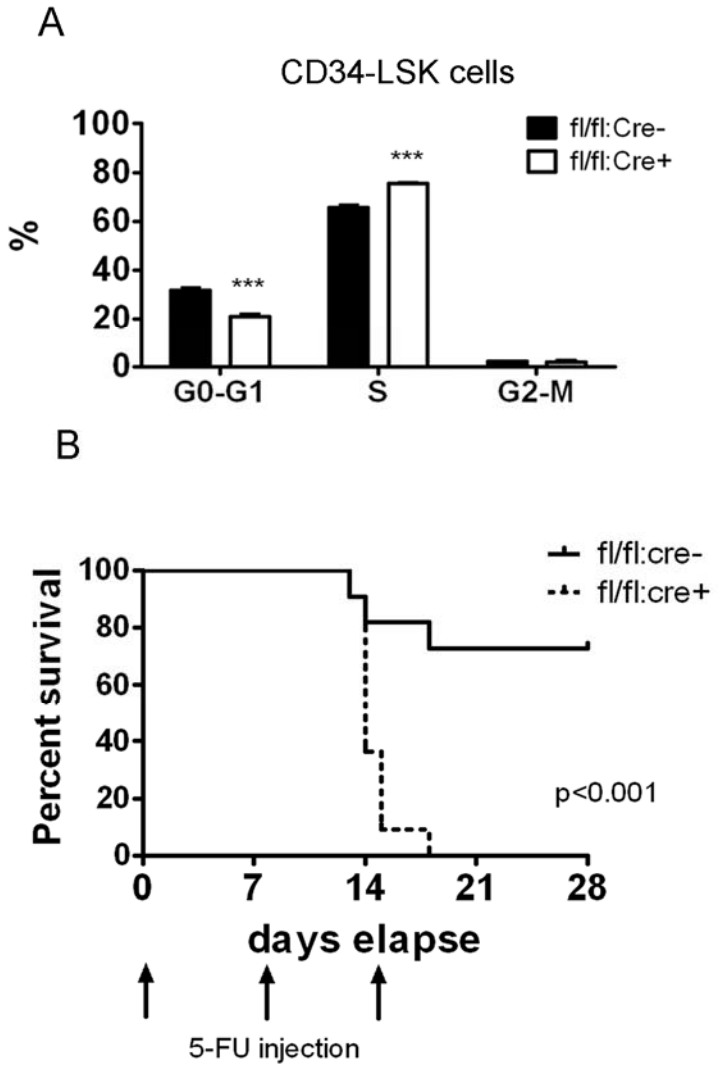

Figure 12. Shp2 ablation causes elevated HSC cell cycle entry.
(A) Quantification of G_0-G_1, S and G_2-M phase cell percentage in 34^-LSK subpopulation.
(B) Increased mortality rate to 5-FU myeloablation in Shp2 knockout animals. Percentage of survived animals was shown. Data were analyzed by log-rank (Mantel-Cox) test (n=11).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

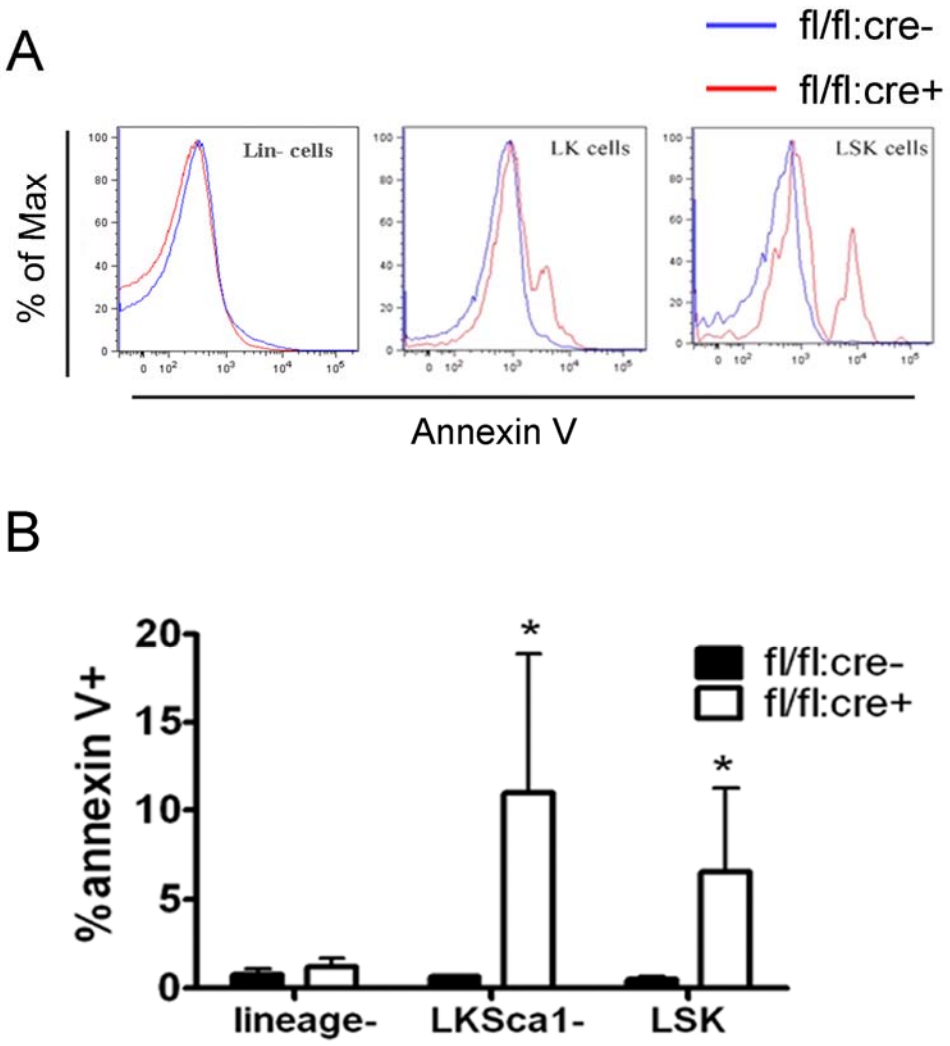

Figure 13. Increased apoptosis in Shp2$^{\Delta/\Delta}$ bone marrow progenitor cells.
(A) Representative FACS plots illustrate the Annexin V staining of different bone marrow compartments.
(B) lin$^-$ckit$^+$Sca1$^-$ and LSK cell but not lin$^-$ population contained more Annexin V$^+$ cells.
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

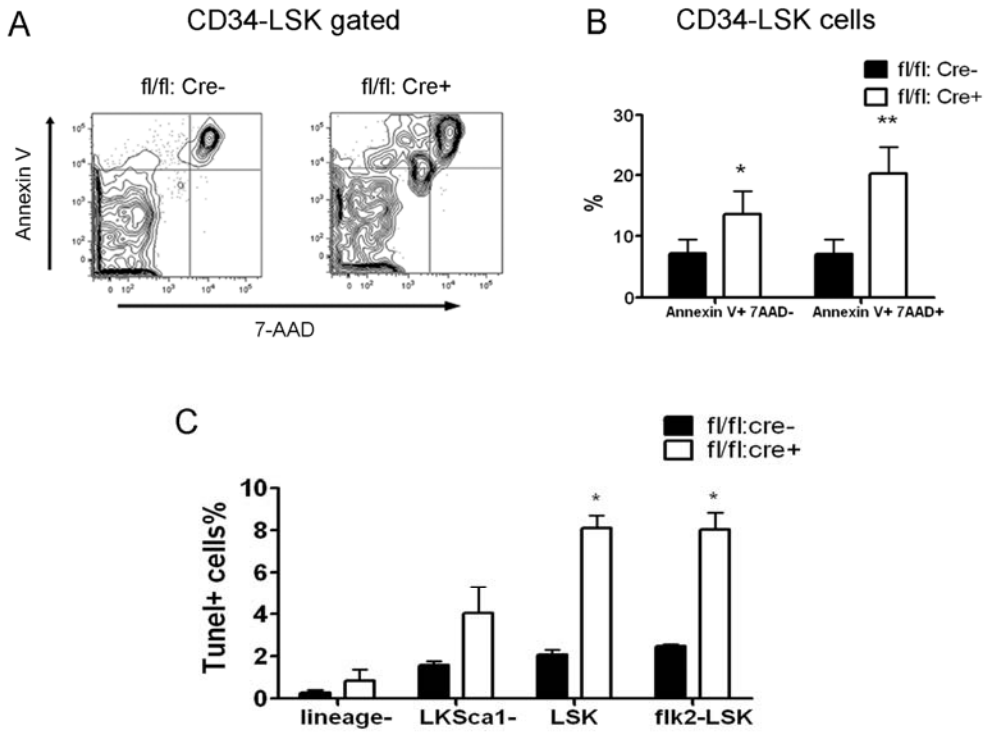

Figure 14. Shp2 deficient HSCs are more apoptotic, relative to controls.
(A) Representative FACS plots illustrate the Annexin V/ 7AAD staining of 34^-LSK cells.
(B) Elevated Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ percentage in *Shp2* $^-$ 34^- LSK cells (n=4). (*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)
(C) Increased number of $Tunel^+$ LSK and $flk2^-$LSK cells was detected in knock-out bone marrow. Results were analyzed from 2 independent experiments (error bars are S.E.M.).

3. Role of Shp2 in HSC and bone marrow niche interaction

3.1. The reconstitution defect is LSK cell-autonomous

The next question we asked was whether Shp2 is required intrinsically for HSCs, or if Shp2 regulates the micro-environmental cues in the bone marrow niche. To address this issue, we conducted reciprocal transplantation in which unfractionated wild-type bone marrow cells or isolated LSK cells were transplanted into recipients with wild-type or Shp2-deficient bone marrow microenvironments (Figure 15). Successful reconstitution was observed in both *fl/fl:cre*$^{-}$ and *fl/fl:cre*$^{+}$ recipients that received healthy bone marrow cells (Figure 16A) or LSK cells (Figure 16B), suggesting that Shp2 deficiency in niche cells did not compromise donor HSC reconstitution capacity.

We also tested functionally retroviral Cre-infected bone marrow cells in a transplantation assay. Control (+/+) or *fl/fl* bone marrow cells infected with Cre-IRES-human-CD2 retrovirus were stained with anti-human-CD2 (hCD2) antibody to distinguish the positively infected cells. As *hCD2* transcription is under control by the same promoter for *Cre*, hCD2 detection on the cell surface was used as a marker for Cre-expressing cells. When examined 48 hours after infection, around 50% of +/+ or *fl/fl* cells were hCD2^{+} (Figure 17, right panel). The mixture of hCD2^{+} and hCD2^{-} bone marrow cells were transplanted into lethally irradiated recipients. Strikingly, while hCD2^{+} control bone marrow cells maintained a chimerism higher than 50%, hCD2^{+} cells of *fl/fl* origin were barely detectable (Figure 17, left panel). These results are consistent with the data obtained in poly-I:C-treated *fl/fl:cre*$^{+}$ mice (Figure 10A, B)

and support the notion that Shp2 removal suppressed HSC reconstitution capacity, independent of the bone marrow microenvironment.

To unequivocally determine if the profound reconstitution defect is HSC-autonomous, 500 LSK cells from poly-I:C-treated *fl/fl:cre*$^{+}$ or *fl/fl:cre*$^{-}$ mice were transplanted into lethally irradiated recipients in competition with 5 x 10^5 CD45.1^{+} bone marrow cells. Consistent with the whole bone marrow cell data, *Shp2*$^{\Delta/\Delta}$ LSK cells derived from *fl/fl:cre*$^{+}$ mice contributed to almost no peripheral blood cells in recipients, although approximately 50% chimerism was detected in recipients that received *fl/fl:cre*$^{-}$ CD45.2^{+} LSK and CD45.1^{+} bone marrow cells (Figure 10F). Taken together, these results indicate that Shp2 acts in a cell-autonomous manner in the regulation of the HSC pool.

3.2. Shp2 controls HSC engraftment by governing homing process

To understand the transplantation failure, we investigated if Shp2 deficiency affected HSC homing and engraftment, by inducing Shp2 deletion before (Figure 9) or after transplantation (Figure 18). Untreated bone marrow cells of *fl/fl:cre*$^{+}$ or *fl/fl:cre*$^{-}$ origin (CD45.2^{+}) were transplanted into lethally irradiated recipients together with CD45.1^{+} bone marrow cells at ratio of 1:1 or 4:1. Four weeks post transplantation, 5 doses of poly-I:C were given to the recipients (wild-type), and the peripheral chimerism was determined at different time points. At 1:1 donor versus competitor ratio, *fl/fl:cre*$^{-}$ cells constituted the expected half of the peripheral blood initially and increased modestly later (Figure19A), similar to data shown in Figure 10B, obtained

in the *injection first/transplantation later* experiment. Approximately 20% of peripheral blood cells were derived from *fl/fl:cre*$^{+}$ bone marrow in the *transplantation first/injection later* experiment (Figure 19A), which stands in contrast to almost no *fl/fl:cre*$^{+}$-derived blood cells in recipients in the *injection first/transplantation later* setting (Figure 10B). 16 weeks after transplantation, we sacrificed the recipients and stained for CD45.2^{+} LSK cells. We found that LSK cells of *fl/fl:cre*$^{+}$ origin decreased to less than 5%, reinforcing the notion that Shp2 is required for self-renewal/maintenance of HSCs/progenitors in the *transplantation first/injection later* scenario (Figure 19C). Similar results were obtained when we used a 4:1 ratio in transplantation (Figure 19D). B cell, T cell or Myeliod cell specific markers were used to evaluate donor-derived cells in different lineages. *Shp2*$^{\Delta/\Delta}$ bone marrow cells achieved significant lower chimerism of B and Myeloid cells (Figure 19B), but the suppression was not as dramatic in the *injection first/transplantation later* setting (Figure 10C). We also determined the competitive reconstitution capacity of purified LSK cells in the *transplantation first/injection later* setting. Around 20% of *Shp2*$^{\Delta/\Delta}$ LSK cell-derived chimerism was again observed in the peripheral blood (Figure 20A), as compared to the more severe phenotype in the *injection first/transplantation later* experiment (Figure 10F). When the isolated CD45.2^{+} bone marrow cells from the primary recipients (injection later) were mixed with CD45.1^{+} competitor cells at 1:1 ratio for secondary transplantation, less than 5% of the CD45.2^{+} cells were detected in second recipients that received *fl/fl:cre*$^{+}$ cells (Figure 20B), confirming the reconstitution failure of *Shp2*$^{\Delta/\Delta}$ HSCs.

The improved reconstitution efficiency observed when *Ptpn11/Shp2* was deleted after transplantation suggests a possible role of Shp2 in engrafted HSC homing and the establishment of new HSC-niche interaction. In the *transplantation first/injection later* experiment, untreated *fl/fl:cre*$^{+}$ bone marrow cells were able to home and occupy the bone marrow niche in the first place, thus compromising the effect of Shp2 deletion by subsequent poly-I:C treatment. To test this hypothesis, we evaluated directly the homing capacity of Shp2$^{\Delta/\Delta}$ bone marrow cells *in vivo*. Purified control and *Shp2*$^{\Delta/\Delta}$ lineage^{-} bone marrow cells were labeled with CDFA-SE and injected into lethally irradiated recipients. At 16 hour post transplantation, recipient mice were euthanized for bone marrow isolation and CDFA-SE^{+} cell quantification. As shown in Figure 21A, Shp2 removal resulted in a dramatic decrease in the number of donor lineage^{-} bone marrow cells in the recipients' bone marrow. To further quantify the homed stem/progenitor cells, we collected the recipients' bone marrow for CFU assay, by using bone marrow cells from lethally irradiated mice without transplantation as negative control. Consistent with the CDFA-SE staining data, bone marrow cells from Shp2 mutant transplantation recipients gave rise to significantly fewer colonies than the wild-type controls (Figure 21B). These results suggested a critical role of Shp2 in regulating signals required for homing of engrafted stem/progenitor cells.

We next assessed the impact of Shp2 ablation on cell motility *in vitro* by performing a cell migration assay using a Boyden Chamber. Purified lineage^{-} bone marrow cells from poly-I:C-treated *fl/fl:cre*$^{+}$ or *fl/fl:cre*$^{-}$ mice were seeded into the

upper chamber and were allowed to migrate toward chemokine SDF-1α (5 nM) for 3 hours. Compared to controls, *Shp2*$^{\Delta/\Delta}$ bone marrow cells showed a significantly impaired response to SDF-1α-mediated chemotaxis (Figure 21C).

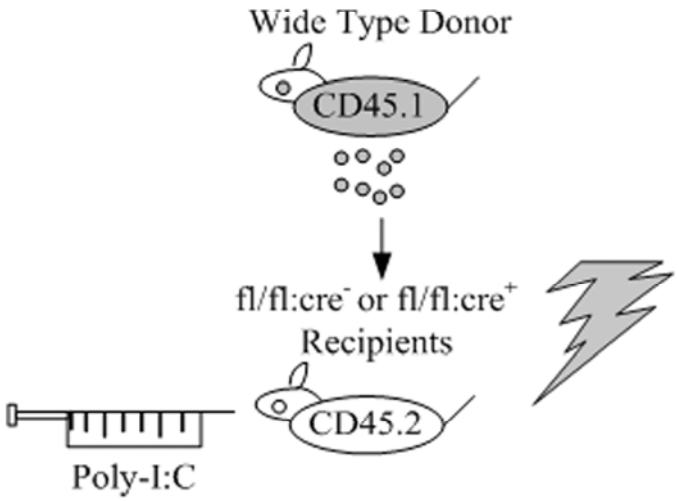

Figure 15. Reciprocal bone marrow transplantation.
Poly-I:C treated *fl/fl:cre*$^{-}$ or *fl/fl:cre*$^{+}$ mice were lethally irradiated and used as recipients. Wild-type whole bone marrow or sorted LSK cells from CD45.1 mice were used as donor cells. Hematopoiesis in recipient mice was monitored at different time points post transplantation.

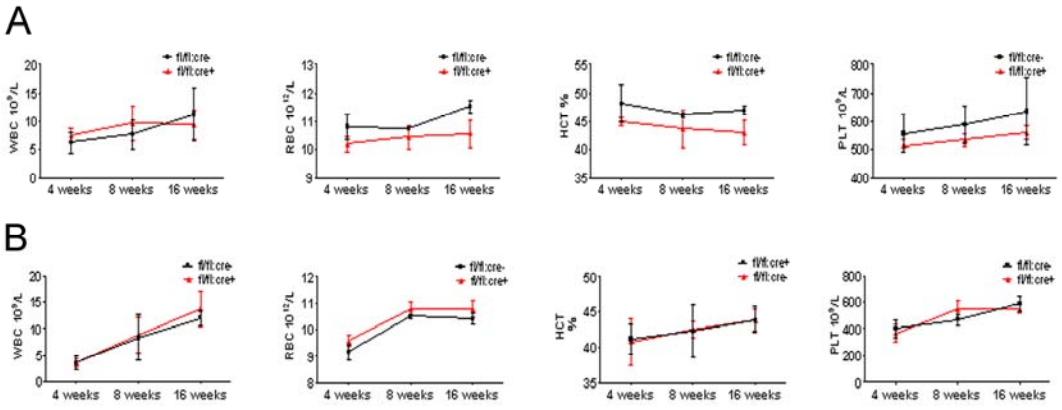

Figure 16. Normal hematopoietic parameters in Shp2 deficient reciprocal transplantation recipient.
(A) CBC counts in the recipients of whole bone marrow reciprocal transplantation. No difference was detected between wild-type and knockout groups (n = 5).
(B) CBC counts in the recipients of LSK cell reciprocal transplantation. No difference was detected between wild-type and knockout groups (n = 5).

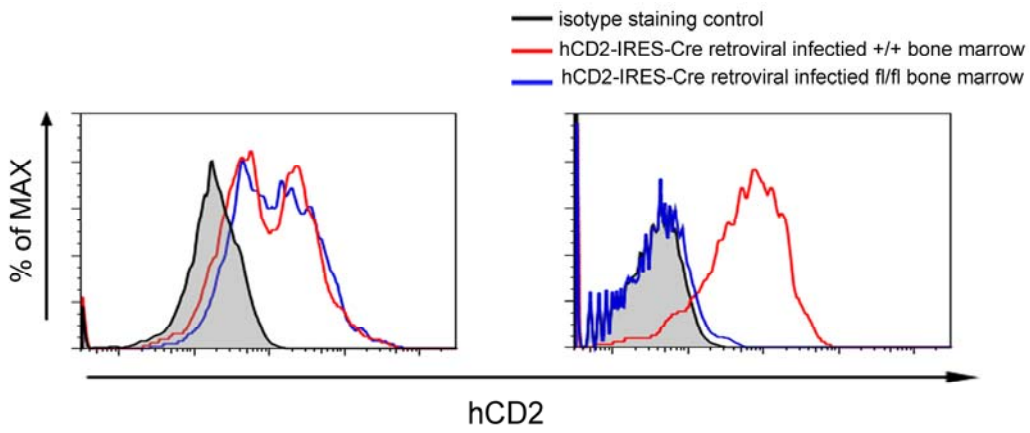

Figure 17. The suppression of HSC reconstitution by Shp2 removal is independent of the bone marrow microenvironment.
Control +/+ or *fl/fl* bone marrow was infected with Cre-IRES-hCD2 retrovirus and transplanted into lethally irradiated mice. As *hCD2* transcription is under control by the same promoter for *Cre*, hCD2 detection on cell surface was used as marker for Cre-expressing cells. The right panel is a representative FACS plot for hCD2 on day 0 of transplantation and left panel is representative FACS plot for hCD2 on day 30 of transplantation. On day 0, around 50% of bone marrow cells either from +/+ or *fl/fl* mice were hCD2-positive. On day 30, hCD2-positive *fl/fl* bone marrow cells were barely detectable, while the chimerism of +/+ cells remained higher than 50%.

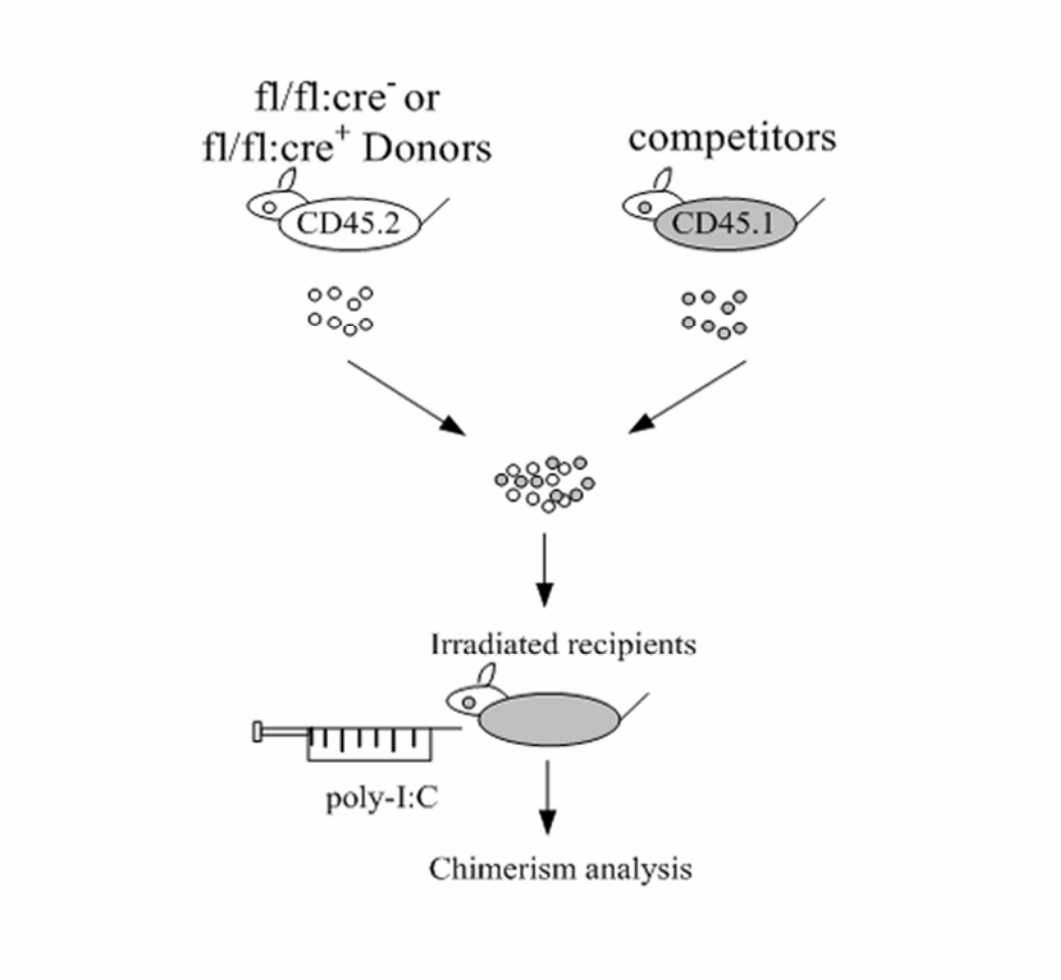

Figure 18. Competitive reconstitution strategy 2: transplantation first/injection later.
The recipient mice were treated with poly-I:C post bone marrow transplantation.

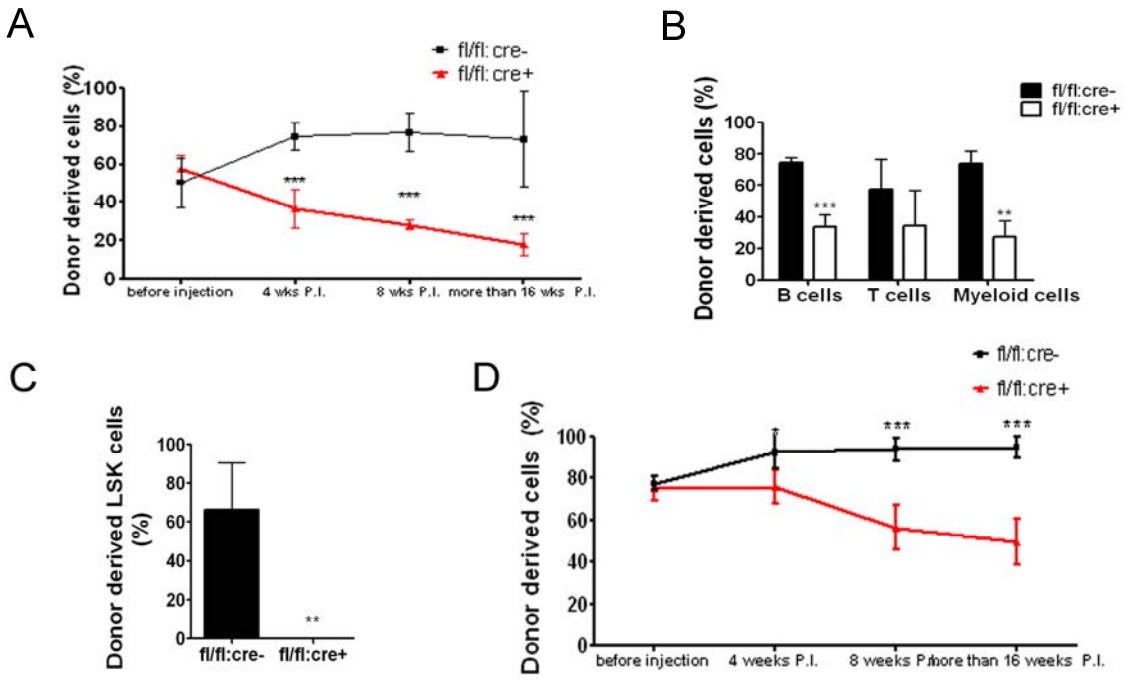

Figure 19. Decreased bone marrow reconstitution capacity of Shp2$^{\Delta/\Delta}$ bone marrow in transplantation first/injection later experimental setting.

(A)Percentage of CD45.2 donor-derived cells using 1:1 donor to competitor ratio with poly-I:C injection after transplantation (*n*=4-7).

(B) Percentage of CD45.2 donor-derived cells within different lineages in 1:1 ratio competitive reconstitution (n=3).

(C) *Shp2*$^{\Delta/\Delta}$ bone marrow contributed significantly less LSK cells in the 1:1 ratio competitive reconstitution experiment (*n*=3).

(D) Competitive reconstitution assay with donor to competitor ratio at 4:1 in the *transplantation first/injection later* setting (*n* = 4-9).

(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

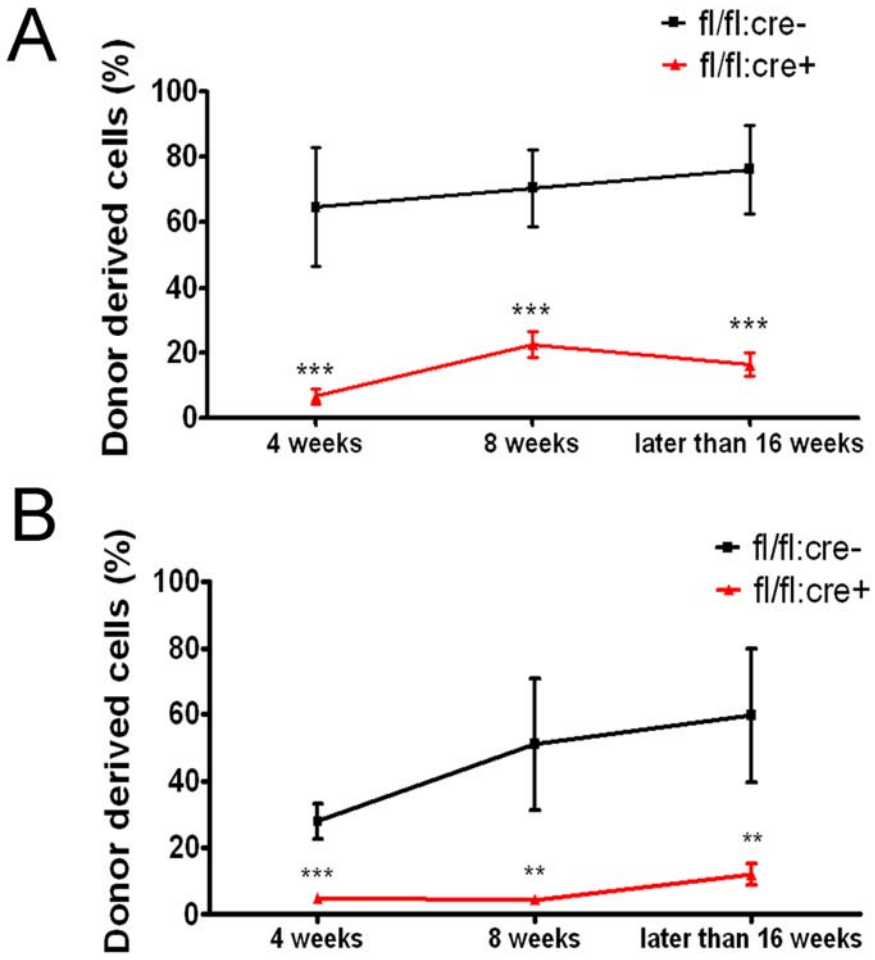

Figure 20. Reconstitution failure of Shp2$^{\Delta/\Delta}$ HSCs

(A) Sorted LSK cells were used as donor cells in the *transplantation first/injection later* competitive reconstitution assay. Peripheral chimerism was analyzed at indicated time points (n=4).

(B) Declined reconstitution efficacy of *Shp2* bone marrow cells in the secondary transplantation (n=4-6).

(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

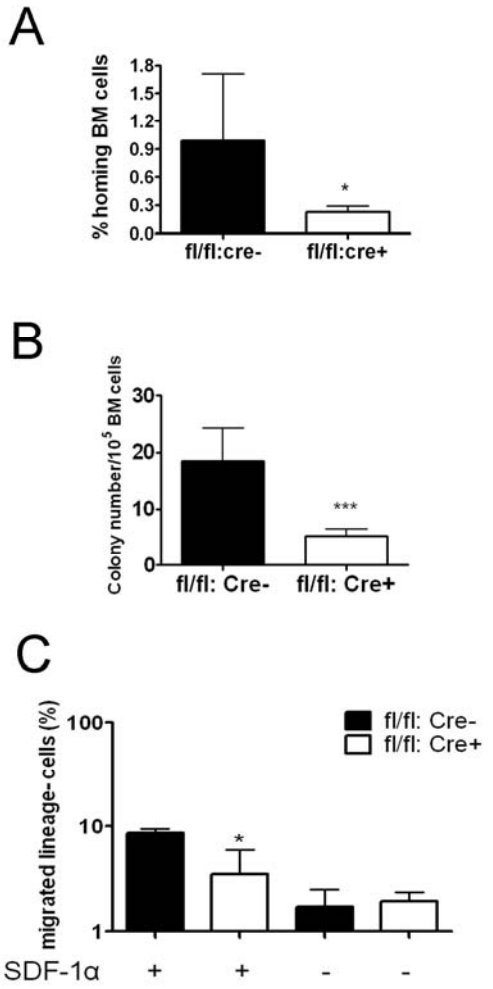

Figure 21. Homing defect in Shp2$^{\Delta/\Delta}$ bone marrow cells.
(A) *In vivo* homing assays. Results were shown with percentage of homed bone marrow cells 16 hours post-transplantation (n=4, 5).
(B) Homing defects in *Shp2* $^{\Delta/\Delta}$ progenitors. 5x10^6 wild type or *Shp2* $^{\Delta/\Delta}$ bone marrow cells were transplanted to lethally irradiated mice. Recipient bone marrow was harvested 24 hours later and seeded in Methocult 3434 medium (StemCell Technologies Inc.). Colonies were counted on day 8 (n=7). We used lethally irradiated but non-transplanted recipients as negative control and no colonies were detected from those bone marrows. Significantly fewer colonies were formed from *Shp2* $^{\Delta/\Delta}$ marrow transplantation, suggesting that the homing defect resides in primitive progenitors.
(C) Shp2 deletion severely reduces the chemotactic response of lin$^-$ cells to SDF-1α. 10^6 lineage depleted bone marrow cells were plated in the upper chamber for migration toward 5 nm SDF-1α in the lower chamber (performed in triplicate).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

4. Molecular Mechanism of Shp2 mediated signaling in the regulation of HSC function.

4.1. Shp2 removal results in downregulation of Kit expression.

In exploring the molecular mechanism underlying the HSC deficiency induced by Shp2 deletion, we noticed severe ablation of Kit^+ cells in the $lineage^-$ cell pool (Figure 5A), which could not be simply explained by the moderate increase in the progenitor cell apoptosis. Therefore, we tested the hypothesis that Shp2, while operating downstream of the Kit receptor in cell signaling, may also mediate intracellular signaling pathways controlling Kit expression. To examine Kit expression in a stem cell pool, we used the SLAM markers CD150 and CD48 staining for further enrichment of HSCs ($lin^-Sca1^+CD150^+CD48^-$). As shown in Figure 22A, wild-type $lin^-Sca1^+CD150^+CD48^-$ cells were Kit^+, as expected. In contrast, Kit expression was significantly lower in $lin^-Sca1^+CD150^+CD48^-$ cells isolated from $Shp2^{\Delta/\Delta}$ bone marrow (Figure 22A, B).

To determine whether acute deletion of Shp2 results in downregulation of Kit expression directly, we measured the kinetics of Kit expression decrease at different time points after poly-I:C injection. *fl/fl:cre*$^-$ and *fl/fl:cre*$^+$ mice were given 3 to 5 doses of poly-I:C at 2-day intervals and bone marrows were collected starting from 7 days after the first injection. As shown in Figure 23B, immunoblotting of marrow samples showed a progressive increase of Shp2 deletion from day 7 (around 50%) to almost 100% on day 16. Interestingly, the decrease in Kit expression of lin^-

$Sca1^{+}CD150^{+}CD48^{-}$ cells correlated well with the Shp2 deletion extent (Figure 23A). These data support the hypothesis that Shp2 is positively required for Kit expression in HSCs. To define an immediate effect of Shp2 removal on Kit expression, we made use of a retrovirus expressing Cre recombinase to delete Shp2 *in vitro*. As shown in Figure 24, fl/fl bone marrow exhibited loss of Kit expression in $lineage^{-}$ gated cells 48 hours after infection with the Cre-expressing virus. This result excludes the possibility of a poly-I:C toxicity or bone marrow microenvironment effect on Kit expression.

4.2. Shp2 modulates Kit expression through Gata2

We then asked at which level Shp2 modulates Kit expression. qRT-PCR analysis demonstrated significantly reduced *c-kit* mRNA levels in $Shp2^{\Delta/\Delta}$ primary $lineage^{-}$ bone marrow cells and also in Shp2 knockdown bone marrow progenitor EML cells, as compared to their respective controls (Figure 25A, B). These results point to a role of Shp2 in mediating intracellular signaling pathways that regulate *Kit* gene transcription. To test this possibility, we conducted a promoter reporter assay, using two constructs p13kit and p70kit, which contain an EGFP reporter gene under control of *Kit* promoter only or promoter plus an enhancer sequence (Cairns et al., 2003). We co-transfected HEK-293 cells with Shp2-specific siRNA or scramble siRNA with p13kit, p70kit and control EGFP constructs, respectively, and examined the reporter expression. As shown in Figure 27A, B, C, Shp2 knockdown strongly repressed GFP expression in cells co-transfected with p13kit and p70kit constructs but not the control vector. We also examined the Shp2 effect on *Kit* expression by co-

transfection of p13kit with constructs expressing wild-type (WT-Shp2), dominant-active (DA-Shp2) or dominant negative mutants (DN-Shp2). As expected, expression of WT-Shp2 and DA-Shp2 enhanced the *Kit* reporter expression, and DN-Shp2 severely suppressed the GFP intensity (Figure 27D, E). Consistently, ectopic expression of a truncated Shp2 that contains the two SH2 domains did not increase the *Kit* reporter GFP expression, suggesting the requirement of the PTPase activity (Figure 27F). Together, these data indicate that Shp2 modulates the transcription of *Kit* in a phosphatase-dependent manner.

Previous biochemical analyses suggest Shp2 participation in intracellular signaling events immediately downstream of the Kit receptor (Tauchi et al., 1994). We assessed the impact of Shp2 deletion on various signaling pathways following c-kit activation by its ligand SCF. As shown in Figure 28, SCF-stimulated p-AKT and p-Erk signals were impaired in EML cells following Shp2 knockdown. Shp2 silencing also decreased the basal level of pY-STAT3. p-JNK was unchanged while p-p38 was modestly enhanced in Shp2 knockdown cells. Consistently, previous data from this and other groups have demonstrated positive roles of Shp2 in regulation of Erk and PI3K/AKT pathways (Chan and Feng, 2007; Neel et al., 2003). We then evaluated the effects of pathway-specific inhibitors, to determine if these signaling defects are responsible for decreased Kit expression. Treatment of EML cells with PI3K inhibitor LY294002 or MEK inhibitors U0126 led to Kit downregulation in a dose-dependent manner. Moderate reduction of Kit expression was also detected when using a dosage

(25 μm) of JAK2 inhibitor AG490. Combined treatment of these three inhibitors resulted in suppression of Kit expression at a very low dosage (Figure 29).

The defect in *Kit* gene transcription in Shp2-deficeint cells must be due to altered activity/expression in transcription factors. To address this issue, we performed qRT-PCR analysis to assess the expression of *Gata1, Gata2, Scl* and *Sp1*, transcription factors known to regulate *Kit* promoter, in both primary lineage$^-$ bone marrow and EML cells (Kroon et al., 1998; Lecuyer et al., 2002; Liu et al.; Munugalavadla et al., 2005). *Gata2* mRNA was decreased in Shp2$^{\Delta/\Delta}$ lineage$^-$ bone marrow and Shp2 knockdown EML cells (Figure 25). We further performed the *q*RT-PCR with sorted 34$^-$LSK and 34$^+$LSK cells, and consistent reduction of *Gata2* mRNA was observed in Shp2$^{\Delta/\Delta}$ 34$^-$LSK cells (Figure 26). Immunoblotting results confirmed the decrease of Gata2 protein in Shp2 knockdown EML cells (Figure 30A). We further performed a CHIP assay to measure directly the amounts of Gata2 associated with the cis-regulatory elements of *Kit* gene (Jing et al., 2008; Maeda et al.). Shp2 knockdown did not affect Gata2 binding to -114kb and +5 kb region of Kit gene but led to decreased Gata2 binding to promoter region (Figure 31), and similarly impaired Gata2 binding was observed when the three chemical inhibitors (LY294002, U0126, AG490) were used alone or in combination (Figure 32). Furthermore, enforced Gata2 expression rescued the phenotype of defective Kit expression in EML cells induced by Shp2 knockdown (Figure 30B). Consistently, a recent report documented regulation of Gata2 activity by AKT (Menghini et al., 2005), suggesting a Shp2/PI3K/AKT/Gata2/Kit pathway. Indeed, combined deletion of Shp2 and Pten, a

negative regulator of the PI3K/AKT pathway, partially restore the Kit expression in lineage$^-$ cells and SLAM marker enriched HSCs. (Figure 33). Therefore, while involvement of other transcription factors are not excluded, data presented here suggest a role of Shp2 in promoting *Kit* expression at least partially via Gata2.

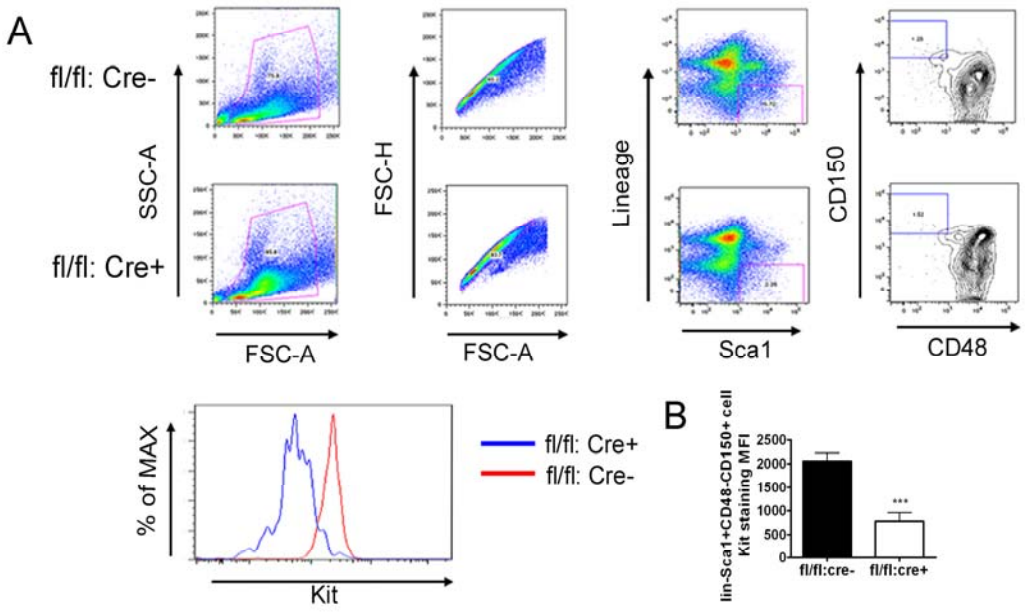

Figure 22. Kit downregulation in SLAM marker-enriched HSCs.
(A) Representative FACS plots for lin$^-$Sca1$^+$CD48$^-$CD150$^+$ staining 4 weeks P.I. indicated severe reduction of Kit expression in SLAM marker-enriched HSCs from *fl/fl:cre*$^+$ bone marrow.
(B) Median fluorescence intensity (MFI) for Kit staining of lin$^-$Sca1$^+$CD48$^-$ CD150$^+$ cells (n=4).
(*** $p<0.001$, ** $p<0.01$,* $p<0.05$, error bars are S.D.)

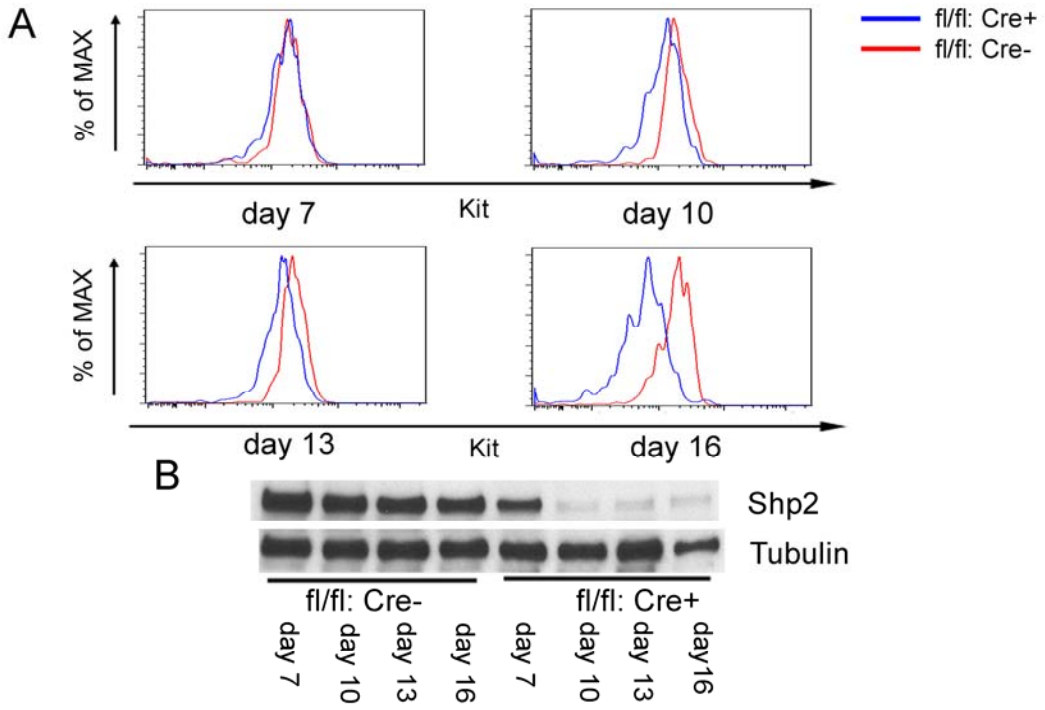

Figure 23. kinetics of Kit expression at different time points after poly-I:C injection

(A) FACS histogram plots illustrating Kit staining of $lin^{-}Sca1^{+}CD48^{-}CD150^{+}$ cells at different time points after first poly-I:C injection, indicating immediate Kit downregulation following acute Shp2 deletion.

(B) Immunoblotting of bone marrow cells after first poly-I:C injection showed gradually increased deletion from day 7 to 16. Tubulin was used as loading control.

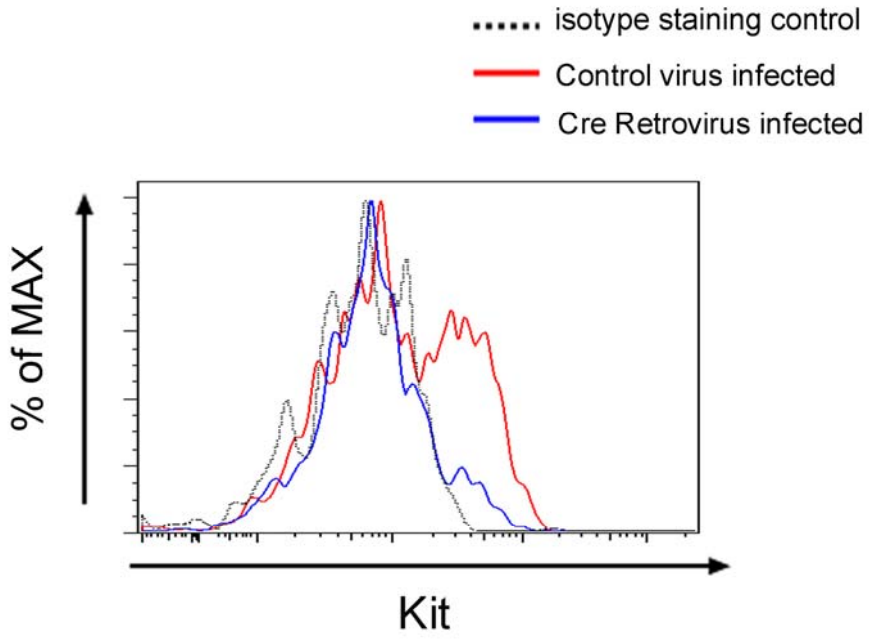

Figure 24. Retroviral cre expression led to loss of Kit$^+$ cells in lineage$^-$ gated population.
The flow cytometric plot illustrates Kit-staining profile of lineage$^-$ *fl/fl* bone marrow infected with control retrovirus or Cre expressing retrovirus.

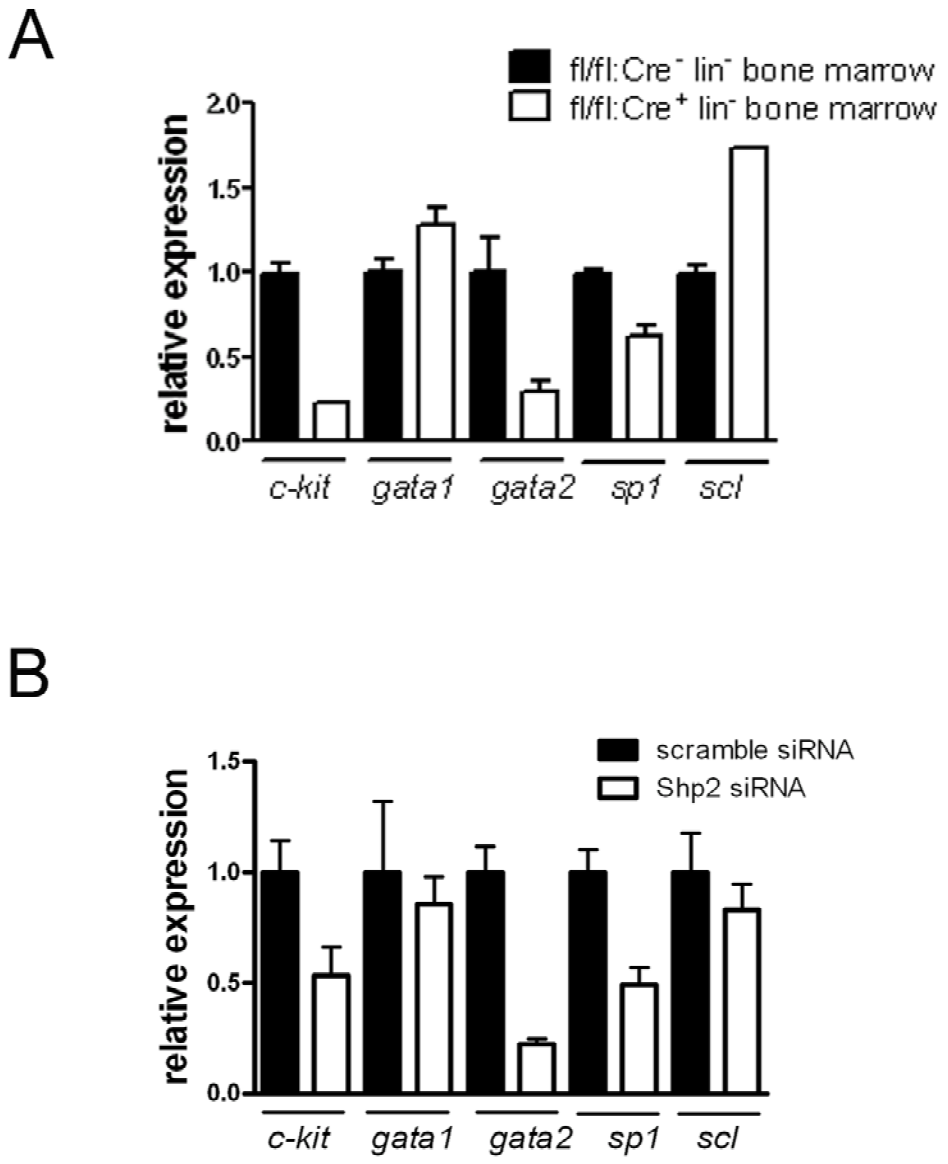

Figure 25. qRT-PCR analysis of *kit* and *kit* regulatory transcriptional factor in lineage⁻ bone marrow cells and EML cell line.
(A, B) qRT-PCR of *Kit* and *Scl, Sp1, Gata2, Gata1* mRNA demonstrated consistent suppression of *Kit* and *Gata2* transcription in primary *Shp2* lineage⁻ cells and Shp2 knockdown EML cells. β-actin was used as reference gene for normalization.

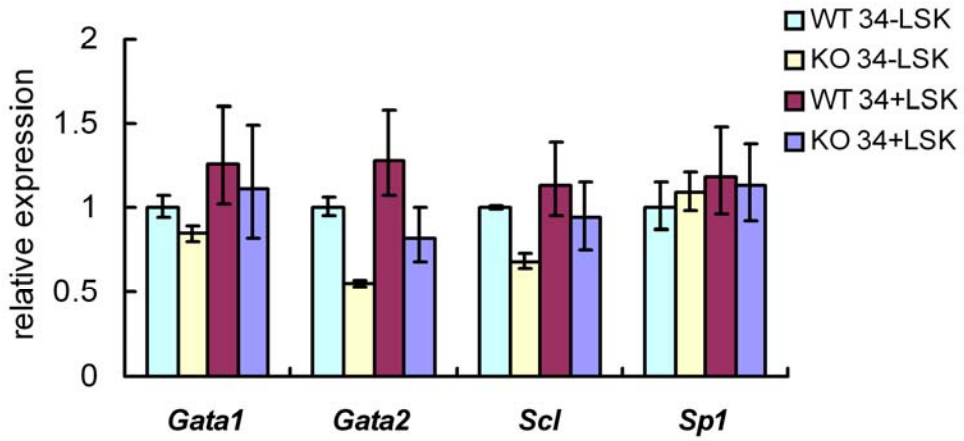

Figure 26. *q*RT-PCR of *Gata1, Gata2, Scl* and *Sp1* mRNA in sorted 34^-LSK and 34^+LSK cells.
β-actin was used as reference gene for normalization.

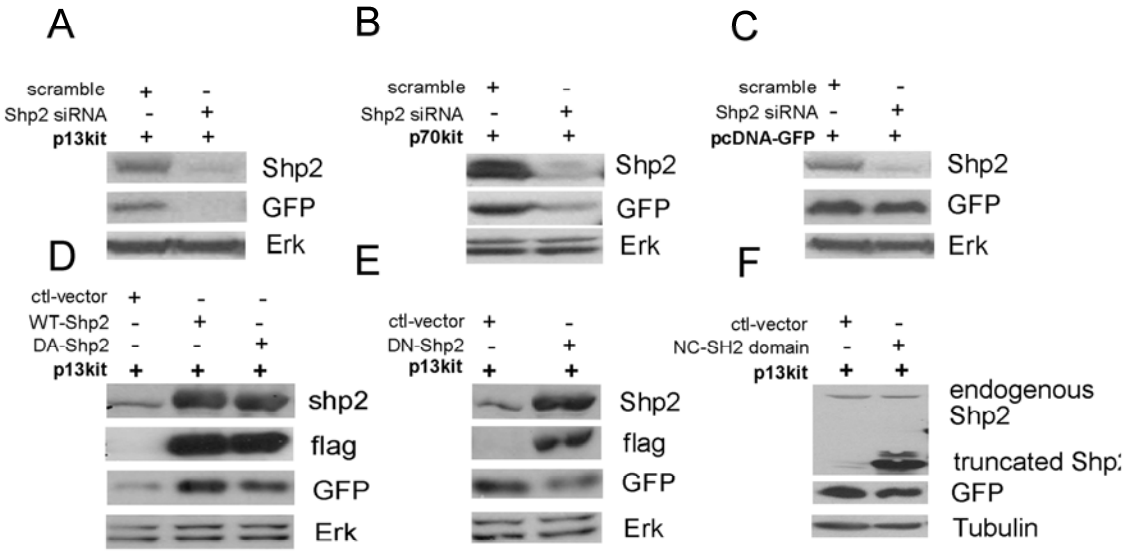

Figure 27. Shp2 knockdown repressed *Kit* promoter activity.
(A, B, C)Constructs with *Kit* regulatory elements fusion to GFP reporter were transfected into HEK293 cells. Shp2 knockdown by specific siRNA lead to GFP downregulation in both p13kit(A) and p70kit transfectants(B) but not GFP vector transfectant(C). Erk was used as loading control.
(D, E, F) Enforced expression of flag-tagged wild-type (WT) and dominant active (DA) Shp2(D) enhanced, dominant negative (DN) Shp2(E) suppressed *Kit* promoter activity, with no effect for truncated Shp2 containing the two SH2 domains(F). Erk or Tubulin was used as loading control.

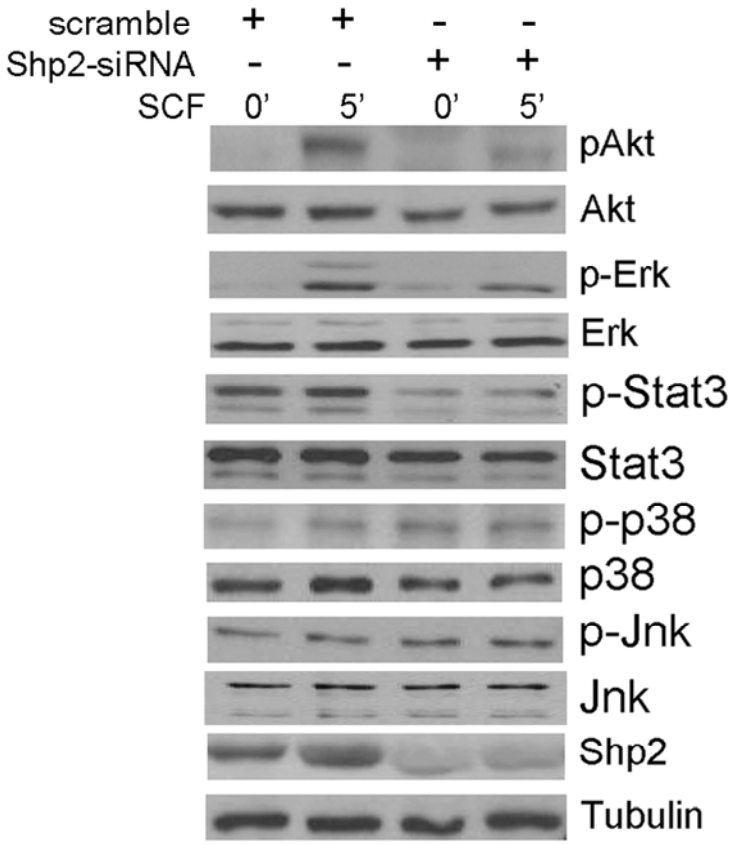

Figure 28. Signaling modification in Shp2 knockdown EML cells elicited by SCF. Tubulin was used as loading control.

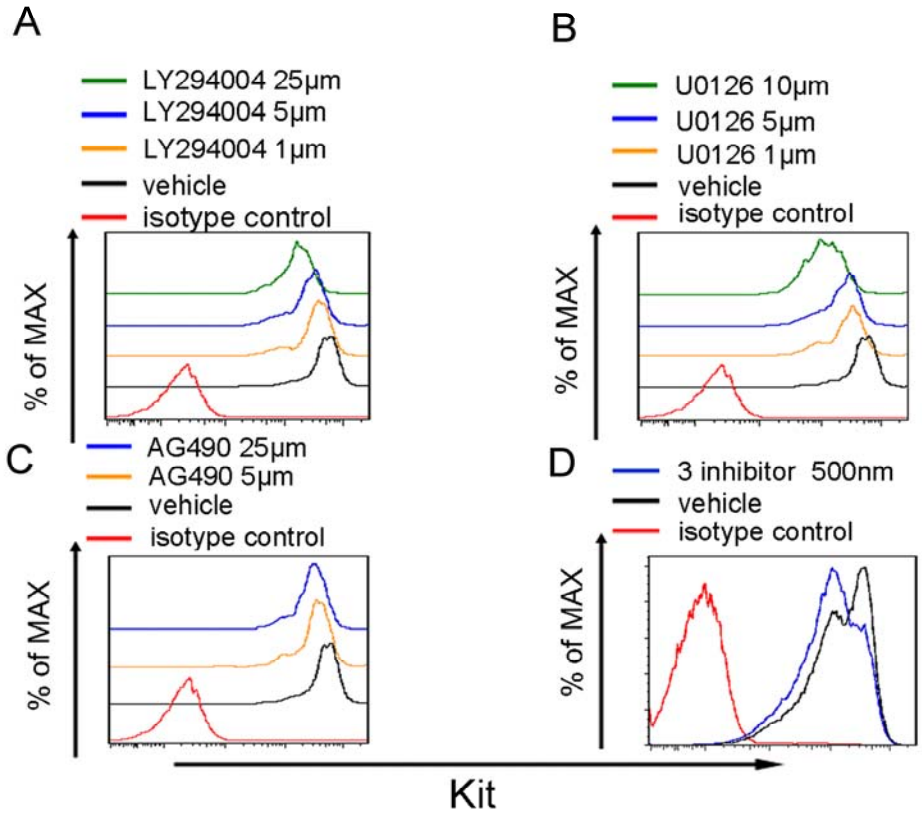

Figure 29. Effect of small molecule inhibition of AKT, ERK and STAT3 activation on surface Kit expression.
Treatment of EML cells with PI3K inhibitor LY294004 (A), MEK inhibitor U0126 (B)or low dose (500 nm) of LY294004, U0126 and STAT3 inhibitor AG490(D) but not STAT3 inhibitor AG490 alone(C) led to downregulation of Kit expression on cell surface.

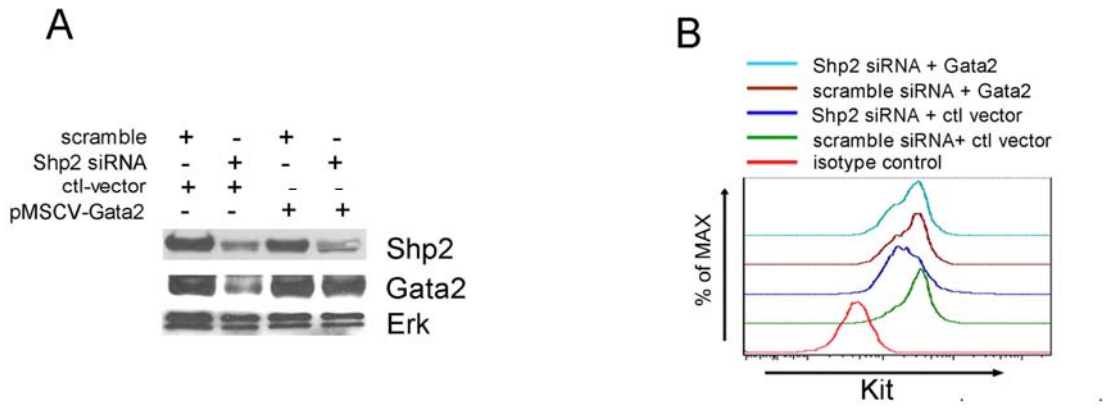

Figure 30. Gata2 acts downstream of Shp2 in the regulation of Kit expression.
(A) Shp2 knockdown in EML cells suppressed Gata2 protein expression. Tubulin was used as loading control.
(B) Ectopic expression of Gata2 rescued Kit downregulation in Shp2 knockdown EML cells.

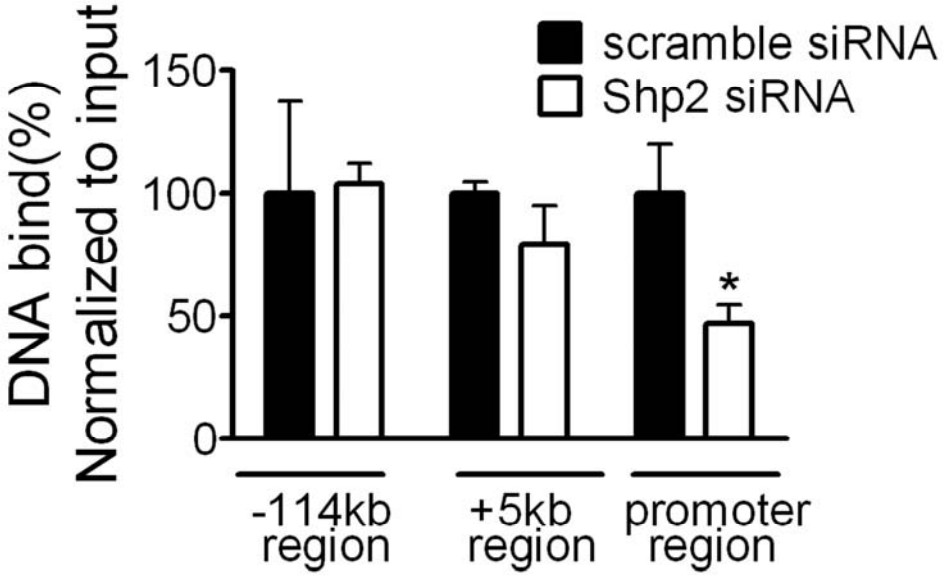

Figure 31. Quantitative analysis of Gata2 binding to the -114 kb, +5 kb and promoter region of *Kit* gene by the CHIP assay.
Binding of Gata2 to *Kit* promoter but not -114kb or +5kb region was significantly reduced in Shp2 knockdown EML cells. (* $p < 0.05$, error bars are S.D.)

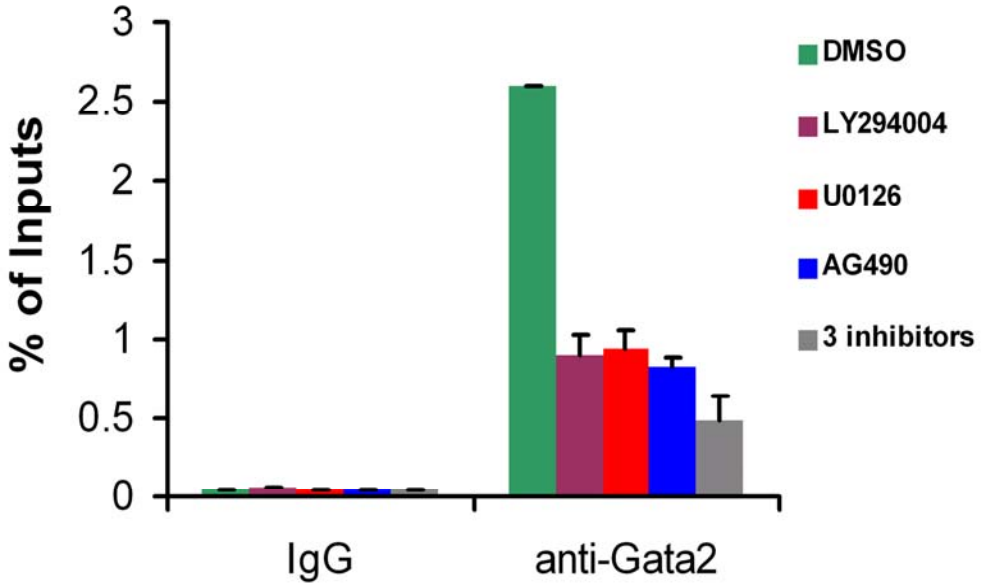

Figure 32. Effect of specific signaling inhibitors on Gata2 occupancy of *Kit* promoter.
CHIP assay demonstrated the binding of Gata2 to *Kit* promoter was significantly reduced in single (25 μM LY294002, 10 μM U0126 and 25 μM AG490) or combined (500nm) chemical inhibitors treated EML cells

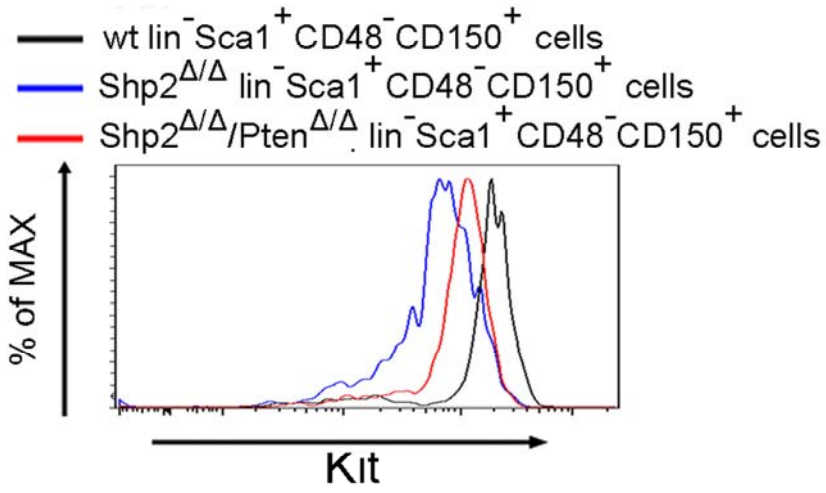

Figure 33. combined deletion of Shp2 and Pten partially restore the Kit expression in lineage$^-$ cells and SLAM marker enriched HSCs.
Kit staining profile of wt lin$^-$Sca1$^+$CD48$^-$CD150$^+$ cells, Shp2 $^{\Delta/\Delta}$ lin$^-$Sca1$^+$CD48$^-$CD150$^+$ cells, Shp2 $^{\Delta/\Delta}$ /Pten $^{\Delta/\Delta}$ lin$^-$Sca1$^+$CD48$^-$CD150$^+$ cells. At least 3 animals were examined in each group. Representative FACS plot was shown.

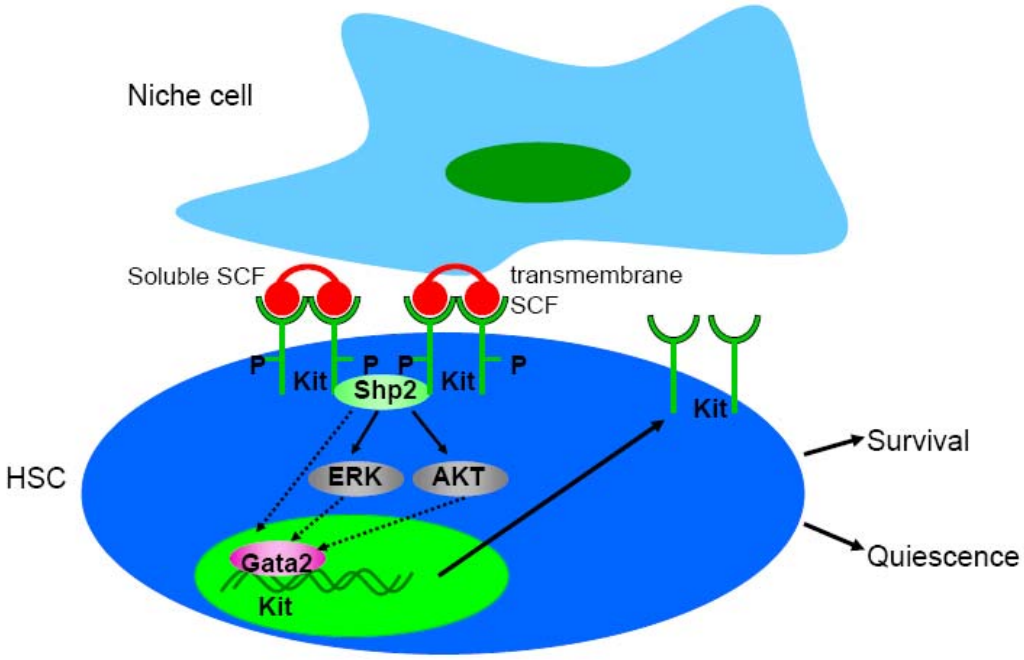

Figure 34. Kit-Shp2-Kit signaling axis in hematopoietic stem and progenitor cells. Cytoplasmic tyrosine phosphatase Shp2 acts downstream of ligand-activated receptor tyrosine kinase, such as SCF-evoked Kit, and promotes AKT and ERK signaling, resulting in upregulation of *Kit* gene expression via transcriptional factors including Gata2.

Acknowledgement

Chapter three, in part, is a reprint of the paper Zhu, H.H., Ji, K., Alderson, N., He, Z., Li, S., Liu, W., Zhang, D.E., Li, L., and Feng, G.S. (2011). Kit-Shp2-Kit signaling acts to maintain a functional hematopoietic stem and progenitor cell pool. Blood *117*, 5350-5361. Figure 34 is adapted and modified from paper Zhu, H.H. and Feng, G.S. (2011) The dynamic interplay between a PTK (Kit) and PTP (Shp2) in hematopoietic stem and progenitor cells. *Cell Cycle.* 10, 2241-2242.

The author of this dissertation was the major investigator and first author of both papers.

IV. Discussion

Previous work has shown that the cytoplasmic enzyme Shp2 physically associates with SCF-activated Kit via docking of the two SH2 domains on autophosphorylated tyrosyl residues in hematopoietic cells (Pati et al., 2010; Tauchi et al., 1994), suggesting its participation in signal relay immediately downstream of Kit. But the biological significance of Kit-Shp2 signaling is unclear. Here we present data suggesting a novel signaling mechanism in which Shp2 regulates HSC/progenitor activities at least in part via mediating signals controlling Kit gene expression.

1. A Kit-Shp2-*Kit* regulatory axis in HSCs and progenitors

It is interesting to note that depletion of LSK cells in $Shp2^{\Delta/\Delta}$ bone marrow was accompanied by selective loss of Kit^+, but not $Sca1^+$, a subpopulation in the lin^- pool. We further observed defective Kit expression in SLAM marker-labelled $lin^-Sca1^+CD150^+CD48^-$ HSCs following Shp2 ablation. This downregulation is an acute effect and corresponds well with the kinetics of Shp2 deletion. Loss of Kit expression likely contributes to the survival defect in $Shp2^{\Delta/\Delta}$ HSCs/progenitors, as the SCF/Kit pathway is required to prevent HSC apoptosis (Domen and Weissman, 2000; Keller, 1995; Li and Johnson, 1994). The aberrant cell cycle entry of $Shp2^{\Delta/\Delta}$ HSCs also phenocopied the disturbed HSC quiescence in viable *White Spotting 41* ($Kit^{W41/W41}$) mice, as reported recently (Thoren et al., 2008). Therefore, this study elucidates a novel molecular signaling mechanism involving a circuit of Kit-Shp2-*Kit* in adult

HSCs, in which the receptor tyrosine kinase Kit controls its own gene expression via signaling through Shp2, an intracellular tyrosine phosphatase (Figure 34).

The biochemical mechanisms underlying functions of Shp2, a widely expressed enzyme, has been extensively investigated recently, leading to identification of multiple substrates in its regulation of Erk, PI3K and STATs pathways (Chan and Feng, 2007; Neel et al., 2003). Molecular data shown here suggest that Shp2 mediates signaling through these pathways to transcription factors such as Gata2 in control of *Kit* mRNA transcription. It is likely that other transcription factors such as Egr1 and Ets1 may also be involved in Shp2 regulation of *Kit* expression (our unpublished data).

Kit expression is regulated by internalization and ubiquitination, extracellular domain cleavage, transcriptional activation or repression by cell specific transcriptional factors, and alteration of mRNA translation by microRNAs. Consistent with a lot of other growth factor receptors, Kit is rapidly internalized after SCF binding (Gommerman et al., 1997; Miyazawa et al., 1994; Yee et al., 1993). SCF induced internalization and trafficking of Kit happens rapidly, which is ligand concentration dependent and requires intact Kit kinase activity (Yee et al., 1994; Zandstra et al., 1999). PI3K and Src family kinases both play positive roles in this process (Broudy et al., 1999; Yee et al., 1994). Ubiquitin-assisted degradation proceeds after the surface protein internalization to avoid obsessive signaling activation (Baghestanian et al., 1996; Chen et al., 1989; Shimizu et al., 1996; Wells et al., 1990). On the other hand, there is evidence suggesting that internalization and trafficking of cell surface receptors is indeed a mechanism to activate the full spectrum

of downstream signal transduction pathways (Vieira et al., 1996; Xiang et al., 2007). Another identified regulatory pathway of surface Kit expression is proteolytic cleavage of its extracellular domain. G-CSF administration to mice or human subjects leads to mobilization of hematopoietic progenitors and simultaneous down-regulation of cell surface Kit intensity. Detailed analysis of bone marrow extracellular fluids from G-CSF treated animals reveals that different proteases, including neutrophil elastase, cathepsin G, proteinase-3 and MMP-9, are capable of shedding Kit *in vitro* (Levesque et al., 2003). Large efforts have been made to identify *cis* acting elements and *trans* regulatory factors in *Kit* gene transcriptions. Scl occupies the *Kit* promoter region and forms a transcriptional complex with Sp1, E2A, Lim-only 2 (LMO2), Gata1/Gata2 in hematopoietic cells (Lecuyer et al., 2002). Myb and Ets2 were also proposed to be candidate regulators of c-kit expression in blood cells (Ratajczak et al., 1998). In addition, Plzf serves as a Kit transcription repressor in both hematopoietic progenitors and germ cells (Filipponi et al., 2007; Spinello et al., 2009). Ap2 was identified to be a specific transcriptional activator for Kit in melanocytes, while Sp1 and Gata2 were found to regulate Kit transcription in mast cells (Huang et al., 1998; Maeda et al., 2010). MicroRNAs are small non-coding RNAs that operate on the gene translational level and cause degradation of messenger RNA. Kit is found to be the target of MicroRNA-221 and MicroRNA-222 in erythropoiesis (Felli et al., 2005). Liu et al. demonstrated recently, by elegant experimental results, that activated Kit in leukemic cells downregulates MicroRNA-29b, leading to enhanced translation of its target Sp1 which in turn forms a complex with NFκB/ histone deacetylase 3 (HDAC3)

that suppress transcription of MicroRNA-29b. Upregulated Sp1/ NFκB then occupy *Kit* promoter and trans-activate the *Kit* gene (Liu et al., 2010). Our model of the Kit-Shp2-*Kit* positive feedback loop in HSCs shares similarities with Sp1/NFκB/HDAC3/MicroRNA29b signaling in leukemogenesis, indicating a common signal amplification mechanism in cells that require constant Kit activation for the maintenance of self-renewal, survival or proliferation.

2. Conserved requirement of Shp2 in embryonic emergence, neonatal expansion and adult maintenance of HSCs.

While much has been learned about transcription factors required for HSC commitment, self-renewal and differentiation, relatively little is known about intracellular signaling pathways in control of HSC activities. Our previous studies suggested a critical role of Shp2 in HSC fate specification/differentiation during mouse embryogenesis, using mouse ES cells homozygous for exon 3 deletion (Chan et al., 2003; Qu et al., 2001; Qu et al., 1998; Qu et al., 1997b). The heterozygous mice (exon $3^{+/-}$) did not display hematopoietic defects, but compromised reconstitution capacity of LSK cells was seen under stress condition following transplantation (Chan et al., 2006), which could be due to a developmental problem arisen during embryonic hematopoiesis. In this study, we developed a new mutant mouse line with *Ptpn11/Shp2* deleted in the hematopoietic compartment in adults, and uncovered a functional requirement for Shp2 in maintaining a functional HSC/progenitor pool in adult mammals. Together, these results suggest a concerted mechanism for control of

embryonic and adult hematopoiesis by Shp2 in mammals. This is in contrast to the distinct requirement for Runx1 in the emergence and maintenance of HSCs in embryos and adults, respectively (Chen et al., 2009; Growney et al., 2005; Ichikawa et al., 2004). Deletion of Shp2 not only resulted in reduced survival of HSCs but also led to impaired differentiation capacity of surviving HSCs/progenitors, as indicated by the CFU assay *in vitro*. Combined defects in survival and function of HSCs clearly contribute to the severe defects observed in multiple lineages of peripheral blood cells in poly-I:C treated *fl/fl:cre*$^{+}$ mice.

Several mechanisms are likely involved in Shp2 function in maintaining the adult HSC pool. Increased apoptosis rate is evidently responsible for the reduced number of HSCs in Shp2-deficient bone marrow. Impaired survival capacity may force quiescent HSCs into the cell cycle, as revealed by decrease of *Shp2*$^{\Delta/\Delta}$ LSK cells in G_0 accompanied by increase in the S phase. Thus, the enhanced cell cycle entry of *Shp2*$^{\Delta/\Delta}$ LSK cells is likely a compensatory mechanism, and this observation may not be contradictory to previous data suggesting a positive role of Shp2 in mitogenic signaling (Lai et al., 2004; Neel et al., 2003).

Interestingly, Chan et al, in a recent study, used a similar conditional gene deletion strategy, by breeding the commonly used Mx1-Cre transgenic mouse strain with mice containing loxP sites flanking exon 11 at the PTPN11/Shp2 locus (Chan et al., 2011). They observed similar defective phenotypes, including severely lower peripheral blood cell counts, reduced cellularity of multiple hematopoietic organs, and loss of phenotypic as well as functional HSCs. Of note, Chan et al. initiated the

inducible Shp2 deletion in newborn mice, while we did it 4 weeks after birth. It was shown previously that murine HSCs undergo active cycling within 3 weeks after birth and switch to an adult quiescent state after 4 weeks of age (Bowie et al., 2006). Taken together, Shp2/Ptpn11 has a conserved role in hematopoiesis, from the early stage of embryonic HSC emergence, to fetal and neonatal HSC expansion stage, and adult HSC maintenance.

3. Shp2 governs HSCs homing and engraftment efficiency.

Another interesting finding is the discrepant engraftment efficiency caused by different Shp2 gene deletion time, before or after transplantation. This inconsistency is hypothesized to result from a homing defect in Shp2 deficient HSCs. We presented here direct *in vivo* evidence supporting this hypothesis by monitoring hematopoietic progenitors trafficking shortly after transplantation. We also showed that Shp2 enhances HSC homing partially by regulating the chemotactic response to SDF-1α. Consistently, Shp2 has been shown to regulate cell motility by modulating FAK signaling (Manes et al., 1999; Oh et al., 1999; Yu et al., 1998). Although spontaneous HSC mobilization from bone marrow to peripheral blood or spleen is not increased in poly-I:C treated *fl/fl:cre*$^{+}$ mice, it is interesting to explore whether Shp2 deficient HSCs respond defectively in cytokine induced mobilization *in vivo*. Previous studies suggested a role of the SCF-Kit activation in hematopoietic progenitors and HSC mobilization. HSCs from *W/W* mice mobilize poorly in response to G-CSF (Cynshi et al., 1991). In addition, mobilization by anti-very late antigen 4 (VLA4) or anti- anti-

vascular cell adhesion molecule 1(VCAM1) does not require functional receptors of GM-CSF, IL-7, or IL-3alpha but are dependent on Kit, because *W/W* mice responded minimally to either antibody treatment (Papayannopoulou et al., 1998). By regulating surface Kit expression, Shp2 may also contributes to HSCs mobilization through SCF-Kit signal pathways. Therefore, it is very likely that Shp2 coordinates multiple aspects of the homing process, including migration back to bone marrow, extravasation to the hematopoietic cord and establishment of the HSC/Niche interactions.

4. Distinct effects of Shp2 in the regulation of embryonic and adult stem cells self-renewal

The Shp2 function in HSC self-renewal and maintenance is also conserved in neural stem cells (NSCs) (Ke et al., 2007). Conditional deletion of Shp2 in the brain driven by *nestin-cre* almost abolished the self-renewing expansion of NSCs *in vitro*. Thus, this signaling molecule plays a critical role in modulating pathways required for sustaining adult stem cells, such as NSCs and HSCs. Interestingly, the role of Shp2 in supporting these adult stem cells is opposite to its action in ESCs. We and others have found that homozygous Shp2 deletion or disruption of Shp2 signaling severely suppressed mouse ESC differentiation, accompanied by increased self-renewal (Burdon et al., 1999; Chan et al., 2003; Qu et al., 1997b; Wu et al., 2009). A similar function of Shp2 in promoting differentiation has been found in human ESCs, as Shp2 knockdown also suppressed differentiation with improved self-renewal (Wu et al., 2009). Therefore, distinct intracellular signaling mechanisms exist in regulating self-

renewal and maintenance of embryonic or adult stem cells, which are yet to be elucidated.

5. Activating mutants of *PTPN11* are leukemogenic, but wild-type Shp2 is indispensable in normal hematopoiesis.

The majority of leukemia-associated *PTPN11* mutations disrupt the auto-inhibitory interactions between its N-terminal SH2 and the phosphatase domain, resulting in elevated catalytic activity. Expression of these mutants in bone marrow cells induced hyperproliferation and hypersensitivity of myeloid progenitors to GM-CSF and interleukin-3. Transplantation of $Shp2^{E76K}$- or $Shp2^{D61Y}$-expressing bone marrow cells elicited fatal myeloid proliferative disease in recipient mice (Mohi et al., 2005), and knock-in mice bearing an inducible $Shp2^{D61Y}$ allele displayed a similar phenotype (Chan et al., 2009). It has been recently shown that LSK cells, but not lineage$^-$committed progenitors from $Shp2^{D61G}$ knock-in mice, are able to initiate MPD in recipient mice (Xu et al., 2010). Although PTPN11/Shp2 is not frequently mutated in neither pediatric nor adult leukemia, over-expression of Shp2 has been detected in different types of adult human leukemia including AML, ALL and CML. Despite the leukemogenic property of activating *PTPN11* mutations, we show in this study that Shp2 plays an essential role in maintaining the normal function of HSCs. Therefore, precise regulation of Shp2 level is pivotal for the hematopoietic homeostasis. Effective treatment of leukemia requires specific targeting of a drug to the mutant but not the wild-type Shp2 enzyme, to minimize the side effect of bone marrow suppression.

6. Future direction.

As Albert Einstein said, "as our circle of knowledge expands, so does the circumference of darkness surrounding it." We have focused this dissertation work on the function of Shp2 in adult HSC maintenance and have uncovered an essential role of Shp2 in this type of stem cells. This observation opens up a series of very interesting unanswered questions. In *Shp2*$^{flox/flox}$*: Mx1-cre* mice, Shp2 was deleted equally among different hematopoietic cell types, which prevented us from examining its function in a specific lineage compartment. Additionally, We have found a differentiation bias from HSCs to CMPs and CLPs in *Shp2*$^{flox/flox}$*: Mx1-cre* mice, which led to the question of how Shp2 governs the cell fate decision during hematopoiesis. Both of the above questions can be studied in lineage-specific knockout models by breeding *Shp2*$^{flox/flox}$ animals with various Cre transgenic mice such as lysM-cre mice that express Cre recombinase in myeloid cells.

We identified a positive feedback loop consisting of Kit-Shp2-*Kit* in HSCs and progenitors that sustain stem cell survival and quiescence. It has been evidently demonstrated before that SCF-Kit signals are required in normal hematopoiesis, melanogenesis, gametogenesis, functions of mast cells and interstitial cells of Cajal, and different malignancies. In leukemias, *Kit* is expressed by a majority of myeloblasts from AML patients (Ikeda et al., 1991). *Kit* mutations were reported in 17% of all AML cases, with a high incidence of 52% in CBF AML subtype (Boissel et al., 2006; Corbacioglu et al., 2006; Goemans et al., 2005; Paschka et al., 2006).

Elevated expression of Kit is also considered a poor prognostic factor for AML disease progression and survival in adults (Schnittger et al., 2006; Shimada et al., 2006). It will be interesting to determine whether a similar Kit-Shp2-*Kit* signaling loop operates in a broader range of cell types and is implicated in tumorigenesis.

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Chapter four, in part, is a reprint of the paper Zhu, H.H., Ji, K., Alderson, N., He, Z., Li, S., Liu, W., Zhang, D.E., Li, L., and Feng, G.S. (2011). Kit-Shp2-Kit signaling acts to maintain a functional hematopoietic stem and progenitor cell pool. Blood *117*, 5350-5361.

The author of this dissertation was the major investigator and first author of the paper.

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