REQUIREMENT FOR FIBROBLAST GROWTH FACTOR RECEPTOR 1 IN HEMATOPOIETIC STEM CELLS UNDER STRESS BUT NOT DURING HOMEOSTASIS

BY

Jason T. Ross

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Co-Chair, Linheng Li, Ph.D.

Committee Members

Co-Chair, Jay L. Vivian, Ph.D.

Joan Conaway, Ph.D.

Patrick E. Fields, Ph.D.

Michael J. Werle, Ph.D.

Leanne M. Wiedemann, Ph.D.

Date defended: 6/4/10

The Dissertation Committee for Jason T. Ross certifies that
this is the approved version of the following dissertation:

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Co-Chair, Linheng Li, Ph.D.

Co-Chair, Jay L. Vivian, Ph.D.

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Abstract

Fibroblast growth factor (FGF) signaling promotes hematopoietic stem cell (HSC) expansion *in vitro*; however, its *in vivo* function remains unknown. Conditional deletion of FGFR1, predominantly expressed in HSCs, did not affect homeostatic hematopoiesis, but led to defects in mobilization of HSCs in response to induced bone marrow damage. Mechanistically, loss of FGFR1 caused defective expression of CXCR4, a receptor for the chemoattractant SDF-1, in HSCs, as well as impaired migration in response to SDF-1 by *in vitro* assay. This is consistent with failure of HSC mobilization by disruption of SDF-1 signaling with AMD3100. Additionally, defects in proliferation of HSCs prior to bone marrow egress, and subsequent extramedullary expansion of HSCs within the spleen was observed *in vivo*. In total, this dissertation has characterized a role for FGFR1 in the mobilization of HSCs *in vivo* and may represent a rarely engaged signaling program that promotes stress reponse and hematopoietic recovery.

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Abbreviations

AER	apical ectodermal ridge
AGM	aorta/gonad/mesonephros
AML	acute myeloid leukemia
Ang-1	angiopoietin-1
BFU-E	burst forming unit-erythroid
BM	bone marrow
BMP	bone morphogenetic protein
BMPR1A	bone morphogenic receptor type 1A
bp	base pair
BW	body weight
°C	degrees centigrade
CAR	CXCL12 abundant reticular
CD	cluster of differentiation
CFU	colony forming unit
CFU-G	CFU-granulocyte
CFU-GM	CFU-granulocyte, macrophage
CXCL12	chemokine (C-X-C) motif ligand 12
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DN	double negative
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
E	embryonic (day)
EDTA	diaminoethanetetra-acetic acid
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMS	8p11 myeloproliferative syndrome
ES	embryonic stem

FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR1	fibroblast growth factor receptor 1
FOXO	Forkhead box transcription factor
FZ	frizzled
g	gram
GAP	GTPase activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GEMM	granulocyte, erythroid, macrophage, megakaryocyte
GSK3β	glycogen synthase kinase 3β
hh	hedgehog
HPRT	hypoxanthine phosphoribosyl transferase
HRP	horseradish peroxidase
HS	horse serum
HSC	hematopoietic stem cell
HSPG	heparan sulfate proteoglycan
IACUC	Institutional Animal Care and Use Committee
IFN	interferon
Ig	immunoglobin
IGF	insulin growth factor
IL	interleukin
kb	kilobase
KO	knockout
L	liter
LK	Lineage$^-$, c-Kit$^+$
LSK	Lineage$^-$, Sca-1$^+$, c-Kit$^+$
LT-HSC	long term-hematopoietic stem cell
M	molar
MAML1	mastermind-like protein
MCAM	melanoma-associated cell adhesion molecule

MMP-9	matrix metalloproteinase-9
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mTOR	mammalian target of rapamycin
NIC	Notch intracellular domain
PB	peripheral blood
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFβ	platelet-derived growth factorβ
PH	Plekstrin homology
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PPR	PTH/PTHrP receptor
Ptc	Patched
PTEN	phosphatase and tensin homologue
PTH	parathyroid hormone
PTHrP	PTH related protein
RBC	red blood cell
RBM	Raf binding motif
RNA	ribonucleic acid
RPM	revolutions per minute
RTK	receptor tyrosine kinase
SCF	stem cell factor
SCFR	stem cell factor receptor
SD	standard deviation
SE	standard error
SDF-1	stromal cell-derived factor-1
Sec	second
Shh	Sonic hedgehog

Smo	Smoothened
SOP	standard operating procedure
SPL	spleen
Spred	Sprouty-related EVH1 domain protein
Spry	Sprouty
ST-HSC	short term-hematopoietic stem cell
TGF-β	transforming growth factor-β
TPO	thrombopoietin
xg	times gravity
WBC	white blood cell
WT	wildtype
μM	micromolar
μg	microgram
5FU	5-Fluorouracil

Chapter 1

Introduction

Hematopoietic stem cells (HSCs) were first characterized more than 40 years ago by studies that led to a proposal for defining the key features of all stem cells: self-renewal capacity and the ability to generate differentiated progeny (Till, McCulloch et al. 1964). It was 20 years until HSCs in the mouse were definitively identified and isolated in the Lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cell population of the bone marrow (BM), which refers to their lack of expression of various antigens found on mature cell types and their positive expression of two cell-surface proteins, Sca-1 and c-kit;(Spangrude, Heimfeld et al. 1988; Ikuta and Weissman 1992) since that time HSCs have been widely studied.

The process of hematopoiesis in adult organisms is ongoing and involves the differentiation of the most primitive, long-term HSCs (LT-HSCs) into more restricted, short-term stem cells (ST-HSCs). These differentiate into less potent progenitors and ultimately into all of the mature, committed cell types of the blood, at least in part through coordinated expression of cell-specific genes (Shivdasani and Orkin 1996; Cantor and Orkin 2001; Shizuru, Negrin et al. 2005). These phenotypically different cells carry out various functions such as the transport of oxygen via red blood cells (RBCs) and the establishment and maintenance of innate and acquired immunity via white blood cells (WBCs), which include neutrophils, monocytes, macrophages, and lymphocytes (Cantor and Orkin 2001).

1.1 Embryonic Hematopoiesis

In mammalian species such as the mouse and human HSCs are predominantly found within the BM throughout adult life. HSCs associate with various niche components of the BM that regulate stem cell activities (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003;

Kiel, Yilmaz et al. 2005; Sugiyama, Kohara et al. 2006). In contrast, during embryonic development HSCs are a dynamic population that expands and migrates through multiple tissues. Prior to the formation of true stem cells, hematopoietic progenitors are spawned in blood islands of the yolk sac around embryonic day 7.5 in the mouse (E7.5). These progenitors are responsible for primitive hematopoiesis, which consists primarily of erythroid development (Palis and Yoder 2001).

HSCs first arise around E9 within the yolk sac and later at E10.5-11.5 within the intraembryonic aorta/gonad/mesonephros (AGM) region (Wood, May et al. 1997; Yoder, Hiatt et al. 1997; Delassus, Titley et al. 1999). At E11.5-12.5 HSCs generated in these tissues migrate through the circulation to the liver, which is the major site of definitive hematopoiesis in the embryo where all of the differentiated lineages are formed (Baron 2003). In order to sustain the rapidly growing fetal blood supply, HSC numbers increase in the liver until E15.5-16.5. Subsequently, HSCs mobilize from the liver to the spleen, which serves as a minor hematopoietic organ, and later to the BM near the time of birth (Morrison, Hemmati et al. 1995). The spatio-temporal shifts in embryonic hematopoiesis are not discrete as might be expected with large numbers of HSCs migrating in waves from one organ to the next. Rather, low numbers of HSCs circulate constitutively from E12-17, facilitating a steady colonization of the various organs and tissues as new niches become viable and available (Christensen, Wright et al. 2004).

1.2 HSC Niches

Ray Schofield first proposed the hypothesis that a physically limited microenvironment or niche exists *in vivo* where stem cells normally reside and stably maintain self-renewal potential. After cell division, one daughter cell remains in the niche (self-renewal)

and the other is forced out of the niche, where it loses its capacity to self-renew and commits to differentiation (Schofield 1978). Evidence supporting this hypothesis came from *in vitro* studies of HSC-supporting stromal cell lines and from *in vivo* identification of germline stem cell-supporting niche cells in the *Drosophila* ovary and testis (Wineman, Nishikawa et al. 1993; Wineman, Moore et al. 1996; Lin and Spradling 1997; Moore, Ema et al. 1997; Xie and Spradling 2000; Kiger, Jones et al. 2001; Tulina and Matunis 2001; Hackney, Charbord et al. 2002).

1.2.1 Endosteal Niche

In 2003 two separate reports put forth evidence supporting what came to be known as the HSC endosteal niche. Conditional deletion of the bone morphogenic protein receptor type 1A (BMPR1A), or constitutive activation of the parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor (PPR) each resulted in expansion of osteoblastic cells within BM capable of supporting expansion of HSCs. Specifically, a sub-population of bone-lining, osteoblast cells that express N-cadherin appeared to function as niche cells (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003). Furthermore, studies into the role of osteopontin, a glycoprotein synthesized by osteoblasts, found it to be a key regulator of HSC localization in BM and inhibitor of HSC proliferation (Nilsson, Johnston et al. 2005; Stier, Ko et al. 2005). Additionally, investigation into the HSC-expressed receptor tyrosine kinase Tie2 demonstrated it as a vital niche signal. Interaction of Tie2 with its ligand angiopoietin-1 (Ang-1), expressed by osteoblasts, promoted HSC quiescence and adhesion to bone (Arai, Hirao et al. 2004).

1.2.2 Vascular Niche

The HSC niche concept was recently adjusted to include the vascular niche. In particular, Kiel, et al, in their endeavor to find an alternative strategy for HSC identification, found that HSCs closely associate with endothelial cells of the vasculature in the BM and spleen, suggesting they could act as niche cells (Kiel, Yilmaz et al. 2005; Kopp, Avecilla et al. 2005). Furthermore, it has been shown that matrix metalloproteinase-9 (MMP9) is upregulated within BM after stress, leading to release growth factors that stimulate recruitment of HSCs to the vascular niche, believed to be more permissive for stem cell expansion and stress recovery (Heissig, Hattori et al. 2002). Yet in spite of these findings, it remained unclear if vascular endothelium truly served as a specific stem cell niche, or instead functions in a more general capacity in delivery of oxygen and nutrients, vital to all cells. The work of Butler, et al offered some resolution to this question by inhibiting the angiocrine function of endothelial cells while preserving their perfusion capacity. Under such conditions, endothelial cells were unable to maintain the HSC pool, though when angiocrine signaling was intact, endothelial cells were capable of supporting expansion of HSCs *in vitro* and *in vivo* (Butler, Nolan et al. 2010).

1.2.3 Other Niche Components

Other stromal cells of the BM microenvironment have been put forth as potential niche cells for HSCs. One example is a population of reticular cells that are scattered throughout the BM and express high levels of the chemokine CXCL12 (also known as SDF-1). HSCs, whether close to the bone surface or to blood vessels, were found to be in close contact with these CXCL12 abundant reticular (CAR) cells. Additionally, data indicated that CAR cells may play an important part in maintaining the quiescent HSC pool via

signaling of CXCL12 through its receptor CXCR4, which is expressed on HSCs (Sugiyama, Kohara et al. 2006).

Alternatively, investigation into a distinct population of stromal progenitors indicated they have niche capability. Study of this population, characterized by their sub-endothelial localization and expression of melanoma-associated cell adhesion molecule (MCAM, also known as CD146) showed that these stromal progenitors had the ability to form hematopoietic microenvironments in extramedullary sites upon transplantation and were capable of supporting hematopoiesis. Also, these cells were found to express HSC-supporting factors such as Ang-1 and SDF-1 (Sacchetti, Funari et al. 2007).

Non-hematopoietic, stromal cell types are not the only potential niche components. Recent work has also found that megakaryocytes may serve a niche function. A radioresistant population of megakaryocytes was identified within BM that could survive marrow-ablating levels of irradiation. These megakaryocytes were found to migrate from their normal parasinusoidal location and associate with the bone surface. There, the megakaryocytes secreted growth factors, including basic fibroblast growth factor (bFGF) and platelet-derived growth factor β (PDGFβ), which stimulated expansion of osteoblasts, which in turn supported engraftment of donor HSCs (Dominici, Rasini et al. 2009).

1.3 Extrinsic Regulation of HSCs

1.3.1 Introduction

Identification of the stem cell niche in this system has highlighted the various roles of external signals that regulate HSCs. These include cytokines and growth factors such as thrombopoietin, stem cell factor, and the various interleukins, and well-studied developmental signaling pathways such as the Wnt, Notch, and BMP pathways (Figure 1.1). Extensive amount of research over the years has shown the importance of these various signals in regulating HSC activities that include self-renewal, proliferation, migration, differentiation, and more. The inability to maintain HSCs for long periods or greatly expand them without loss of stem cell identity *in vitro* highlights the continued need for research into their *in vivo* regulation.

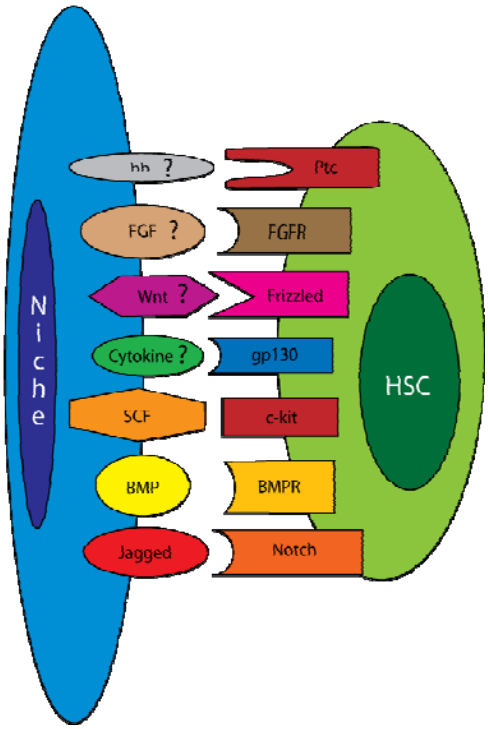

Figure 1.1. HSC/Niche Signals. Depicted here are some of the various extrinsic signals originating from the niche (or more generally, the bone marrow microenvironment) that regulate the hematopoietic stem cell (HSC). While all of the cell surface receptors for these signals have been found on HSCs, and it is known that some of the ligands like SCF, BMP, and Jagged are expressed by osteoblastic cells of the endosteal bone niche, the origin of the other signaling proteins is less certain. Some, such as FGF, may be expressed by endothelial cells of the vascular niche, or as the case may be with hh and Wnt, by other cells of the bone marrow stroma. Furthermore, ligands may be secreted into the extracellular space/matrix and not expressed directly on niche cells.

1.3.2 Cytokines and Growth Factors

Historically cytokines and growth factors have been the primary targets of study as regulators of HSCs. Steel (also known as stem cell factor or SCF) is a growth factor that interacts with its receptor, c-kit, and is important for proliferation and differentiation of

HSCs, specifically myeloerythroid lineages (Ikuta and Weissman 1992; Broudy 1997). The cytokine thrombopoietin (TPO) is key in regulating megakaryocyte and platelet production, though later work pointed to a role for TPO in stimulating proliferation of HSCs by interacting with SCF (Ikuta and Weissman 1992; Ku, Yonemura et al. 1996). Other cytokines that favor myeloerythroid fates such as interleukins (IL) 3, 6, and 11 similarly affect proliferation when acting in concert, such as TPO and IL-3 (Ikebuchi, Wong et al. 1987; Ikebuchi, Clark et al. 1988; Musashi, Yang et al. 1991; Tsuji, Zsebo et al. 1991; Ku, Yonemura et al. 1996). The consequences of inactivating the TPO receptor (c-Mpl) or TPO itself support its role in HSC self-renewal (Solar, Kerr et al. 1998; Fox, Priestley et al. 2002). Additionally it is known that gp130, a shared cell-surface receptor for cytokines that include IL-3 and IL-11, functions in promoting self-renewal as well as driving differentiation of HSCs, establishing it as an important regulator (Audet, Miller et al. 2001). Whether these various factors are considered individually or in concert, it becomes apparent that though many can stimulate proliferation of HSCs, they tend to do so at the cost of a loss of stem cell identity. This suggests that additional signals such as developmental signaling pathways play important roles in regulating HSC function.

1.3.3 Wnt Signaling

The Wnt pathway is a well defined signaling cascade first identified in *Drosophila* and later in mice and humans. Wnt signaling acts during embryonic development as well as in the adult, directing events such as cell proliferation and differentiation. This pathway is activated by binding of a Wnt protein to its cell-surface receptor complex, frizzled (FZ) and LRP5/6. In the absence of Wnt ligand, β-catenin is continually targeted to the proteosome for degradation. A Wnt ligand-bound receptor blocks the degradation of β-catenin and al-

lows it to accumulate and translocate into the nucleus, where it binds LEF/TCF transcription factors (Figure 1.2) to activate target genes (Reya and Clevers 2005; Staal and Clevers 2005).

In addition to its roles during development, Wnt signaling has been linked to hematopoiesis and found to affect the self-renewal of HSCs. Austin, et al. were the first to establish that several Wnt ligands and FZ are expressed in hematopoietic tissues, and went on to show activation of Wnt signaling increased HSC proliferation with increased levels of both myeloid and lymphoid lineages (Austin, Solar et al. 1997). Reya, et al. went on to show that expression of β-catenin maintains HSCs in an immature state both phenotypically and functionally and allows this stem cell pool to expand. By using Wnt inhibitors, they also demonstrated that HSCs require Wnt signaling to undergo self-renewing cell divisions (Reya, Duncan et al. 2003). Additional work to isolate active Wnt proteins for the first time resulted in the demonstration that purified Wnt3a can induce the self-renewal of HSCs *in vitro* (Willert, Brown et al. 2003).

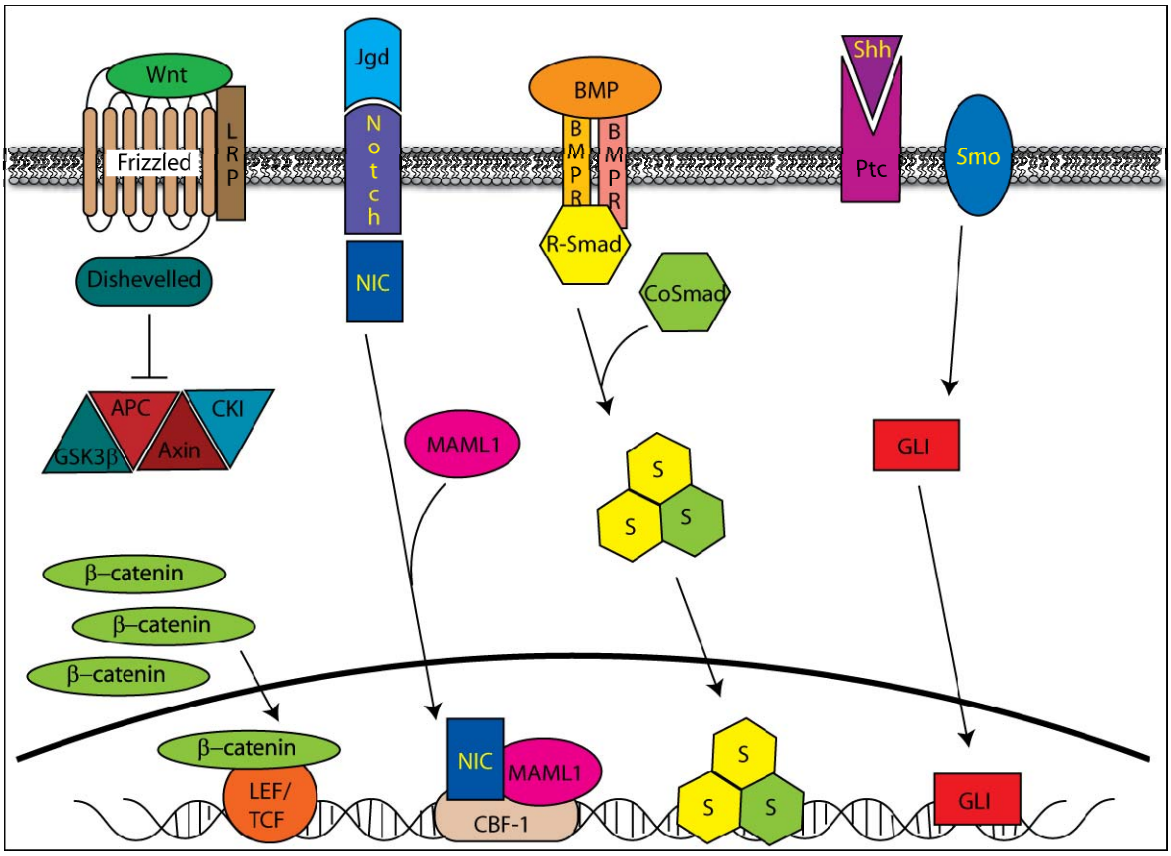

Figure 1.2. Active Developmental Signaling Pathways.
Depicted here are the various developmental signaling pathways discussed in this chapter, from left to right: Wnt, Notch, Bone morphogenic protein (BMP), and Sonic hedgehog (Shh), each shown in a state of active signaling. Binding of a Wnt protein to the LRP/Frizzled (FZ) receptor complex inhibits the phosphorylation of β-catenin by glycogen synthase kinase 3β (GSK3β) via DVL (mammalian homolog of disheveled). Accumulated β-catenin translocates into the nucleus and interacts with LEF/TCF transcription factors (TFs) to affect transcription of target genes. Binding of Jagged (Jgd) to the Notch receptor results in proteolytic cleavage of the intracellular domain of Notch (NIC), which translocates into the nucleus along with mastermind-like protein (MAML1) to bind the CBF-1 TF and regulate target genes. Binding of BMP to its receptor complex results in phosphorylaton of regulatory Smad proteins (R-Smads) which then form a complex with CoSmad and translocate into the nucleus to affect gene transcription. When bound by (Shh the patched (Ptc) receptor's inhibition on smoothened (Smo) is blocked, allowing Smo to regulate TFs through GLI proteins.

Efforts in cancer research have led to a focus on a histone deacetylase inhibitor, valproic acid, due to its potential use as a differentiation therapy of acute myeloid leukemia (AML). Clinical findings provoked Bug, et al to investigate if valproic acid has any potential activity in HSCs. In addition to inducing proliferation of human HSCs they found that valproic acid is able to increase the self-renewal potential of mouse HSCs *in vitro* and

in vivo. In providing a mechanism for this action, the authors biochemically showed, in human and mouse HSCs, that β−catenin is upregulated, indicating active Wnt signaling is occurring, which correlates with earlier studies of the role of Wnts in HSCs (Bug, Gul et al. 2005). Yet the importance of Wnt signaling alone was challenged by the work done by Cobas, et al, who showed that conditional inactivation of β-catenin in BM progenitors did not impact their self-renewal potential, impair their ability to reconstitute the hematopoietic systems of lethally irradiated recipients, or negatively affect T- or B-cell development. These results indicate that β-catenin is not essential for hematopoiesis or lymphopoiesis, in contrast to previous findings (Cobas, Wilson et al. 2004). The reason for these contrary findings could be due to actions by Wnts through non-canonical signaling, or perhaps due to redundancy with other pathways.

1.3.4 Notch Signaling

The Notch pathway is a highly conserved signal transduction pathway that functions as a master regulator of cell fate decisions in a wide range of developmental processes and systems. Notch is a cell-surface receptor that is capable of binding two transmembrane protein ligands: Delta and Jagged (Artavanis-Tsakonas, Rand et al. 1999). Binding of either ligand to the receptor results in proteolytic cleavage of Notch, releasing the Notch intracellular domain (NIC). The NIC then translocates into the nucleus where it binds to CBF-1 and RBP-Jκ, converting these normally repressive transcription factors into transcriptional activators of Notch target genes (Figure 1.2) (Radtke, Wilson et al. 2005).

Studies in which Notch signaling was activated using various approaches indicate this pathway has roles in regulating HSCs. Li, et al established a role for Jagged1-Notch1 signaling as a mediator of cell fate decisions during hematopoiesis (Li, Milner et al. 1998).

(Stier, Cheng et al. 2002) reported activation of Notch1 resulted in expanded self-renewal of HSCs and favored lymphoid over myeloid progenitor lineages. (Varnum-Finney, Brashem-Stein et al. 2003) expanded HSCs *in vitro* while maintaining *in vivo* reconstituting ability by activating Notch signaling with an engineered Delta ligand. (Calvi, Adams et al. 2003) showed that osteoblasts producing high levels of Jagged1 supported increased numbers of HSCs via active Notch signaling.

Duncan, et al illustrated that Notch signaling is highly active in HSCs but is down-regulated after they differentiate. They also used colony forming unit assays to functionally demonstrate that Notch signaling delineates a more primitive cell population within the LSK population both *in vitro* and *in vivo*. This group then used several known inhibitors of the Notch pathway and provided evidence that a block of Notch signaling increases the rate of differentiation of HSCs in the BM environment. The authors also established a connection between Wnt and Notch signaling in the regulation of HSCs. Through Notch/Wnt double reporter mice they found evidence that the majority of cells in the stem cell niche use both pathways simultaneously, and they observed that HSCs stimulated with Wnt3a protein upregulated certain Notch target genes. Finally, they demonstrated the Notch pathway is the dominant signal that maintains HSCs in their undifferentiated state and must be intact for the Wnt pathway to enhance self-renewal of these stem cells (Duncan, Rattis et al. 2005).

As was the case with the Wnt pathway, there are findings which deemphasize the role of Notch in the regulation of HSCs. Work by Han, et al indicates Notch is required for T vs. B lineage commitment but is nonessential for maintaining HSCs as evidenced by normal reconstitution of RBP-Jκ defective BM donor cells (Han, Tanigaki et al. 2002).

Also, Mancini, et al observed that conditional inactivation of Jagged1, Notch1, or both simultaneously had no significant phenotype in the HSC compartment, even when the hematopoietic systems of these mice were challenged by 5-fluorouracil (5-FU). Their work indicates that Jagged1-mediated Notch1 signaling is dispensable for both self-renewal and differentiation of HSCs. One reason for such contradictions may be the redundancy inherent in the mammalian Notch pathway, where four different Notch receptors (Notch1-4) can bind five different ligands (Jagged1-2, Delta-like 1-3-4) (Mancini, Mantei et al. 2005).

1.3.5 BMP Signaling

Bone morphogenic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily of signaling molecules. Binding of a BMP ligand to its cell-surface, heterodimeric receptor complex (ALK2, -3, or -6 and BMPRII) activates its kinase activity and results in phosphorylation and complex formation of the Smad proteins that allows them to translocate into the nucleus to regulate gene transcription (Figure 1.2). The TGF-β superfamily has roles in regulation of cell proliferation, differentiation, and apoptosis (Massague 1998), and evidence has linked it and the BMP pathway specifically to regulation of various stem cells (Zhang and Li 2005). Bhatia, et al provided the first evidence that BMPs have a role in the regulation of hematopoietic stem cells *in vitro* by their work with these cells from human sources. They first observed expression of BMP receptors and Smad proteins in isolated human HSCs and went on to show that treatment of these cells with BMP-4 preserved their stem cell activity (Bhatia, Bonnet et al. 1999). Zhang, et al demonstrated the key role BMP signaling plays in the maintenance of mouse HSCs through its control of the HSC endosteal niche size (Zhang, Niu et al. 2003). A recent review by Larsson and Karlsson provides an in depth look at the role of TGF-β signaling and signal-

ing mediated by BMP proteins and the Smads in the regulation of HSCs and hematopoiesis, including the critical regulation at multiple points of the establishment and expansion of hematopoietic precursors as well as potent inhibitory growth regulation on adult HSCs by TGF-β signaling (Larsson and Karlsson 2005).

1.3.6 Hedgehog Signaling

The hedgehog (hh) proteins are a family of related signaling molecules first implicated in patterning events in the *Drosophila* embryo (Perrimon 1995). Sonic hedgehog (Shh), one of the human homologs of the *Drosophila* hh protein, is a transmembrane protein that upon binding its receptor, Patched (Ptc) blocks the inhibition by Ptc of another transmembrane protein, Smoothened (Smo), thereby freeing Smo to ultimately regulate transcription of target genes (Figure 1.2). Efforts to characterize this pathway in the mouse have been hindered due to embryonic lethality of targeted inactivation of either Shh or Ptc. Bhardwaj, et al provided evidence for a role for Shh in the regulation of human HSCs and demonstrated that a connection between Shh and BMP signaling known to exist in *Drosophila* is present in mammalian HSCs. They first isolated human HSCs and showed they highly express Shh, Ptc, and Smo, indicating Shh signaling is active in this cell population. They proceeded to show that in combination with certain cytokines, proliferation of these cells could be inhibited by blocking Shh signaling, or induced by activating this pathway *in vitro,* which didn't affect their ability to repopulate irradiated immunodeficient mice. They provided evidence indicating Shh is acting upstream of the BMP pathway and requires intact BMP signaling toregulate HSCs (Bhardwaj, Murdoch et al. 2001). Supporting these findings Akashi, et al found by microarray studies that Smo is highly expressed in mouse HSCs (Akashi, He et al. 2003). A study by Gering and Patient in the zebrafish model sys-

tem has implicated hh signaling in the formation of adult HSCs. Through the use of hh mutants and inhibitors of this pathway, they demonstrated its requirement for HSC generation, and similarly showed that intact Notch signaling is also a prerequisite for HSC production, supporting other findings indicating its role in HSC regulation (Gering and Patient 2005).

1.3.7 FGF Signaling

Fibroblast growth factors (FGFs) are a large family of proteins that act in concert with their receptors, fibroblast growth factor receptors (FGFRs), as signaling molecules. They do so throughout the lives of certain organisms, with key roles in growth and development in a variety of tissues. FGFs are evolutionarily conserved from *Drosophila* and *C. elegans* to mammalian species (Itoh and Ornitz 2004). Three FGF ligands have been identified in *Drosophila* (*branchless*, *thisbe*, and *pyramus*) and two have been identified in *C. elegans* (EGL-17 and LET-756) (Forbes and Lehmann 1999; Borland, Schutzman et al. 2001; Stathopoulos, Tam et al. 2004). In contrast 22 FGFs have been identified in mouse and human (Thisse and Thisse 2005). These molecules can be secreted from cells by signal peptides located on their amino termini. Once secreted, they will diffuse into the extracellular space or, conversely, they can be found on the surface of cells or deposited onto extracellular matrix. FGF molecules bind to their receptors, which requires the involvement of heparin/heparan sulfate proteoglycans (HSPGs) to form a stable complex and achieve full activation (Lin, Buff et al. 1999; Ornitz and Itoh 2001).

Receptors of the FGF pathway are fairly typical receptor tyrosine kinases, similar to platelet-derived growth factor (PDGF) receptor or stem cell growth factor receptor (SCFR or more commonly known as c-Kit). As is the case with the ligands, FGFRs are evolutionarily conserved, with two receptors identified in *Drosophila (breathless* and *heartless*), one

(EGL-15) identified in *C. elegans,* and five receptors (FGFR1-5) found in mice and humans (Forbes and Lehmann 1999; Borland, Schutzman et al. 2001; Sleeman, Fraser et al. 2001; Eswarakumar, Lax et al. 2005). The typical structure includes a large extracellular domain, a single-pass transmembrane domain, and a cytoplasmic domain. The extracellular domain is composed of three immunoglobin (Ig)-like domains arranged linearly. It is the two Ig-like domains closest to the plasma membrane (designated D2 and D3) that are responsible for ligand binding. Alternative splicing at the c-terminal half of the D3 domain is a determinant of both tissue expression and ligand specificity for FGFR1-3 (alternative splicing has not been observed in FGFR4 or -5) (Groth and Lardelli 2002; Eswarakumar, Lax et al. 2005). Also the D2 domain contains the sequence responsible for heparin binding. The cytoplasmic domain contains the catalytic tyrosine kinase domain in addition to several regulatory elements (Ornitz 2000; Olsen, Ibrahimi et al. 2004; Eswarakumar, Lax et al. 2005).

As stated ligand binding induces dimerization and activation of the receptor tyrosine kinase. This enzymatic activity stimulates auto-phosphorylation at multiple tyrosine residues present in the intracellular domain. In contrast to other growth factor receptors, such as EGFR, that can activate downstream signaling cascades directly, FGFRs largely rely on FRS2, an adapter protein that is constitutively associated with the receptor and is a primary kinase target. Once phosphorylated, FRS2 recruits a host of intracellular proteins to the receptor to initiate downstream signaling. Two isoforms of this protein have been identified; FRS2α is believed to be the predominant form and is broadly expressed, while FRS2β is restricted to the nervous system during embryonic development. One of the most well characterized pathways that is activated by the FGFR through FRS2 is the Ras/MAPK cas-

cade; another major mediator of FGF signaling is the PI3K/AKT pathway (Figure 1.3) (Eswarakumar, Lax et al. 2005). The large size and complexity of the FGF pathway has hindered efforts to characterize its roles within the hematopoietic system, but some advances have been made.

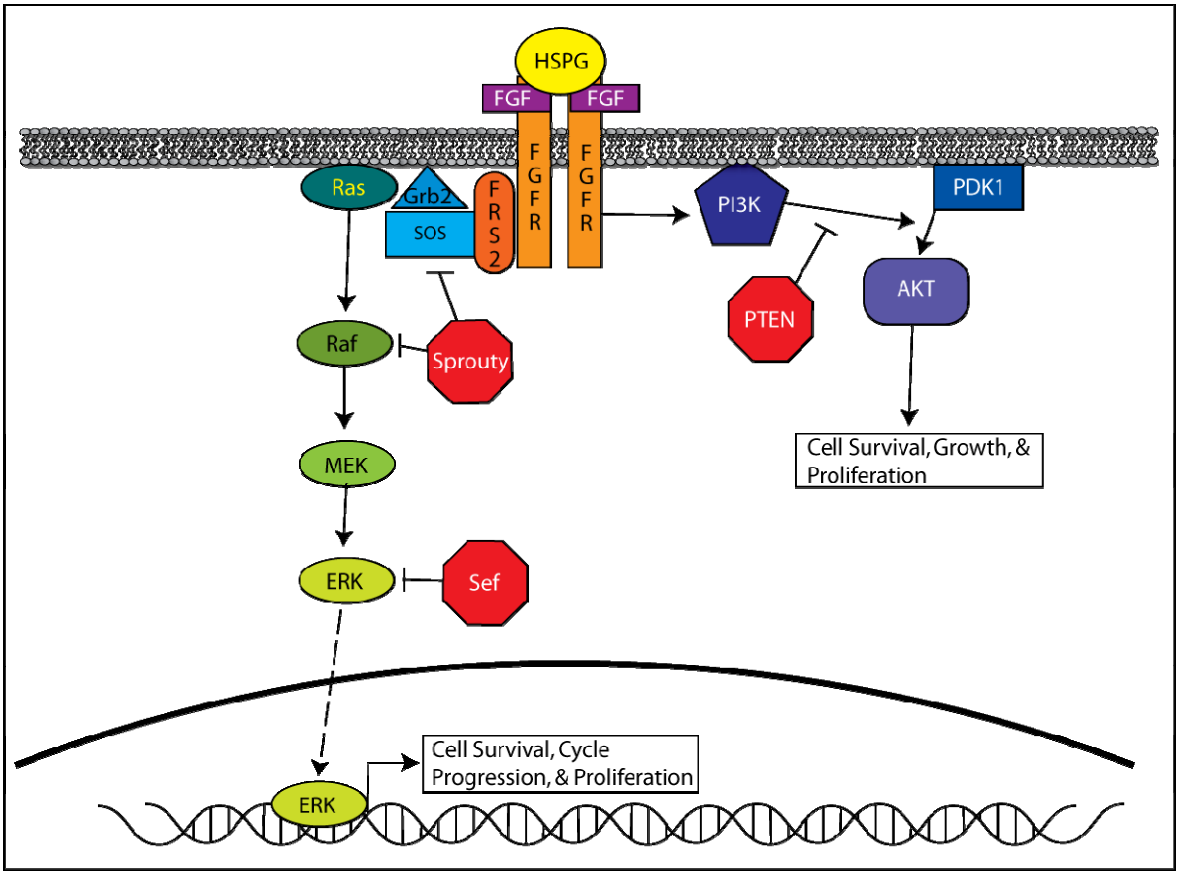

Figure 1.3. Fibroblast Growth Factor Signaling Pathway
FGF ligand binding to its receptor complex, mediated by heparin sulfate proteoglycans (HSPGs), recruits proteins such as Grb2 and SOS to transduce its signal via the Ras/MAP kinase pathway. Alternatively, activated FGF receptor can activate PI3K and subsequently activate AKT via PDK1. These signaling cascades mediate processes of cell survival, proliferation, and growth.

1.3.7.1 PTEN/PI3K/AKT Signaling

Central to this pathway is phosphatidylinositol-3-kinase (PI3K), which can phosphorylate both protein and lipid substrates, and is composed of both a regulatory subunit,

p85, and a catalytic subunit, p110. While there are 3 different classes of PI3Ks, class Ia molecules have been the most well described and remain the most relevant in cancer development. The p85-p110 complex is normally inactive and localized in the cytoplasm. The p85 subunit contains two SH2 domains flanking a p110 binding domain, as well as an N-terminal SH3 domain and a BCR domain, both thought to inhibit the catalytic activity of p110. The p110 subunit contains p85 and Ras binding regions, a kinase domain and a C2 domain that facilitates attachment to the plasma membrane.

Upon binding of a ligand to its respective receptor tyrosine kinase (RTK) at the cell surface, the RTK will undergo autophosphorylation facilitating activation of class Ia PI3K molecules by several mechanisms. The p85 subunit can bind to either phospho-tyrosine residues on RTKs or phospho-tyrosines on adaptor proteins bound to RTKs via p85 SH2 domains, thereby localizing PI3K near its primary lipid substrate, PtdIns (4,5)P_2 (PIP_2), in the plasma membrane. Furthermore, p85-RTK binding is thought to relieve inhibition of the catalytic p110 subunit. In addition, Ras - often activated downstream of RTKs, can directly bind and activate the p110 subunit. Once active, PI3K phosphorylates PIP_2 at the 3-position on the inositol ring, resulting in the formation of PtdIns (3,4,5)P_3 (PIP_3). PIP_3 levels are tightly controlled and mediate recruitment of the Ser/Thr kinases AKT (protein kinase B or PKB) and PDK1 (phosphatidylinositol-dependent kinase 1) via their respective PH (Plekstrin homology) domains. Once localized to the membrane, PDK1 phosphorylates AKT at Thr308. Additional phosphorylation at Ser473 results in complete activation of AKT, the main effector kinase of this pathway. Interestingly a single lipid and protein phosphatase, PTEN (phosphatase and tensin homologue), serves as a focal control point for PI3K-mediated signaling. When unphosphorylated, PTEN is active and converts PIP_3 into PIP_2,

thereby inhibiting AKT activation. Upon phosphorylation PTEN becomes inactivated and allows AKT activation to proceed (Figure 1.3) (reviewed in (Vivanco and Sawyers 2002; Cully, You et al. 2006)).

AKT has multiple targets through which it elicits many of the cellular effects attributed to the PI3K pathway, specifically anti-apoptotic effects, cell cycle progression, and increased protein translation. AKT mediates several processes leading to increased cell survival. AKT can phosphorylate the pro-apoptotic factors BAD and Caspase 9, thereby inhibiting their induction of cell death. Additionally, AKT can phosphorylate and inactivate FOXOs (Forkhead box transcription factors) known to drive the expression of pro-apoptotic genes such as BIM. Furthermore, AKT can activate Mdm2 resulting in decreased levels of p53, a protein with well characterized roles in mediating DNA damage-induced cell cycle arrest and apoptosis.

With regard to promoting cell cycle progression, AKT inhibits GSK3β (glycogen synthase kinase 3 β), known to phosphorylate and promote the degradation of CyclinD1 and c-MYC, two molecules involved in progression through the G_1/S phase of the cell cycle. In addition, AKT can lead to increased levels of CyclinD1 and decreased levels of cell cycle inhibitors such as $p27^{KIP1}$ via inhibition of FOXO activity. AKT can also directly inactivate $p27^{KIP1}$. Finally, AKT can lead to increased activity of mTOR (mammalian target of rapamycin). When unphosphorylated, TSC2 binds to TSC1 forming a complex. This complex functions as a GAP (GTPase activating protein) to decrease the amount of GTP-bound RHEB, the primary activator of mTOR. AKT phosphorylates TSC2 thereby inhibiting the GAP function of the TSC1/2 complex and leading to increased mTOR activity, resulting in increased translation and subsequent cell growth (reviewed inVivanco and Sawyers 2002;

Cully, You et al. 2006)).

Stem cells in adult tissues must successfully balance the need for continually generating new daughter cells to replenish those lost in the normal maintenance of that tissue with the necessity for preserving the stem cell pool over the life of the organism. To attain this balance it is widely held that adult stem cells are predominantly quiescent, entering the cell cycle very infrequently such that at any given time only a small fraction of the stem cell pool is actively dividing while the vast majority is held under cell cycle arrest. This balance is partly maintained by the interplay of positive and negative influences on the state of stem cells including various signaling pathways such as Wnt and Notch. *In vivo* studies into the functional loss of PTEN have revealed it as a major negative influence on hematopoietic stem cells, and PI3K/AKT signaling as the opposing, positive influence.

Genetic deletion of PTEN in the bone marrow of mice initially results in increased proliferation of HSCs. This overstimulation is evidenced by a shift of HSCs from the G0 to G1/S/G2/M phases of the cell cycle. The expansion of HSCs is only transient, as the excessive proliferation ultimately exhausts the stem cell pool causing their long-term decline and impaired ability to sustain reconstitution of a depleted hematopoietic system. This deregulated proliferation of HSCs primes these animals for tumorigenesis (Yilmaz, Valdez et al. 2006; Zhang, Grindley et al. 2006).

1.3.7.2 Regulators of FGF Signaling

A crucial aspect of any signaling pathway is how such signaling is controlled. This is certainly true for the FGF pathway, and several factors have been characterized that provide negative and positive regulation at many levels, some common to all RTKs, and others specific to the FGFR. Sprouty (Spry) was the first such regulator identified in *Drosophila*.

It was found to antagonize FGF signaling during tracheal development. There are four homologs of this protein (Spry1-4) and three Sprouty-related EVH1 domain proteins (Spred1-3) in mammals; they all share a conserved cysteine-rich domain in their carboxy terminus and have variable amino-termini. Sprouty proteins have been found to act as negative regulators of RTK-induced MAPK signaling (Figure 1.3), though it remains undetermined at what level they act, at or upstream of Ras, or at the level of Raf. Yet the Sprouty proteins are also capable of positive regulation, as evidenced by their ability to potentiate, rather than repress growth factor-mediated MAPK signaling in some cell types. A mechanism of action for the positive effects of Sprouty on growth factor signaling involves a highly conserved residue in the n-terminus, Tyr 55. Phosphorylation of Sprouty proteins at this conserved site allows for binding of c-Cbl, an E3 ubiquitin ligase. In the absence of Sprouty c-Cbl targets an activated RTK for internalization, thus Sprouty can compete for c-Cbl binding, allowing for sustained RTK signaling. However, this same residue (Tyr 55) mediates negative regulation by Sprouty; specifically once phosphorylated at this site Sprouty can bind the adaptor Grb2, preventing its role in activation of the Ras/MAPK cascade. An additional mechanism for Sprouty-mediated repression has been identified. A highly conserved domain in the C-terminus called the Raf Binding Motif (RBM) allows Sprouty proteins to bind Raf and block Ras-independent activation. Though these mechanisms have been elucidated, it remains to be determined how Sprouty proteins are regulated and incorporated into growth factor signaling (Christofori 2003).

Additional regulators include the transmembrane proteins Sef and FLRT3 (Figure 1.3). Both have been found to be expressed in patterns similar to FGFs and to be regulated by FGF signaling. Although the mechanism of Sef's action remains controversial, it is

known to inhibit FGF signaling specifically through the receptors FGFR1 and FGFR4. Some findings show Sef acting to block phosphorylation of the receptor and/or FRS2, thereby inhibiting recruitment of Grb2 and any downstream signaling through this protein. Other studies have found Sef acts at or below the level of MEK, specifically in the Ras/MAPK cascade, and prevents disassembly of MEK-ERK complexes, thus sequestering ERK in the cytoplasm and preventing activity in the nucleus. FLRT3, a leucine-rich transmembrane protein, in addition to independent activities has been found to bind to FGFRs and modulate signaling, though the mechanism of this action is not known (Bottcher and Niehrs 2005; Thisse and Thisse 2005).

1.3.7.3 Biological Functions of FGF Signaling

Much has been learned about the importance of FGF signaling, both during embryonic development and in the adult, and the functions it carries out in various model organisms. This information can lead to clues about its roles within the hematopoietic system and in HSCs. In *Drosophila,* FGF signaling through both of the known receptors is essential for directing migration of several cell types during embryonic development. *breathless* is a FGFR expressed by all tracheal cells of the *Drosophila* respiratory system, and signaling through it is required for proper formation of tracheal branches. Loss of function mutations in *breathless,* or its ligand *branchless* result in failed branch formation. Similarly signaling through *heartless* is required for proper mesodermal patterning. During gastrulation mesodermal cells migrate to a position adjacent to ectoderm and rely on signals from the ectoderm for induction of dorsal fates such as the heart and visceral mesoderm. Mutations in *thisbe*, *pyramus*, or *heartless* result in a lack of migration as well as fate specification, indicating their requirement for this process. Signaling through EGL-15 has also been shown

to have roles in migration in *C. elegans.* Mutations in either EGL-15 or one of its ligands, EGL-17, result in defects in the migration of sex myoblasts, causing infertility in animals due to defects in egg laying. In both organisms the FGF ligands are believed to function as chemoattractive guidance cues for migrating cells (Forbes and Lehmann 1999; Borland, Schutzman et al. 2001).

FGF signaling is also capable of directing cell migration during development in the mouse. Knockout of FGFR1 results in an embryonic lethal phenotype at late gastrulation resulting from defects in cell migration, fate specification, and patterning. It is thought these defects may be due to a concomitant reduction in *snail* and increase in E-cadherin that prevents proper exit from the primitive streak. Similarly, a lack of both FGF4 and -8 cause defects in migration of cells out of the primitive streak, but does not affect fate specification within it, indicating a more specific role for the ligands in migration. This is supported by additional findings of direct chemotactic potential for FGFs. FGF2 and -8 are strong chemoattractants for migrating mesencephalic neural crest cells, FGF2 and -4 attract mesenchymal cells during limb development, and FGF10 directs proper movement of lung epithelial buds (Bottcher and Niehrs 2005; Thisse and Thisse 2005).

In addition to conserved roles in directing cell migration, FGFs function in other biological processes within the mouse. In particular, FGFs are involved in both the initiation and maintenance of limb development. Initiation is triggered by coordinated actions of FGF8 and –10 that results in formation of the apical ectodermal ridge (AER). FGFs from the AER drive proliferation of mesenchymal cells contributing to formation of the developing limb. Additionally, FGF2, -4, and –8 coordinate with sonic hedgehog to control outgrowth and patterning of the limb. Furthermore, FGFs have been found to contribute to

bone formation. This began with the discovery that the most common form of human dwarfism is caused by activating mutations in FGFR3. Thereafter, an extensive number of human disorders including skeletal dysplasias and craniosysnostosis syndromes were linked to missense mutations in ligands and receptors; subsequent study has led to more defined roles for FGF signaling in this system. FGF2, -9, and –18 are expressed in mesenchymal cells and osteoblasts at varying times during development. FGFR1 and FGFR2 are also expressed in mesenchymal cells at early developmental time points, and later in osteoblasts along with FGFR3. Mutational studies have revealed a trend for FGF signaling in bone cells as a positive regulator of proliferation and differentiation, as well as influencing apoptosis (Thisse and Thisse 2005).

Some existing evidence points to a role for FGF signaling generally in hematopoiesis and also within stem cells. Work involving targeted deletion of *fgfr1* in embryonic stem (ES) cells resulted in defects in hematopoietic lineage development. Moreover when bFGF ligand was added to ES cells in culture it generally enhanced proliferation and/or differentiation as well as promoted hematopoietic progenitor development (Faloon, Arentson et al. 2000). Supporting this latter finding, when FGF1 (aFGF) was used to supplement culture of HSCs it stimulated their expansion without loss of multipotentiality (de Haan, Weersing et al. 2003). In addition to roles in normal hematopoiesis, abnormal FGF signaling has been implicated in various cancers including leukemia. Several distinct FGFR1 genetic translocations that all result in constitutive activation of its tyrosine kinase activity cause a particular kind of myeloproliferative syndrome called “8p11 myeloproliferative syndrome” (EMS). This syndrome is characterized by myeloid hyperplasia, eosinophilia, and lymphoblastic lymphoma (Eswarakumar, Lax et al. 2005).

Taken together these findings demonstrate that FGF signaling is a vital component of multiple processes both during development and in the adult, and specifically can impact the activity of HSCs. They prompt an in-depth *in vivo* study to discern to what extent, and by what mechanisms the FGF pathway may be contributing to the regulation of HSCs.

Chapter 2

Materials and Methods

Fgfr1fx/fx mice were provided by Chu-Xia Deng; *Mx1-Cre* mice were supplied by Klaus Rajewski; *HSC-SCL-CRE-ER^T* mice were provided by Joachim Goethert; Z/EG reporter mice and C57J/B6 mice were supplied by the Stowers Institute for Medical Research (SIMR) Animal Facility. Oligonucleotides were supplied by Integrated DNA Technologies (IDT). All the oligonucleotide sequences are listed in 5' to 3' orientation. Buffer solutions and media were supplied by the Core Facility of SIMR, unless otherwise stated. Statistical significance was determined by the student t-test, unless otherwise stated.

2.1 Standard solutions and reagents

PBS + 2% FBS

Phosphate buffered saline, pH adjusted to 7.2, with 2% fetal bovine serum sterilized through a 22 μM filter, stored at 4°C

RBC Lysis Buffer

8.3 g/L ammonium chloride, 1.0 g/L sodium bicarbonate, 0.037 g/L EDTA dissolved in water, stored at 4°C

PolyI:C

4 mg/ml in DPBS/DEPC, stored at -20°C

5-Fluorouracil

20 mg/ml in phosphate buffered saline, stored at -20°C

AMD3100

1 mg/ml in water, stored at -20°C

G-CSF

25 μg/ml in phosphate buffered saline with 0.1% murine serum albumin, stored at 4°C

2.2 Transgenic Animals

2.2.1 Animal Husbandry

Fgfr1$^{fx/fx}$ mice (Xu, Qiao et al. 2002) were mated with *Mx1-Cre* mice (Kuhn, Schwenk et al. 1995) or *HSC-SCL-CRE-ER*^{T} mice (Gothert, Gustin et al. 2005) to generate heterozygous progeny that were further crossed with mice homozygous for the floxed allele and Z/EG (*lac*Z/EGFP) mice(Novak, Guo et al. 2000) to ultimately obtain *Mx1-Cre*$^{+}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (FGFR1 KO) and *Mx1-Cre*$^{-}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (FGFR1 WT) littermates or *HSC-SCL-Cre-ER*$^{T-}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (SCL/FGFR1 WT) and *HSC-SCL-Cre-ER*$^{T+}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (SCL/FGFR1 KO) littermates. Genotyping was performed on tail biopsies using a proprietary PCR-based method developed by Transnetyx, Inc. (Cordova, TN). All mice used in this study were housed in the animal facility at SIMR and handled according to SIMR and National Institutes of Health (NIH) guidelines. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of SIMR.

2.2.2 Induction of Gene Deletion

To induce gene deletion, PolyI:C (GE Healthcare, United Kingdom) was injected intraperitoneally every other day at a dose of 25 μg/g body weight (BW) per injection to FGFR1 WT and KO mice for a total of five injections. SCL/FGFR1 WT and KO mice were placed on a tamoxifen diet for two weeks to induce gene deletion. Induction of gene deletion was initiated 4-6 weeks after birth.

2.2.3 Animal Euthanasia

All mice used in this study were sacrificed by asphyxiation with carbon dioxide followed by cervical dislocation to confirm death according to IACUC standard operating procedure (SOP).

2.3 Harvest of Hematopoietic Cells

2.3.1 Bone Marrow

Femurs and tibias were dissected from mouse hindlimbs. Muscle and connective tissue were removed mechanically by hand using Kimwipes (Kimtech, Kimberly-Clark Professional). The proximal end of the femur and distal end of the tibia was cut off with scissors, and bone marrow (BM) cells were harvested by flushing PBS + 2% FBS through the bones from the distal end of the femur and proximal end of the tibia using a 3 ml syringe equipped with a 22-gauge needle. The resulting cell suspension was triturated through the 22-gauge needle to produce a single-cell suspension. Red blood cells (RBCs) were lysed using a 0.3M ammonium chloride solution at room temperature for 1 minute with gentle agitation. Lysis reactions were stopped with an excess of PBS + 2% FBS, and non-lysed cells were collected by centrifugation at 1500 RPM for 5 minutes at 4°C. The cell pellet was resuspended in PBS + 2% FBS and debris was removed by passing the suspension through a 70 μM cell strainer (BD Biosciences, Bedford, MA).

2.3.2 Spleen

Spleens were dissected from the mouse abdominal cavity and mechanically dissociated by grinding between the frosted ends of two frosted glass microscope slides (VWR International, West Chester, PA). The resulting cell suspension was triturated through the 22-

gauge needle to produce a single-cell suspension. Red blood cells (RBCs) were lysed using a 0.3M ammonium chloride solution at room temperature for 1 minute with gentle agitation. Lysis reactions were stopped with an excess of PBS + 2% FBS, and non-lysed cells were collected by centrifugation at 1500 RPM for 5 minutes at 4°C. The cell pellet was resuspended in PBS + 2% FBS and debris was removed by passing the suspension through a 70 μM cell strainer (BD Biosciences, Bedford, MA).

2.3.3 Peripheral Blood

Peripheral blood samples were collected either from live mice by the submandibular method according to IACUC SOP or after animal euthanasia by cardiac puncture in EDTA-coated tubes (BD Biosciences, Bedford, MA) to prevent coagulation. Red blood cells (RBCs) were lysed using a 0.3M ammonium chloride solution at 37°C for 6 minutes. Lysis reactions were stopped with an excess of PBS+ 2% FBS, and non-lysed cells were collected by centrifugation at 1500 RPM for 5 minutes at 4°C. The cell pellet was resuspended in PBS + 2% FBS and debris was removed by passing the suspension through a 70 μM cell strainer (BD Biosciences, Bedford, MA).

2.4 Analysis of Hematopoietic Cells by Flow Cytometry

HSCs were stained for cell surface phenotyping with a cocktail of lineage antibodies: CD3, CD4, CD8, CD11b (Mac-1), CD45R (B220), Gr-1, IgM, and Ter119 and monoclonal antibodies against Sca-1, c-Kit, Flk2, and CXCR4 (eBioscience, San Diego, CA). Megakaryocytes were identified by staining with a monoclonal antibody to CD41 (eBioscience, San Diego, CA). Cells were stained for 60 minutes at 4°C in the dark and washed with PBS + 2% FBS. Typically 1 x 10^{6} cells were stained with 0.5 μg of antibody for analysis. BM and spleen cell counts were obtained on the Cell Lab Quanta SC (Beckman

Coulter, Brea, CA). PB cell counts were obtained on the Hemavet 950 (Drew Scientific, Dallas, TX). Cell sorting and analysis was done on the Cyan ADP (Dako, Denmark), MoFlo (Dako, Denmark), and/or Influx (BD Biosciences, Franklin Lakes, NJ).

2.5 DNA manipulations

2.5.1 Genomic DNA Isolation

Genomic DNA was isolated by first homogenizing cells in TRIzol (Invitrogen, Carlsbad, CA) by passing the cell lysate through a pipette tip several times. Homogenized samples were incubated for 5 minutes at room temperature, 100 µl of cholorform was added for every 500µl of lysate, and samples were vigorously shaken by hand for 15 seconds. Samples were then incubated for 3 minutes at room temperature before being centrifuged at 12,000xg for 15 minutes at 4°C. The aqueous phase was removed and stored at -80°C for future RNA isolation or discarded. DNA present in the interphase and organic phase was precipitated by adding 150 µl of 100% ethanol for every 500 µl of TRIzol used in the initial homogenization step. Samples were gently mixed, incubated for 3 minutes at room temperature, and centrifuged at 2,000xg for 5 minutes at 4°C. The supernatant was then removed and the DNA pellet was washed by adding a volume of 0.1 M sodium citrate in 10% ethanol equal to the volume of TRizol used in the initial homogenization step. The DNA pellet was stored in wash solution for 30 minutes at room temperature and gently mixed every 10 minutes before being centrifuged at 2,000xg for 5 minutes at 4°C. The supernatant was removed and this washing step was repeated once. After a second wash, the DNA pellet was washed a final time by adding a volume of 75% ethanol 1.5 times greater than the volume of TRIzol used in the initial homogenization step, incubated for 20 minutes at room temperature, gently mixed once during this incubation, and centrifuged at 2,000xg for 5

minutes at 4°C. After removal of the supernatant the DNA pellet was allowed to dry at room temperature for 10-15 minutes. The DNA pellet was dissolved in 8 mM sodium hydroxide. Yield and purity was determined by spectrophotometry using the Nanodrop (Thermo Scientific, Wilmington, DE).

2.5.2 Polymerase Chain Reaction (PCR)

Cre-mediated excision of the loxp-flanked region of *Fgfr1* was detected by amplification with recombination specific PCR primers (5' GTATTGCTGGCCCACTGTTC and 5' CAATCTGATCCCAAGACCAC). PCR primers were dissolved in water to a stock concentration of 100µM and a working concentration of 10 µM; primers were stored at -20°C. PCR reactions were set with Taq Polymerase (Bioline, Taunton, MA) and were run on a GeneAMP PCR System 9700 (Applied Biosystems, Carlsbad, CA). PCR reactions were analyzed by assessing amplicon size in 2% agarose gels.

A typical PCR reaction mixture was:

PCR reaction buffer (10x)	2.5µl
50 mM magnesium chloride	0.75µl
dNTPs	0.5µl
primer (forward)	0.5µl
primer (reverse)	0.5µl
Taq Polymerase	0.15µl
template	xµl
water	25- (4.9+x)µl
total volume	25µl

Thermocycling conditions for this reaction were as follows:

94°C	5 mins	
94°C	30 secs	
60°C	30 secs	10 cycles, decrease 0.5°C each cycle
72°C	30 secs	
94°C	30 secs	
55°C	30 secs	30 cycles
72°C	30 secs	
72°C	5 mins	
4°C	hold	

2.6 RNA Manipulations

2.6.1 RNA Isolation

RNA was isolated by first homogenizing cells in TRIzol (Invitrogen, Carlsbad, CA) by triturating the cell lysate through a 27-gauge needle a minimum of 10 times. Homogenized samples were incubated for 5 minutes at room temperature, 100 µl of cholorform was added for every 500µl of lysate, and samples were vigorously shaken by hand for 15 seconds. Samples were then incubated for 3 minutes at room temperature before being centrifuged at 12,000xg for 15 minutes at 4°C. The aqueous phase was removed and stored at -80°C if the RNA was not isolated immediately. RNA present in the aqueous phase was precipitated by adding 250 µl of isopropyl alcohol for every 500 µl of TRIzol used in the initial homogenization step. 1 µl of glycogen was added for to aid precipitation of RNA from a small number of cells. Samples were gently mixed, incubated for 10 minutes at room temperature, and centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was

then removed and the RNA pellet was washed by adding a volume of 75% ethanol equal to the volume of TRizol used in the initial homogenization step, mixed by vortexing, and centrifuged at 7,600xg for 5 minutes at 4°C. After removal of the supernatant the RNA pellet was allowed to dry at room temperature for 10-15 minutes. The RNA pellet was dissolved in RNase-free water. Yield and purity was determined by spectrophotometry using the Nanodrop (Thermo Scientific, Wilmington, DE).

2.6.2 Reverse Transcriptase (RT)-PCR

For gene expression analysis target cells were double-pass sorted directly into 200 μl TRIzol (Invitrogen, Carlsbad, CA). RNA from each sample was chloroform extracted as described and aliquots equivalent to 400 cells were used for each reaction. Primers used for amplification were as follows: CXCR4: 5' ACGGCTGTAGAGCGAGTGTT and 5' AGGGTTCCTTGTTGGAGTCA, FGFR1: 5' TGGAGTTCATGTGCAAGGTG and 5' ATAGAGAGGACCATCCTGTG (856 bp), or 5' CTTGACGTCGTGGAACGATCT and 5' TTCCAGAACGGTCAACCATGCAGA (350 bp), HPRT: 5'GTTCTTTGCTGACCTGCTGG and 5'TGGGGCTGTACTGCTTAACC, GAPDH: 5' TGGCAAAGTGGAGATTGTTGCC and 5'AAGATGGTGATGGGCTTCCCG. Real-time RT-PCR reactions were performed in triplicate using the Quantitect SYBR Green RT-PCR Kit (Qiagen, Germantown, MD) on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Amplification of hypoxanthine phosphoribosyl transferase (HPRT) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize for sample RNA content. Specificity of products was confirmed by melting curve analysis and/or assessing band size in 2% agarose gels.

2.7 Methods for Chapter 3

2.7.1 Mobilization Protocols

To achieve mobilization of HSCs mice were treated with reagents as follows: injected once via tail vein with 5FU (Sigma-Aldrich, St. Louis, MO) at 150 μg/g body weight (BW)(Haug, He et al. 2008), injected once subcutaneously with AMD3100 (Sigma-Aldrich, St. Louis, MO) at 5 μg/g BW(Broxmeyer, Orschell et al. 2005), or injected subcutaneously on consecutive days with G-CSF (Amgen, Thousand Oaks, CA) at 5 μg/g BW(Carlo-Stella, Di Nicola et al. 2002) for a total of five injections. PB, BM, and/or spleen tissue was harvested at various time points after 5FU treatment, 60 minutes after AMD3100 treatment, and within 4 hours after the final G-CSF treatment.

2.7.2 Colony Forming Unit Assay

In vitro colony forming unit (CFU) assays detect a mixture of myeloid progenitors including: erythroid (BFU-E), granulocyte-macrophage (CFU-G, CFU-M, and CFU-GM), and multi-potential granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). The assay was performed using 2×10^5 PB cells per well of a 12-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and 0.9 mls MethoCult™ GF M3434 Media (Stem Cell Technologies, Vancouver, BC, Canada). MethoCult was allowed to thaw overnight at 4^oC the day before the assay was started, or for several hours at room temperature the day the assay was started. Once completely thawed, MethoCult was warmed in a 37^oC water bath. PB samples were collected by the submandibular method according to IACUC SOP. RBCs were lysed as described, and WBCs were resuspended at the appropriate concentration in sterile PBS. Warmed MethoCult was aliquoted using a 1-ml syringe equipped with

a 16-gauge blunt-tipped needle (Becton Dickinson, Franklin Lakes, NJ). $2x10^5$ WBCs suspended in sterile PBS were added to each well, and the MethoCult/cell suspension was triturated through the 16-gauge needle several times to mix the cells throughout the media. Colonies were evaluated according to the manufacturer's guidelines and counted on day 12 of culture using a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany).

2.8 Methods for Chapter 4

2.8.1 Cell Migration Assay

Cell migration was studied using 6.5mm, 5 μm pore size transwell inserts in 24-well cluster plates (Corning-Costar Incorporated, New York, NY). Target cells were suspended in 0.1 ml Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% horse serum (HS) in the upper chamber. Chemotaxis towards various factors added to 0.6 ml DMEM +5% HS in the lower chamber was allowed to continue for 4 hours at 37^oC and 5% CO_2 in a humidified atmosphere. Cells that had migrated to the lower chamber were visualized and enumerated using a Leica DM IL (Leica Microsystems, Wetzlar, Germany). Chemoattractants were tested at the following concentrations: 50, 100, or 300 ng/ml murine CXCL12/SDF-1α (R&D Systems, Minneapolis, MN), 100 ng/ml FGF1 (Cell Signaling Technology, Danvers, MA), 100 ng/ml FGF4 (R&D Systems, Minneapolis, MN), or 100 ng/ml FGF23 (R&D Systems, Minneapolis, MN) 10 ng/ml heparin sulfate proteoglycan (Sigma-Aldrich, St. Louis, MO) was added to media in the lower chambers in assays toward FGF ligands. Klotho was included where indicated at 10 ng/ml (R&D Systems, Minneapolis, MN). Where indicated the following chemical inhibitors were included in the media of both the upper and lower chambers: SU5402 at 25 μM (Calbiochem, La Jolla, CA), LY294002 at 50 μM (BIOMOL Research Laboratories, Plymouth Meeting, PA), and

PD98059 at 50 μM (Sigma-Aldrich, St. Louis, MO).

2.9 Methods for Chapter 5

2.9.1 Cell Culture

LSK/Flk2$^-$ cells were sorted from BM into 96-well U-bottom tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ) at 500 cells/well with 180 μl media/well. Note: since our lab has found that the RBC lysis step may induce slight damage to HSCs that can affect their performance in culture, RBCs were not lysed prior to sorting. Cells were incubated at 37°C, 5% CO_2, 5% O_2 (balance N_2). Defined HSC expansion media was based on previous reports(Zhang and Lodish 2005) and consisted of StemSpan SFEM (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with SCF (10 ng/ml), TPO (20 ng/ml), and heparin (10 μg/ml). Where indicated, IGF-2 (20 ng/ml) and FGF1 (10 ng/ml) were also added to the culture media. Half of the total volume of media was changed every 2-3 days. Cultures were visually inspected and images taken with a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany). Where indicated the chemical inhibitor SU5402 (Calbiochem, La Jolla, CA) was added to the culture media at 1 or 5 μM; an equal volume of DMSO was added as a vehicle control. On the day of analysis, cultures were collected by removing media and cells from each well with a pipette. Wells were washed twice with PBS + 2% FBS, and the washes were pooled with the media containing cells. These suspensions were centrifuged at 1500 RPM for 5 minutes at 4°C to pellet cells. Cell counts were obtained on the Cell Lab Quanta SC (Beckman Coulter, Brea, CA), and the cells were stained for flow cytometry as described in 2.4. Fold expansion of HSCs was determined by dividing the number of LSK/Flk2$^-$ cells determined to be present in the culture on the day of analysis by the input number of LSK/Flk2$^-$ cells.

2.10 Methods for Chapter 6

2.10.1 Immunohistochemistry

Tissues collected for immunohistochemistry were fixed in unbuffered zinc formalin (Richard-Allan Scientific, Kalamazoo, MI) for 24 hours at room temperature. Femurs and tibias were decalcified in Immunocal (Decal Chemical Corp, Tallman, NY) for 24 hours at room temperature. Tissues were embedded in paraffin and 3 μM sections were obtained. Tissues were deparrafinized using a Leica Autostainer XL (Leica Microsystems, Wetzlar, Germany). Antigen retrieval was performed on deparrafinized sections by incubating with a trypsin solution (Invitrogen, Carlsbad, CA) at 37°C for 10 minutes. Sections were washed 3 times with PBS + 0.05% Tween-20. Sections were then blocked with 1X Universal Blocking Buffer (BioGenex, San Ramon, CA) either for 30 minutes at room temperature or overnight at 4°C. Sections were stained with a rabbit polyclonal antibody to FGF basic (Abcam, Cambridge, MA) at a dilution of 1:300 overnight at 4°C and then for 3 hours at room temperature. After 3 washes with PBS + 0.05% Tween-20, secondary staining was done with the EnVision+ System-HRP Labelled Anti-Rabbit Polymer (Dako, Denmark) for 1 hour at room temperature. Sections were washed 3 times with PBS + 0.05% Tween-20, then 3 drops of AEC+ substrate (Dako, Denmark) was applied and color development was allowed to proceed for 4 minutes at room temperature. Sections were washed 3 times with PBS + 0.05% Tween-20 and then counterstained with 2 drops of Hematoxylin (Invitrogen, Carlsbad, CA) for 5 minutes at room temperature. Sections were washed 3 times with PBS + 0.05% Tween-20. Clear Mount (Electron Microscopy Sciences, Hatfield, PA) was applied to each section (to maintain structure) and allowed to dry for 15 minutes at roomtemperature. Coverslips were then applied using Cytoseal (Richard-Allan Scientific,

Kalamazoo, MI). Sections were evaluated and images were taken on a Leica DM 2000 (Leica Microsystems, Wetzlar, Germany).

2.10.2 Enyme-linked immunosorbent assay (ELISA)

BM was collected as described, which included RBC lysis. Supernatants were collected in 1.5mL Eppendorf tubes and subjected to centrifugation at 5000 RPM for 5minutes at 4°C to remove dead cells and debris. Supernatants were transferred to new tubes and stored at -80°C. Protein concentration was measured by the Bradford Assay. For the ELISA, a monoclonal antibody to bFGF (MAB233 clone 10060, R&D Systems, Minneapolis, MN) was used for capture, and a biotinyated anti-bFGF antibody (MAB233 clone 10043, R&D Systems, Minneapolis, MN) was used for detection using SA-HRP. Plates were read with an ELISA reader and analyzed according to the standard curve and Bradford Assay results. The standard curve was made using recombinant mouse bFGF (Peprotech, Rocky Hill, NJ).

Chapter 3

Primary phenotyping of conditional *Fgfr1* knockout mice reveals requirement for FGFR1 in HSCs under stress but not during homeostasis

3.1 Introduction

In the adult hematopoietic system FGF receptor 1 (FGFR1) is predominantly expressed on hematopoietic stem cells (HSCs) as reported by microarray (Akashi, He et al. 2003) and RT-PCR (de Haan, Weersing et al. 2003). Though FGF ligands can help support HSC expansion *in vitro* (de Haan, Weersing et al. 2003; Zhang and Lodish 2005), the role of FGF signaling via FGFR1 *in vivo* has not been elucidated. I set out to understand the potential role of FGFR1 in the biology of adult HSCs by gene knockout studies in mouse models. Complete inactivation of *Fgfr1* results in an embryonic lethal phenotype with embryos dying shortly after gastrulation (Yamaguchi, Harpal et al. 1994), necessitating a conditional knockout approach to study the requirement of FGFR1 in adult tissues. I began my investigation by crossing the $Fgfr1^{fx/fx}$ mouse line (Xu, Qiao et al. 2002) with a PolyI:C-inducible *Mx1-Cre* mouse line (Kuhn, Schwenk et al. 1995) and incorporating a reporter of Cre-mediated excision by mating resulting mice with the Z/EG reporter line (Novak, Guo et al. 2000). The inducible nature of the Mx1 promoter permits control of the timing of Cre induction and gene deletion, and our lab has previously shown high efficiency of knockout in adult hematopoietic tissues (Zhang, Niu et al. 2003; Zhang, Grindley et al. 2006). Therefore, I could directly assess the effects of loss of FGFR1 in the ability of HSCs to maintain a functional hematopoietic system. Given how amenable hematopoietic tissues are to flow

cytometry, I relied heavily on this technology in my initial phenotypic analyses.

Phenotyping of FGFR1 conditional knockout mice revealed defects in the process of mobilization. Throughout adult life HSCs are primarily localized within the bone marrow (BM) where they associate with niches that regulate their activity (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003; Kiel, Yilmaz et al. 2005; Sugiyama, Kohara et al. 2006). While a small proportion of HSCs routinely undergo a process of recirculation from BM to peripheral blood (PB) and back (Goodman and Hodgson 1962; Wright, Bowman et al. 2002), the number of HSCs that emigrate from BM can be markedly increased by certain stimuli in the process of mobilization (Lapidot and Petit 2002; Cottler-Fox, Lapidot et al. 2003; Papayannopoulou 2004). These include chemotherapeutic drugs that induce tissue damage such as cyclophosphamide and 5-fluorouracil (5FU) (Longley, Harkin et al. 2003), various cell signaling molecules such as stem cell factor (SCF), stromal derived factor-1 (SDF-1) (Shen, Cheng et al. 2001), and granulocyte-colony stimulating factor (G-CSF) (Lane, Law et al. 1995), as well as man-made small molecules such as AMD3100 that disrupts the interaction between SDF-1 and its receptor CXCR4 (Broxmeyer, Orschell et al. 2005). All stimulate migration of HSCs out of the BM, but mobilization is a complex and multi-step process that can involve expansion of HSC numbers within the BM prior to emigration to sites of extramedullary hematopoiesis such as the spleen.

3.2 Results

3.2.1 Confirming *Fgfr1* expression and knockout

To begin my investigation I wanted to both confirm expression of *Fgfr1* in HSCs and ensure that my induction of *Mx1-Cre*$^{+}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (FGFR1 knockout (KO)) mice resulted in deletion of *Fgfr1* expression as compared to *Mx1-Cre*$^{-}$ *Fgfr1*$^{fx/fx}$ Z/EG$^{+/-}$ (FGFR1

wildtype (WT)) controls. Toward that end I administered PolyI:C to a cohort of FGFR1 WT and KO littermates and obtained genomic DNA from PB samples collected 4 weeks after the last PolyI:C injection. Two of the mice (L0115 and L0116) were sacrificed and LSK cells (enriched for HSCs) and LK cells (enriched for committed progenitors) were flow sorted from BM of each mouse. The gating strategy for sorting of LSK cells and post-sort purity of this population are shown from a representative experiment (Figure 3.1). Breeding with the *Z/EG* line allowed for reporting of Cre recombination in live cells, and prospective isolation of HSCs known to bear deleted *Fgfr1*.

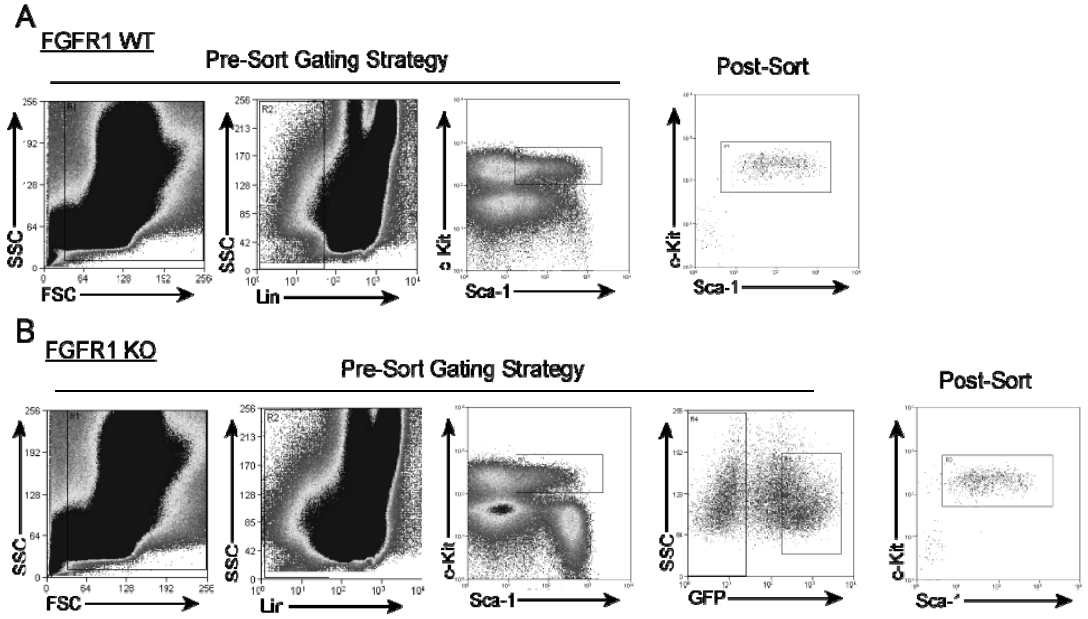

Figure 3.1. Flow Sorting Strategy of LSK Cells from FGFR1 WT and KO Mice
(A) Representative gating strategy for flow sorting of LSK cells from BM of FGFR1 WT mice. Sca-1^{+}/c-Kit^{+} cells (pre-gated on total nucleated cells (TNC or "live" cells) and Lineage^{-} cells were sorted on the MoFlo. (B) Representative gating strategy for flow sorting of LSK cells from BM of FGFR1 KO mice. Mice genotyped positive for the *Z/EG* allele exhibited GFP^{+} cells, indicative of Cre recombination. Post-sort purity is shown for each sample.

Only DNA from mice previously genotyped as FGFR1 KO (data not shown) amplified a recombined *Fgfr1* allele (Figure 3.2A). Furthermore, semi-quantitative RT-PCR of

Fgfr1 from RNA isolated as described in Materials and Methods demonstrated that *Fgfr1* transcripts could be detected in all sorted populations from the FGFR1 WT control (L0116), yet no amplicon was observed in samples from the FGFR1 KO (L0115) (Figure 3.2B), confirming the PCR recombination assay and indicating that *Fgfr1* was successfully deleted. Furthermore, this finding was repeated in additional experiments using an alternate primer pair for FGFR1 and RNA isolated from whole BM (Figure 3.2C). This data indicates that high efficiency of Cre induction was being achieved in FGFR1 KO mice. Though this PCR recombination assay was not performed on every FGFR1 KO mouse, it was routinely employed throughout subsequent experiments and analyses to validate Cre induction.

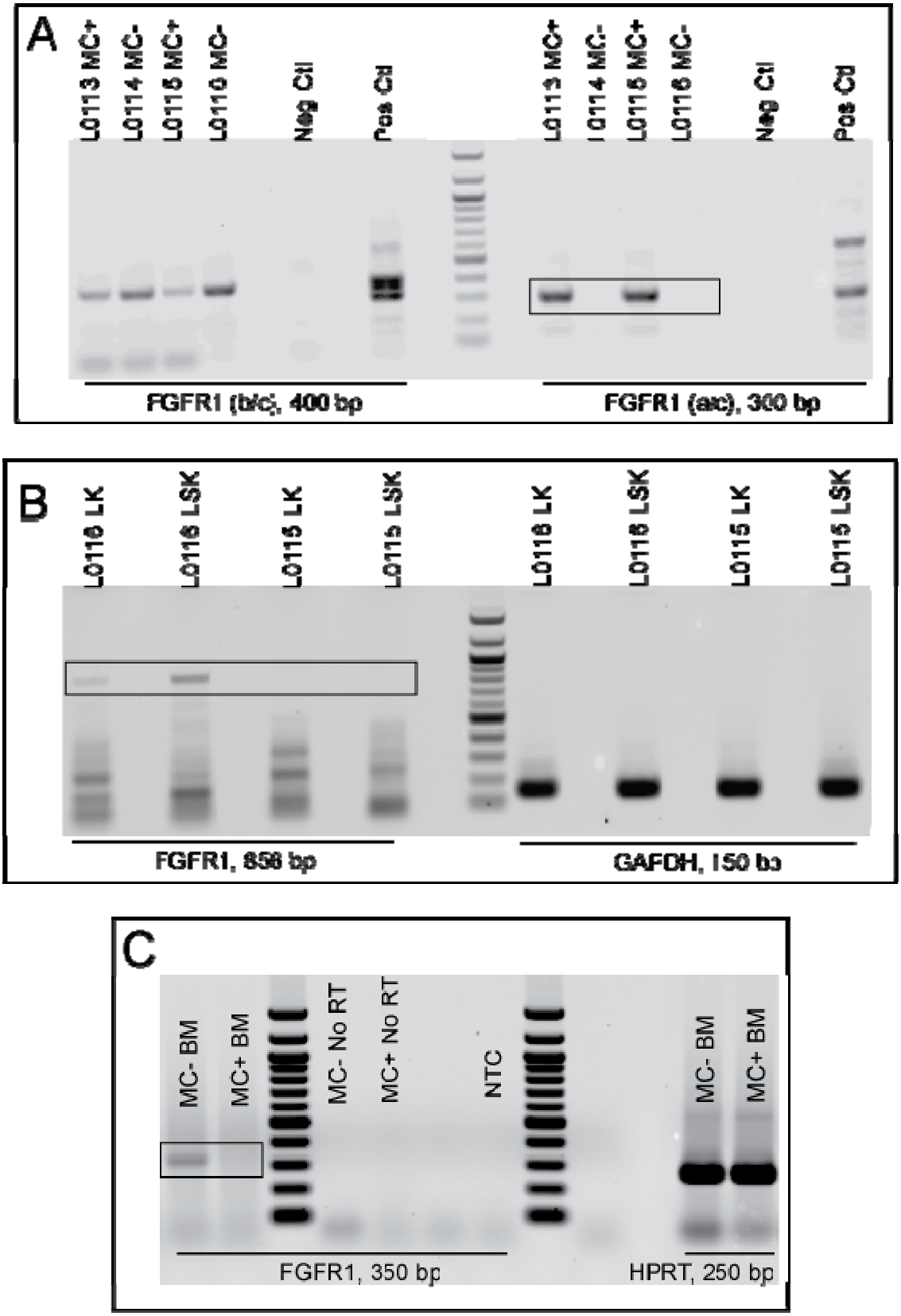

Figure 3.2. Confirmation of *Fgfr1* Deletion in FGFR1 KO Mice

(A) Detection of Cre induction and activity with a recombination sensitive primer pair (FGFR1 a/c) that produces a 300bp amplicon. (**B)** Semi-quantitative RT-PCR for *Fgfr1* (856 bp) showing expression in sorted BM LK and LSK cells from FGFR1 WT (L0116) but not FGFR1 KO (L0115) mice. (**C)** Semi-quantitative RT-PCR for *Fgfr1* (350 bp) showing expression in BM FGFR1 WT but not FGFR1 KO mice.

3.2.2 Deletion of *Fgfr1* during homeostasis does not affect hematopoiesis

Once I was confident that PolyI:C induction deleted *Fgfr1*, my initial phenotypic analyses focused on measuring levels of stem cells as well as various lineages in different hematopoietic tissues of FGFR1 KO mice and comparing them to FGFR1 WT controls. FGFR1 KO mice exhibited similar numbers of HSCs (enriched by the lineage$^-$/Sca-1$^+$/c-Kit$^+$ or LSK immunophenotype) in BM as FGFR1 WT controls (Figure3.3A), as well as normal levels of mature hematopoietic lineages including myeloid cells (Mac1$^+$/Gr1$^+$), B-cells (B220$^+$), and T-cells (CD3$^+$) (Figure 3.3B). Similarly, levels of these same lineages in PB were not significantly different in FGFR1 KO mice compared to WT (Figure 3.3C). Since the spleen serves an important immune function, in part by acting as a repository for B cells(Tan, Xu et al. 2009), I monitored levels of B cells within spleens of FGFR1 WT and KO mice; however, I found no difference in the number of B220$^+$ cells (Figure 3.3D). Finally, I investigated the thymus for levels of mature T cells (Uematsu, Donda et al. 1997) and immature, developing thymocytes (Mulroy, McMahon et al. 2002). FGFR1 KO mice had comparable levels of mature CD4$^+$/CD8$^+$, CD4$^+$, and CD8$^+$ cells as FGFR1 WT controls (Figure 3.3E). Also, I analyzed the four, immature "double negative" (or DN) populations defined as follows: DN1 = CD44$^+$/CD25$^-$, DN2 = CD44$^+$/CD25$^+$, DN3 = CD44$^-$/CD25$^+$, and DN4 = CD44$^-$/CD25$^-$. I found no difference in the levels of these DN populations between FGFR1 WT and KO mice (Figure 3.3F). Collectively, these data indicate that deletion of *Fgfr1* has no major effect on hematopoiesis, in particular differentiation and maintenance of HSCs, under homeostatic conditions.

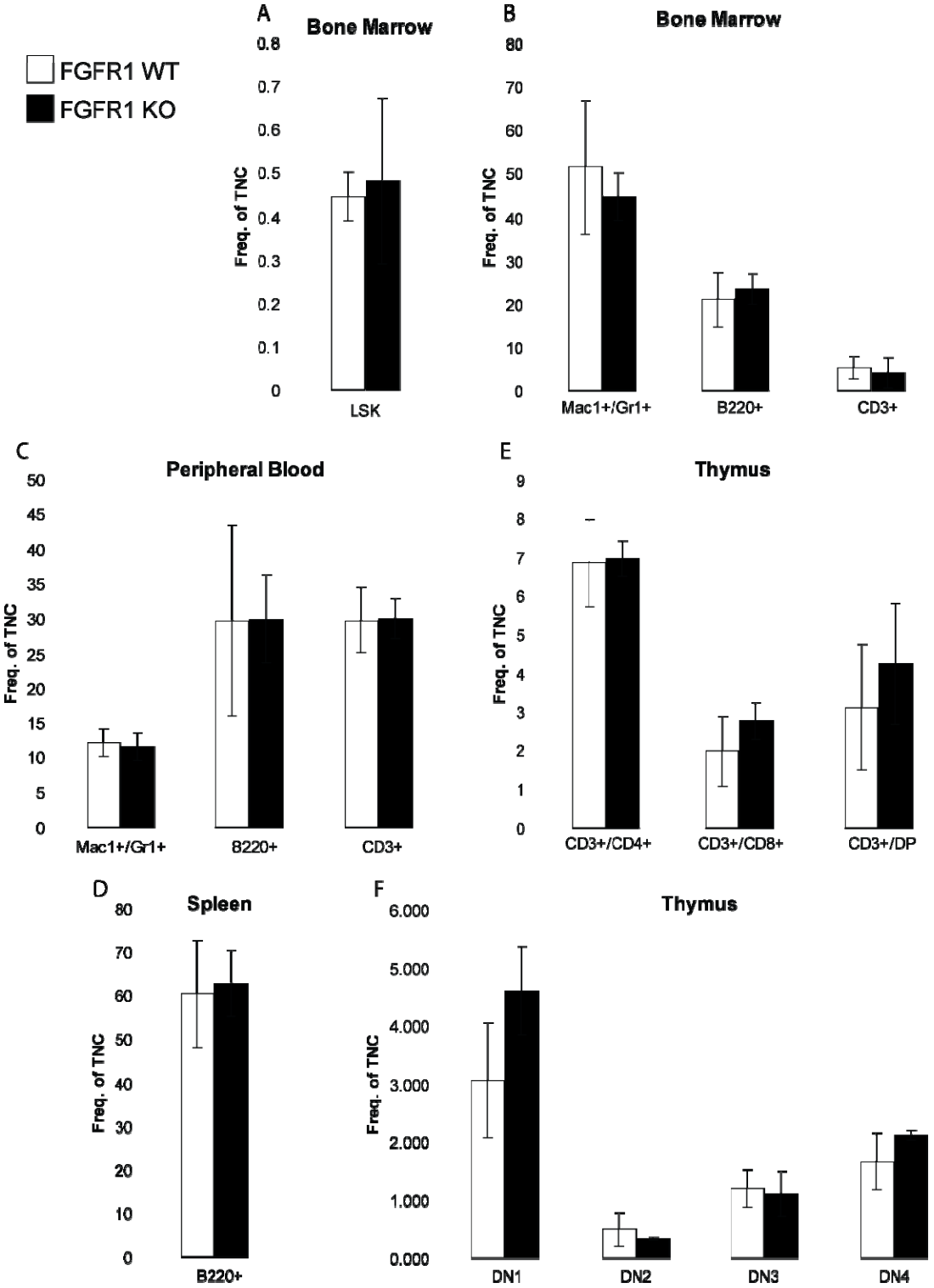

Figure 3.3 Normal Hematopoiesis in FGFR1 KO Mice at Homeostasis.
(A-F) Comparison of the frequency of TNC of the indicated cell population between FGFR1 WT and KO mice of BM (A, B), PB (C), Spleen (D), and thymus (E, F) 4 weeks after PIPC induction of Cre (n=6).

3.2.3 *Fgfr1* is required for mobilization of HSCs in response to stress

Given the lack of any phenotype at homeostasis, and considering known roles for FGF signaling (Eswarakumar, Lax et al. 2005; Thisse and Thisse 2005), I tested the ability of FGFR1 KO mice to respond to cytotoxic stress within BM and reconstitute a compromised hematopoietic system. Initially, I administered 5FU to FGFR1 WT and KO mice and conducted colony forming unit (CFU) assays on PB samples obtained at multiple intervals after 5FU injection (Figure 3.4).

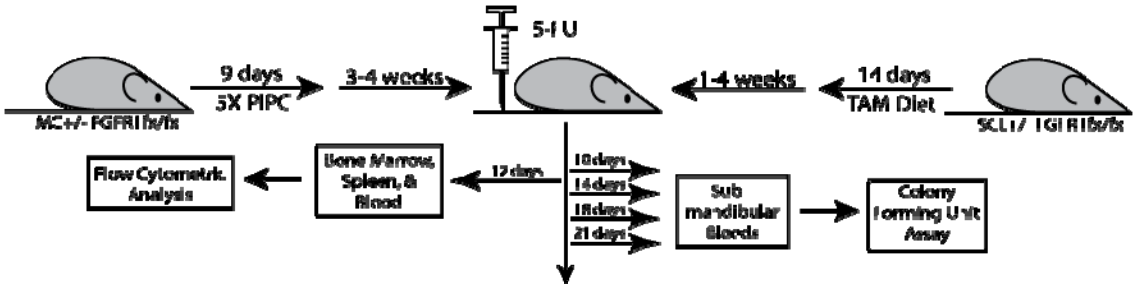

Figure 3.4. Outline for Stress Response Experiments
To achieve Cre induction FGFR1 WT and KO mice were treated with PolyI:C (PIPC) and SCL/FGFR1 WT and KO mice were treated with tamoxifen (TAM) as described in Materials and Methods. Stress was induced by a single injection of 5FU, and tissues were collected for anlaysis as depicted.

I tracked mobilization by changes in total colony counts in the CFU assays. I observed the maximal increase of colonies 14 days post-5FU, in accordance with known kinetics of 5FU-induced mobilization (Heissig, Hattori et al. 2002). FGFR1 WT mice displayed a 3.2 fold increase in colonies over FGFR1 KO mice (p-value = 0.05, Figure 3.5A), indicating a mobilization defect. I did not observe differences in the distribution of colony types present (Figure 3.5B).

In addition, I monitored levels of circulating, phenotypically defined HSCs by flow cytometry (Figure 3.4). Normally, under homeostasis, HSCs in PB are at low to undetectable levels as indicated by the LSK population (Figure 3.5C). 12 days post-5FU treatment, however, circulating LSK cells were 32.8 fold higher in FGFR1 WT mice than in FGFR1

KO mice (p-value = 0.001, Figure 3.3D, 3.5E), demonstrating defective ability to mobilize HSCs in response to stress. Since the Mx-1 promoter driving Cre expression in FGFR1 KO animals is not tissue specific (Kuhn, Schwenk et al. 1995), I mated *Fgfr1*$^{fx/fx}$ animals with the HSC-specific *HSC-SCL-Cre-ER*^{T} mouse line (Gothert, Gustin et al. 2005) to determine whether FGFR1 is required by HSCs or whether it acts indirectly on HSCs through non-

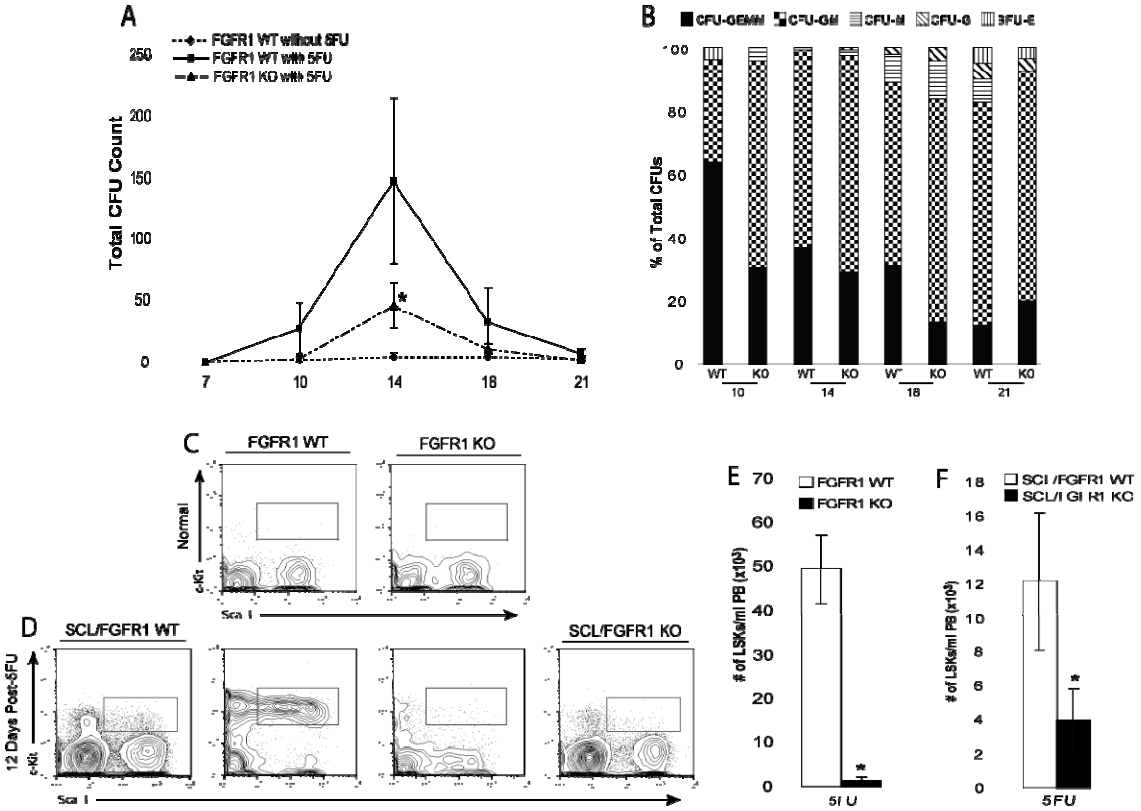

Figure 3.5. Deletion of FGFR1 Results in Defective Mobilization of HSCs in Response to Stress
(A) Comparison of CFU-C from blood samples of FGFR1 WT and KO mice taken on the indicated days after 5FU injection (n=4, *p-value = 0.05). (**B**) Comparison of CFU types between FGFR1 WT and KO mice on the indicated days after 5FU injection. (**C-D**) Representative flow cytometric analyses of the LSK population (pre-gated on live, lineage negative cells) in PB at steady state (**C**), FGFR1 and SCL/FGFR1 WT and KO mice 12 days after 5FU (**D**). (**E-F**) Comparison of absolute numbers of LSKs/ml PB between FGFR1 WT and KO mice (n=4) (**E**) and SCL/FGFR1 WT and KO mice (n=3) (**F**) after 5FU (*p-value = 0.001 (E) and 0.05 (F)). Error bars indicate SD.

hematopoietic stromal components. After tamoxifen induction of Cre expression, I tested the ability of resultant mice to mobilize in response to 5FU (Figure 3.4). *HSC-SCL-Cre-ER*$^{T-}$ *Fgfr1*$^{fx/fx}$ (SCL/FGFR1 WT) mice successfully mobilized 3.1 fold more LSK cells to

PB 12 days post-5FU than did *HSC-SCL-Cre-ER*$^{T+}$ *Fgfr1*$^{fx/fx}$ (SCL/FGFR1 KO) mice (p-value = 0.05, Figure 3.5D, 3.5F). These results show that FGFR1 signaling is indeed necessary for mobilization of HSCs in response to stress, but not necessary for normal hematopoiesis.

3.2.4 *Fgfr1* is required for mobilization of HSCs with the SDF-1 inhibitor AMD3100, but not the cytokine G-CSF

Considering these findings, I tested whether FGFR1 is required for HSCs to mobilize in response to two other clinically used mobilizing reagents. Treatment with the SDF-1 inhibitor AMD3100 resulted in rapid mobilization of HSCs in both FGFR1 and SCL/FGFR1 WT mice; mobilization was less, however, in KO mice (4.8 fold less in FGFR1, p-value = 0.016 and 74.5 fold less in SCL/FGFR1, p-value = 0.0014) (Figure 3.6A, 3.6C-D). Conversely, administration of G-CSF successfully mobilized HSCs to PB at comparable levels in all FGFR1 WT / KO mice and SCL/FGFR1 WT / KO mice (Figure 3.6B-D). These results indicate that FGFR1 is required for HSCs to mobilize in response to AMD3100 but not G-CSF.

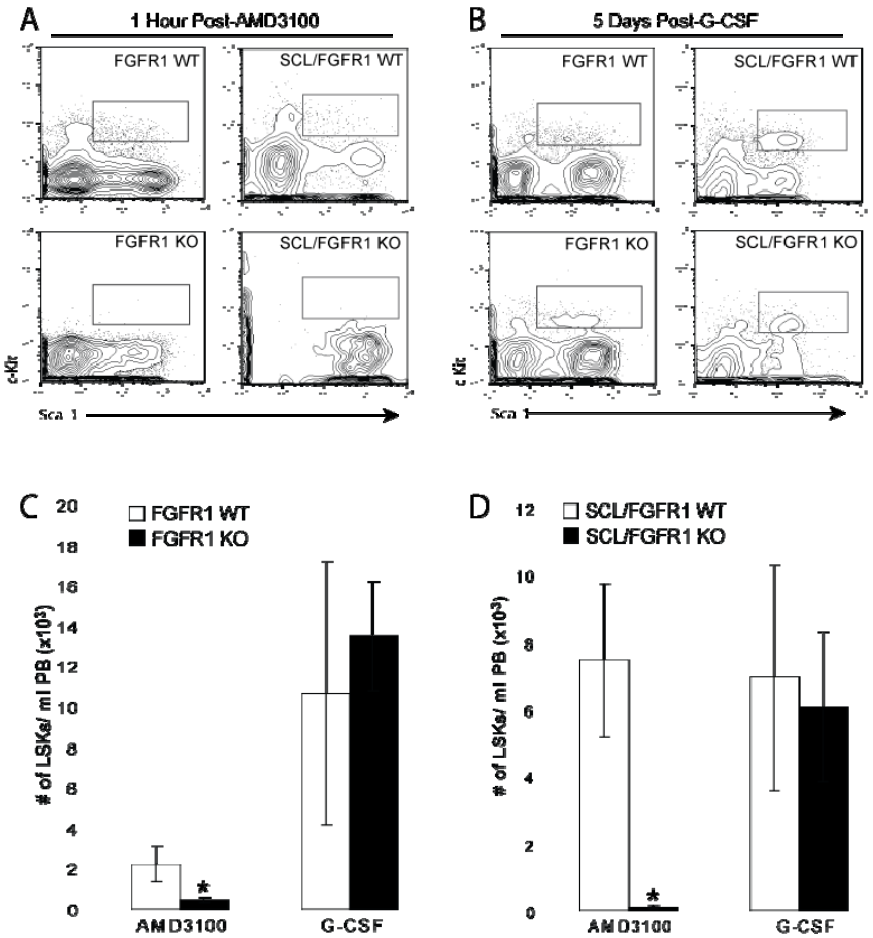

Figure 3.6. FGFR1 KO Mice do not Respond to AMD3100 but Respond Normally to G-CSF
(A-B) Representative flow cytometric analyses of the LSK population (pre-gated on live, lineage negative cells) in PB of FGFR1 and SCL/FGFR1 WT and KO mice1 hour after AMD3100 (A) and 5 days after G-CSF (B). (C-D) Comparison of absolute numbers of LSKs/ml PB between FGFR1 WT and KO mice (n=3) (C) and SCL/FGFR1 WT and KO mice (n=3) (D), after AMD3100 (*p-value = 0.016 (C) and 0.0014 (D)), and G-CSF treatment. Error bars indicate SD.

3.3 Discussion

To begin my study of FGFR1 conditional knockout mice I confirmed previously reported expression of *Fgfr1* in HSCs (Akashi, He et al. 2003; de Haan, Weersing et al. 2003) by performing semi-quantitative RT-PCR. Indeed, similar to published findings *Fgfr1* expression was higher in a population enriched for HSCs than one enriched for more committed progenitors. Furthermore, I was able to demonstrate by PCR on genomic DNA and RT-PCR on RNA that the floxed *Fgfr1* allele in FGFR1 KO mice was recombined by Cre upon

induction and as a result *Fgfr1* expression was abolished. These assays instilled confidence to move forward with preliminary phenotyping of FGFR1 KO mice.

Initial studies of FGFR1 KO mice revealed no obvious effects on hematopoiesis during homeostasis as compared to FGFR1 WT controls, as determined by lineage analyses in multiple hematopoietic tissues. Moreover, HSC numbers within BM of FGFR1 KO mice were comparable to WT controls, indicating there was no effect (positive or negative) on the maintenance of HSCs. However, I was able to observe a requirement for FGFR1 in HSCs during stress response by treating mice with the cytotoxic drug 5FU. 5FU stimulated mobilization of HSCs out of the BM and into the peripheral circulation in FGFR1 WT controls, yet FGFR1 KO mice displayed impaired ability to mobilize HSCs to PB as seen by CFU assays and flow cytometry. Given this result, I tested the requirement of FGFR1 in mobilization of HSCs to two other clinically relevant reagents. Surprisingly, while FGFR1 was required for mobilization in response to AMD3100, it appeared to be dispensable for G-CSF-induced mobilization. Furthermore, I was able to show that the requirement for FGFR1 during stress response and AMD3100-induced mobilization is at the level of the HSC by using the HSC-specific *HSC-SCL-Cre-ER*^{T} mouse line (Gothert, Gustin et al. 2005). Results obtained from SCL/FGFR1 KO mice indicate that FGFR1 does not act indirectly on HSCs through non-hematopoietic stromal components, a possibility in the less specific FGFR1 KO model. Further research was needed to understand the mechanism of FGFR1's involvement in HSC stress response and mobilization.

Chapter 4

Mechanisms of mobilization – deletion of FGFR1 impairs HSC CXCR4 expression and migration

4.1.1 Introduction

As discussesd in the previous chapter, the process of mobilization by definition involves migration of a BM cell population out of the marrow and into the peripheral circulation. In my investigation into the role of FGFR1 in HSCs, I found it to be required for stress-induced mobilization of HSCs, as well as mobilization induced by the SDF-1 inhibitor AMD3100. This is intriguing as HSC mobilization in response to stress (5FU) and AMD3100 both involve the SDF-1/CXCR4 signaling axis, though they are quite different in kinetics and efficacy.

CXCR4 is the only member of the CXC chemokine receptor family that is expressed on HSCs. CXCR4 specifically binds the ligand stromal cell-derived factor-1 (SDF-1), also known as chemokine (C-X-C motif) ligand 12 (CXCL12), and SDF-1 is the only chemokine capable of stimulating chemotaxis of HSCs *in vitro* (Wright, Bowman et al. 2002). Indeed, SDF-1/CXCR4 signaling has been characterized not only in the migration, and more specifically mobilization of HSCs (Lapidot and Petit 2002), but also their retention in BM (Foudi, Jarrier et al. 2006). Directly elevating levels of SDF-1 in blood plasma can stimulate mobilization of HSCs (Hattori, Heissig et al. 2001). Furthermore, BM stress can lead to increases in SDF-1 within the peripheral circulation, which can act to promote egress of HSCs from the BM (Heissig, Hattori et al. 2002). Additionally, forced overexpression of CXCR4 on HSCs increases their migration and response to SDF-1 (Kahn, Byk

et al. 2004). Interestingly, FGF ligands have been reported to regulate CXCR4 expression in other cell types. bFGF was found to upregulate CXCR4 mRNA and protein levels in endothelial cells (Feil and Augustin 1998; Salcedo, Wasserman et al. 1999) and neural glial cells (Sanders, Everall et al. 2000). Given the importance of CXCR4 in migration and mobilization, and the possible regulation of its expression by FGFR1, we investigated expression of this receptor in FGFR1 KO HSCs, and directly tested their ability to migrate.

4.2 Results

4.2.1 HSCs require FGFR1 for expression of CXCR4

To begin investigating the SDF-1/CXCR4 signaling pathway I analyzed gene expression of CXCR4 in HSCs during 5FU stress response. To do this I sorted LSK/Flk2$^-$ cells, a population enriched for LT-HSCs (Christensen and Weissman 2001), from wildtype BM samples at homeostasis (day 0), 3, 5, and 10 days after induction of stress by 5FU, isolated RNA, and performed real-time RT-PCR as described in Materials and Methods. I observed that CXCR4 mRNA expression slightly diminished at day 3, perhaps reflecting the nadir of HSCs after 5FU-induced cell death; peaked at day 5, when HSCs were expanding within BM and preparing to migrate (Lerner and Harrison 1990; Venezia, Merchant et al. 2004); and regressed at day 10, when HSCs were possibly starting to mobilize (Figure 4.1).

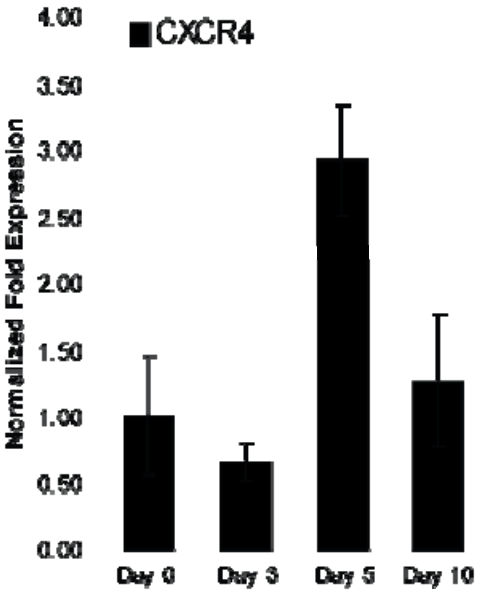

Figure 4.1. CXCR4 Expression in BM HSCs During Stress Response
Gene expression analysis of CXCR4 using real time RT-PCR on LSK/Flk2- cells sorted from BM of WT mice on the indicated days after 5FU injection. Expression is normalized against HPRT.

Then, I used flow cytometry to study surface expression of CXCR4 on HSCs. The percentage of $CXCR4^{+}$/LSK cells at homeostasis (day 0) was 2.6 fold higher in FGFR1 WT than in KO mice (p-value = 0.009), but increased to 12.7 fold at day 7 post-5FU (p-value = 0.0001) and dropped back to 5.8 fold at day 10 post-5FU (p-value = 0.0025) (Figure 4.2A). At day 7 post-5FU, though the intensity of CXCR4 staining on FGFR1 KO HSCs was higher than isotype control staining, it was indeed significantly lower than that of FGFR1 WT controls (Figure 4.2B).

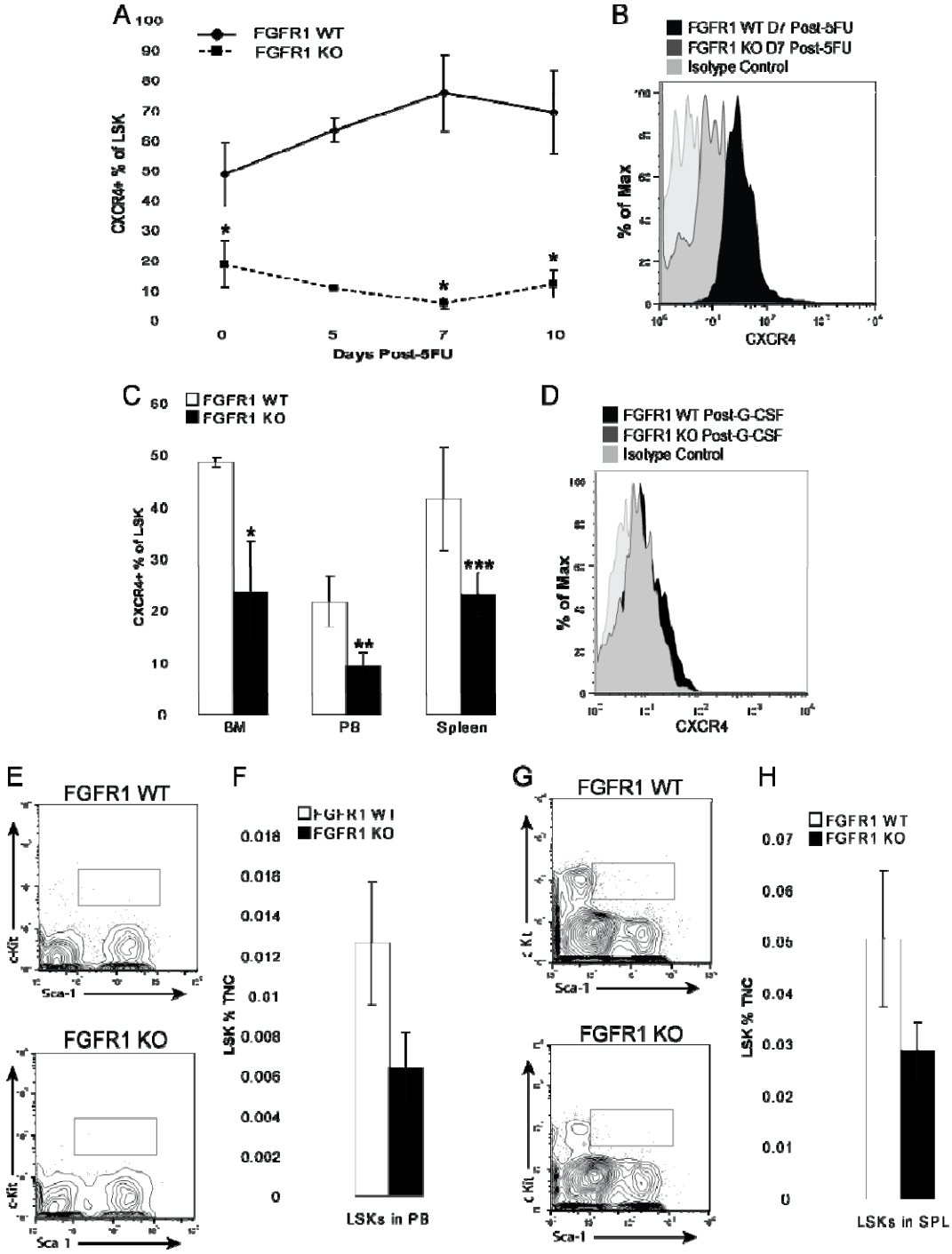

Figure 4.2. FGFR1 KO BM HSCs have Impaired CXCR4 Surface Expression but Normal Retention **(A)** Time course of CXCR4 surface expression as a percentage of LSK cells in BM of FGFR1 WT and KO mice 0 (*p-value = 0.009), 5, 7 (*p-value < 0.0001), and 10 (*p-value = 0.0025) days after 5FU injection. (**B)** Comparison of intensity of CXCR4 signal between FGFR1 WT and KO mice 7 days after 5FU treatment. Signal from a rat-IgG2a isotype control is shown. (**C)** Comparison of CXCR4 surface expression as a percentage of LSK cells in BM, PB, and spleen of FGFR1 WT and KO mice 5 days after G-CSF treatment (*p-value = 0.012, **p-value = 0.019, ***p-value = 0.042). (**D)** Comparison of intensity of CXCR4 signal between FGFR1 WT and KO mice 5 days after G-CSF treatment. Signal from a rat-IgG2a isotype control is shown. (**E)** Flow cytometric analysis of the LSK population (pre-gated on live, lineage-negative cells) in PB of FGFR1 WT and KO mice. (**F)** Comparison of the LSK % TNC in PB between FGFR1 WT and KO mice (n=4). (**G)** Flow cytometric analysis of the LSK population (pre-gated on live, lineage-negative cells) in spleens of FGFR1 WT and KO mice. (**H)** Comparison of the LSK % TNC in spleens between FGFR1 WT and KO mice (n=4). Analyses were carried out 4 weeks after PIPC induction of Cre. Error bars indicate SD.

Additionally, I examined surface expression of CXCR4 on HSCs during G-CSF induced mobilization. The percentage of CXCR4^{+}/LSKs was 2.1 fold higher in BM (p-value = 0.012), 2.3 fold higher in PB (p-value = 0.019), and 1.8 fold higher in spleens (p-value = 0.042) of FGFR1 WT than in KO mice (Figure 4.2C). Yet the intensity of CXCR4 staining after G-CSF (Figure 4.2D) was not as dramatically different between FGFR1 WT and KO mice as after 5FU (Figure 4.2B). Interestingly, we did not observe HSC retention defects in FGFR1 KO mice, despite reduced CXCR4 expression, as there was no difference in the number of HSCs in the PB or spleens of FGFR1 KO mice as compared to FGFR1 WT controls (Figure4.2E-H).

4.2.2 HSCs require FGFR1 for responsiveness to SDF-1

Considering these results and the *in vivo* mobilization data (discussed in Chapter 3), I predicted FGFR1 KO HSCs would be defective in their ability to migrate when placed in transwell migration assays *in vitro*. These assays utilize a two-chambered system separated by a microporous membrane to test migration of a target cell population in response to a chemotactic signal such as SDF-1 (Figure 4.3). In addition to SDF-1, FGF ligands have been reported to direct cell movements and act as chemoattractants for neural crest cells and mesenchymal cells (Thisse and Thisse 2005). Therefore, I began these experiments by comparing the migration of unfractionated, WT BM cells in response to FGF ligands to SDF-1. Though increasing concentrations of SDF-1 could induce migration of BM cells, similar concentrations of FGF1 or FGF4 failed to stimulate migration (Figure 4.4A). Due to the diverse cell types present

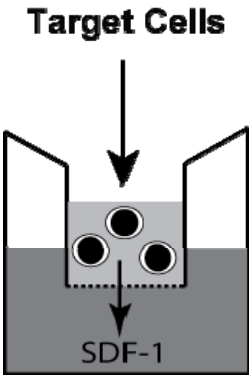

Figure 4.3. Outline of Transwell Migration Assay
A target cell population from BM were placed into the upper chamber, and their ability to migrate in response to various factors (typically SDF-1) was evaluated.

in BM, I chose to optimize transwell migration assays using flow sorted LSK cells as a target population, in order to specifically assay the migration of HSCs. Toward that end, I placed increasing numbers of sorted LSK cells in transwell assays with a concentration of SDF-1 known to stimulate chemotaxis of HSCs (Jo, Rafii et al. 2000). The number of cells that migrated to the lower chamber in response to SDF-1 was significantly higher in every case than the corresponding negative control (Figure 4.4B). I chose a starting input cell number of 2,500-5,000 LSK cells for future transwell migration assays. I continued to investigate the possibility that FGF ligands could directly stimulate migration of HSCs by comparing migration to SDF-1. However, even when using sorted LSKs in these migration assays, FGF1 could not stimulate chemotaxis above negative controls, where as SDF-1 could (Figure 4.4C). Additionally, given findings that FGF23 is expressed by endothelial cells of venous sinusoids within BM (Liu, Zhou et al. 2006), I assayed if this ligand could stimulate migration of HSCs. Recombinant Klotho was included in these assays to test its reported role as a co-factor that promotes FGF23 binding to FGF receptors (Kurosu, Ogawa et al. 2006). However, whether Klotho was present or absent, FGF23 could not stimulate migration of sorted LSK cells above negative controls; again SDF-1 served as a potent chemoattractant (Figure 4.4D). These results indicate that the FGF ligands tested cannot serve as direct chemoattractants for HSCs, at least at the concentrations tested.

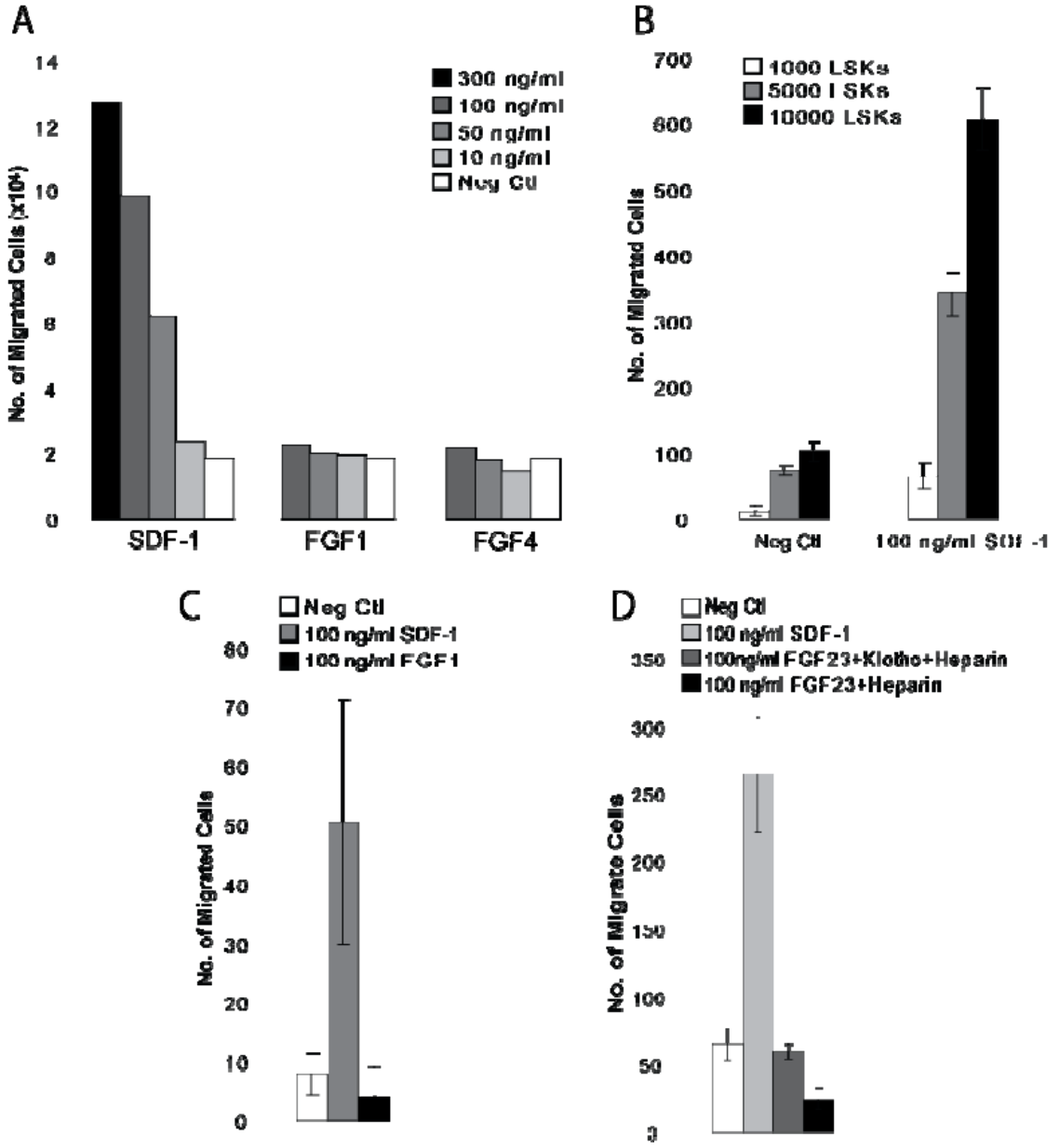

Figure 4.4. FGF Ligands do not Induce Migration of HSCs in Transwell Migration Assays
(A) Comparison of the ability of increasing concentrations of SDF-1, FGF1, and FGF4 to induce chemotaxis of unfractionated BM cells from FGFR1 WT mice. (**B)** Optimization of Transwell migration assays with varying input numbers of sorted LSK cells. (**C)** Comparison of the ability of equal concentrations of SDF-1 and FGF1 to induce chemotaxis of sorted LSK cells. The input for these assays was 2,500 LSK cells. (**D)** Comparison of the ability of equal concentrations of SDF-1 and FGF23 (with and without Klotho) to induce chemotaxis of sorted LSK cells. The input for these assays was 5,000 LSK cells. Error bars indicate S.D.

Given these results and my observation of reduced CXCR4 expression on FGFR1 KO HSCs, I focused on testing the migration of these cells in response to SDF-1. Initial

migration assays demonstrated that 2.2 times fewer FGFR1 KO BM cells migrated in response to 100 ng/ml SDF-1 than FGFR1 WT cells (p-value = 0.022), and 2.9 times fewer migrated in response to 300 ng/ml SDF-1 (p-value = 0.0007, Figure 4.5A). Furthermore, the number of FGFR1 KO LSK cells able to migrate in response to SDF-1 was 2.7 times fewer than FGFR1 WT LSK cells (p-value = 0.0005, Figure 4.5B). I also studied the effects of different chemical inhibitors on LSK cell migration from BM of FGFR1 WT mice. I found that an FGFR inhibitor (SU5402) reduced the number of LSK cells able to migrate 2.9 fold (p-value = 0.0042); a P13K inhibitor (LY294002) reduced the number 18.5 fold (p-value = 0.0001); and a MEK inhibitor (PD98059) reduced the number 2.1 fold (p-value = 0.0023) (Figure 4.5C). As PI3K and MEK are key molecules in the two major downstream pathways of the FGF/FGFR signaling complex, these results support our findings with FGFR1 KO and SU5402-treated WT LSK cells and indicate that FGFR1 is required SDF-1 induced chemotaxis.

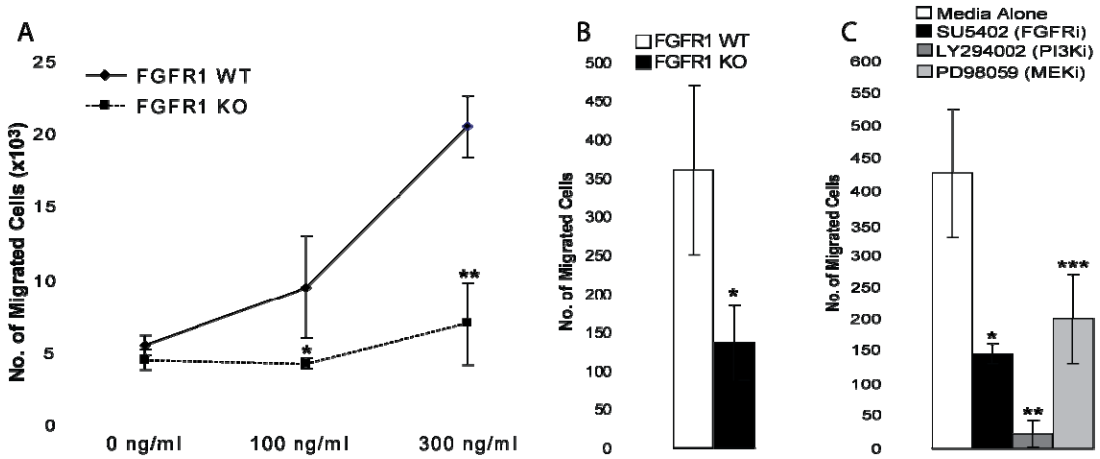

Figure 4.5. FGFR1 is Required for HSC Migration in Response to SDF-1
(A) Comparison of the chemotactic ability of FGFR1 WT and KO BM cells in response to increasing concentrations of SDF-1 (n=3, *p-value = 0.022, **p-value = 0.0007) . **(B)** Comparison of the chemotactic ability of FGFR1 WT and KO LSKs (n=4, *p-value = 0.0005). **(C)** Comparison of the chemotactic ability of wildtype LSKs in the presence of the following inhibitors (i): SU5402 (n=6, *p-value = 0.0042), LY294002 (n=6, **p-value = 0.0001), and PD98059 (n=6, ***p-value = 0.0023). Error bars indicate S.D.

4.3 Discussion

To begin elucidating the mechanism of the function of FGFR1 in stress response and mobilization of HSCs, I examined expression of the receptor CXCR4. These data indicate that surface expression of CXCR4 by HSCs is important for their response to stress-induced changes in SDF-1 gradients (Avecilla, Hattori et al. 2004) that induce HSC migration from BM. Intriguingly, FGFR1 KO mice have significantly fewer HSCs that express CXCR4 than do WT controls, both at homeostasis and during stress response, which may help explain the impaired mobilization of FGFR1-null HSCs to the peripheral circulation in response to 5FU. Indeed, though FGF ligands could not directly induce migration of HSCs, SDF-1 acted as a potent chemoattractant of HSCs *in vitro*, yet FGFR1-null HSCs displayed impaired responsiveness to SDF-1.

Furthermore, these results explain the lack of AMD3100-induced mobilization in

FGFR1 KO mice. Specifically, given the impaired expression of CXCR4 on FGFR1-null HSCs, these cells would be insensitive to AMD3100, as it has been reported that this antagonist of SDF-1 signaling must bind to CXCR4 on the cell surface to be effective (Rosenkilde, Gerlach et al. 2004). It remains to be determined which factors downstream of FGFR1 are mediating this activity, though evidence supports NF-κB (Helbig, Christopherson et al. 2003), c-Myc (Moriuchi, Moriuchi et al. 1999), and Hif-1α (Rahimi, George et al. 2010) as potential effectors.

Finally, though the mechanism of G-CSF remains unclear, the finding that FGFR1 KO mice mobilize normally in response to G-CSF is perhaps not surprising, as it has been reported that G-CSF-induced mobilization involves down-regulation of CXCR4 expression on target cells (Levesque, Hendy et al. 2003; De La Luz Sierra, Gasperini et al. 2007). Indeed, the percentage of FGFR1 WT HSCs in BM that were $CXCR4^+$ after G-CSF treatment was no higher than at homeostasis, and the intensity of staining was only slightly higher than isotype control. While some reports have indicated that upregulation of CXCR4 is important for G-CSF induced mobilization (Petit, Szyper-Kravitz et al. 2002), my results do not support such a claim. However, this does not settle the controversy over the mechanism of G-CSF, as variable among experimental systems could account for the different findings. A more thorough investigation is warranted to fully understand how G-CSF affects mobilization of HSCs. Nevertheless, it is clear that FGFR1-null HSCs are able to respond to G-CSF and mobilize, indicating that G-CSF-mediated mobilization does not require FGF signaling in HSCs.

Chapter 5

Mechanisms of mobilization – deletion of *Fgfr1* impairs HSC expansion *in vivo* and *in vitro*

5.1 Introduction

Migration out of the BM during the process of mobilization is only one of the necessecary steps HSCs must undergo during stress response. Induced BM damage, such as with the chemotherapeutic drug 5FU, leads to extensive loss of hematopoietic cellularity including loss of cycling HSCs, causing their numbers in BM to drop within the first week after treatment (Figure 5.1A). This cell loss leads to activation of remaining, quiescent HSCs and their expansion within BM. However, perhaps due to the inhibitory nature of endosteal niches present in BM, HSCs associate with vascular endothelium and mobilize out of the marrow and temporarily lodge in extramedullary organs such as the spleen. There, in a more permissive microenvironment, HSCs undergo rapid expansion to replenish lost cells and reconstitute the hematopoietic system (Heissig, Hattori et al. 2002; Yin and Li 2006).

I had already observed impaired mobilization of HSCs out of the BM in FGFR1 KO mice and demonstrated that FGFR1 was required for proper expression of CXCR4 and responsiveness to SDF-1, the absence of which resulted in diminished migration of FGFR1-null HSCs. Given that the FGF pathway also has well characterized functions in stimulating proliferation (Schlessinger 2000) and cell growth (Thisse and Thisse 2005), and more specifically, *in vitro* expansion of HSCs (de Haan, Weersing et al. 2003), I sought to assess the ability of FGFR1-null HSCs to expand *in vivo* during stress response as well as *in vitro*.

5.2 Results

5.2.1 FGFR1 is required for HSC expansion *in vivo*

5.2.1.1 HSC Expansion in BM

FGFR1 WT and KO mice showed similar nadirs of HSCs at day 7 post-5FU (Figure 5.1A). While BM HSCs in FGFR1 WT mice expanded dramatically 10 and 12 days post-5FU, FGFR1 KO mice displayed a slight but significant impairment in HSC expansion with 1.7 fold fewer HSCs on day 10 (p-value = 0.031) and 1.6 fold fewer on day 12 (p-value = 0.014) (Figure 5.1A). Similarly, LSK cells in BM of SCL/FGFR1 KO mice expanded 2.7 fold less than in SCL/FGFR1 WT 12 days post-5FU (p-value = 0.0006, Figure 5.1B-C). In contrast, the LSK percentages of total nucleated cells (% TNC) in BM of FGFR1 WT and KO mice treated with AMD3100 were neither significantly different from each other nor significantly different from animals treated with saline, indicating no effect on the proliferative status of HSCs prior to their mobilization with AMD3100 (Figure 5.1D). Unlike AMD3100, G-CSF does induce a slight increase in the proliferation of BM HSCs, though not as dramatic as with 5FU. After 3 and 5 days of G-CSF treatment, FGFR1 WT and KO mice showed similar increases in LSK numbers in BM (Figure 5.1E). These data indicate that FGFR1 is required for expansion of HSCs during stress response. Defects in the ability of FGFR1-null HSCs to expand became more pronounced in the spleens of 5FU-treated mice.

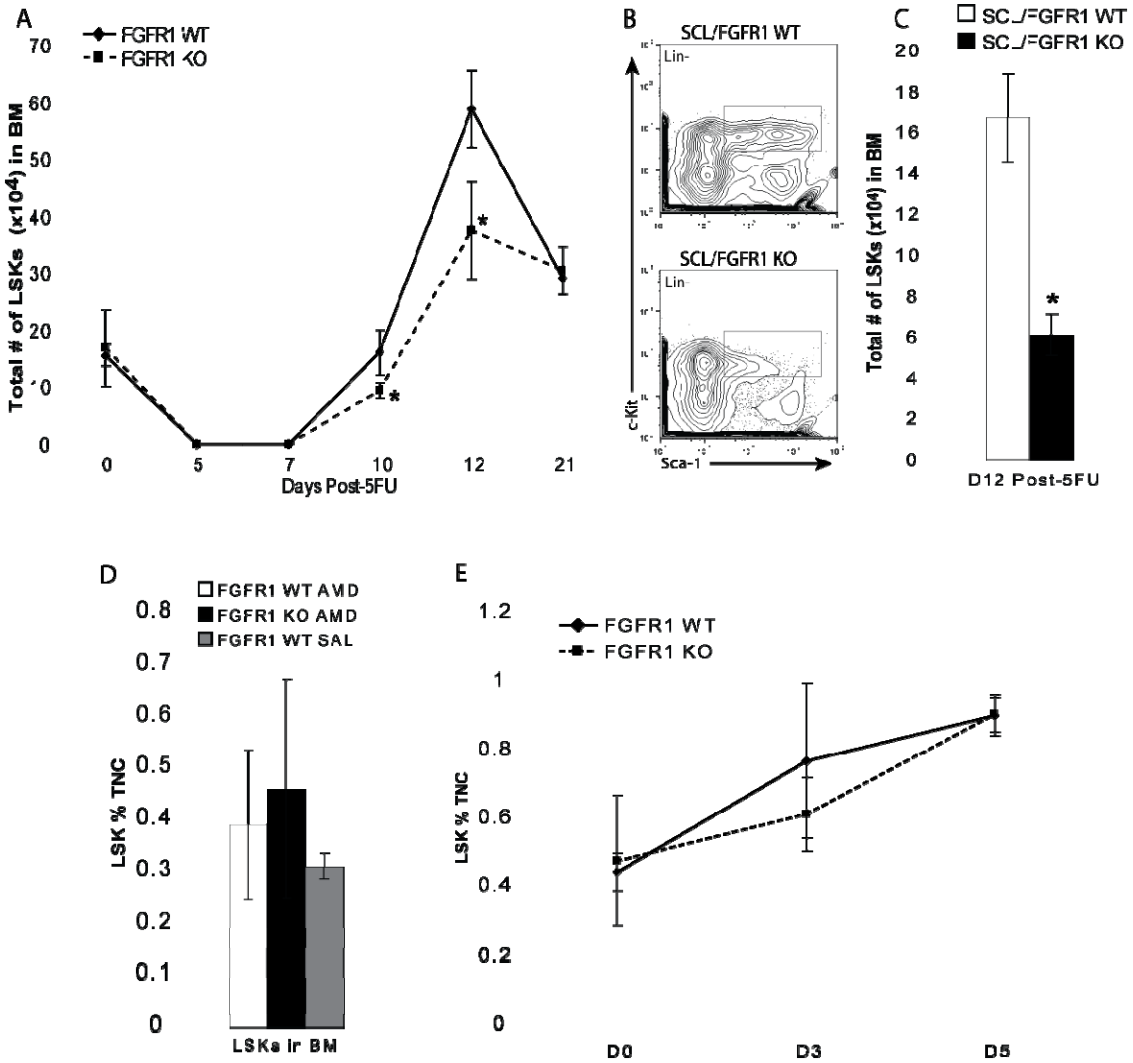

Figure 5.1. FGFR1 is Required for HSC Expansion in BM in Response to Stress
(A) Comparison of LSK numbers in BM of FGFR1 WT and KO mice at the indicated times after 5FU treatment (D10*p-value = 0.031, D12*p-value = 0.014). **(B)** Representative flow cytometric analyses of the LSK population (pre-gated on live, lineage negative cells) in BM of SCL/FGFR1 WT and KO mice after 5FU. **(C)** Comparison of absolute numbers of LSK cells in BM of SCL/FGFR1 WT and KO mice (n=3, *p-value = 0.0006). **(D)** Comparison of the LSK % TNC in BM of FGFR1 WT and KO mice 1 hour after treatment with AMD3100 and saline as a control (n=3). **(E)** Time course of the LSK % TNC in BM of FGFR1 WT and KO mice at steady state, 3, and 5 days after G-CSF treatment (n=3). Error bars indicate S.D.

5.2.1.2 Extramedullary HSC Expansion in Spleen

Normally, HSCs are restricted predominantly to BM and are found in the spleen only at low to undetectable levels (Figure 5.2A). HSCs mobilized to PB by 5FU, however, can transiently lodge in the spleen where they undergo robust expansion. At day 12 post-5FU, LSK cells expanded in FGFR1 WT spleens with 22.7 fold more cells than in KO spleens (p-value = 0.01, Figure 5.2B, 5.2C). Similarly, expansion of splenic LSK cells in SCL/FGFR1 KO mice was reduced 53.4 fold compared to WT (p-value = 0.001, Figure 5.2B, 5.2D).

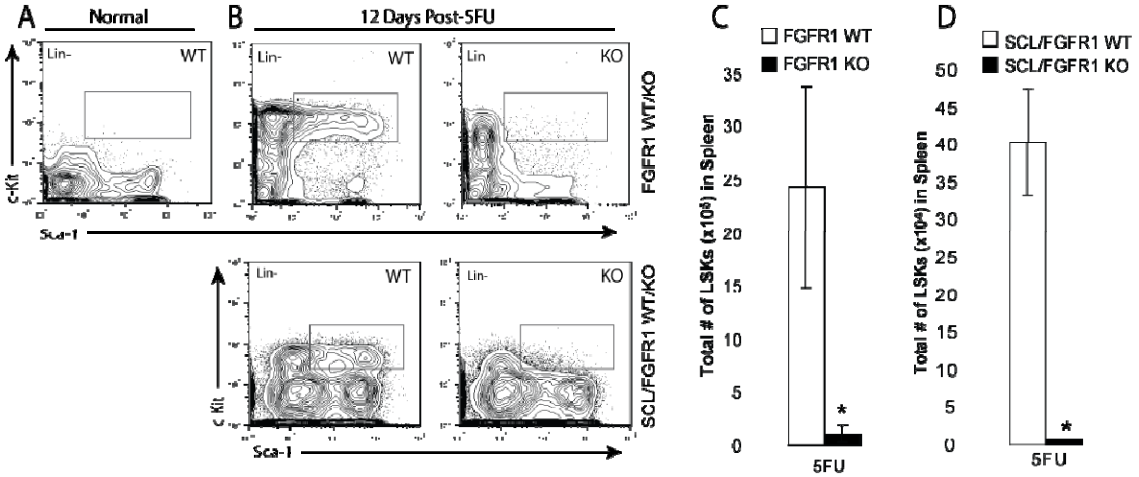

Figure 5.2. FGFR1 is Required for Extramedullary Expansion of HSCs in Spleen
(A-B) Representative flow cytometric analyses of the LSK population (pre-gated on live, lineage negative cells) in spleen at steady state (**A**), and FGFR1 and SCL/FGFR1 WT and KO spleen after 5FU (**B**). (**C-D**) Comparison of absolute numbers of LSK cells in spleens of FGFR1 WT and KO mice (n=4) (**C**) and SCL/FGFR1 WT and KO mice (n=3) (**D**) at the indicated times after 5FU (*p-value = 0.01 (**C**) and 0.001 (**D**)). Error bars indicate S.D.

G-CSF induces proliferation of splenic HSCs but to a lesser extent than 5FU. Following G-CSF treatment, FGFR1 and SCL/FGFR1 KO mice had LSK cell numbers comparable to WT (Figure 5.3A-B), indicating that what limited expansion occurs does not require FGFR1 in HSCs. As expected given AMD3100's rapid turnover,(Bonig, Chudziak et al. 2009) AMD3100 did not mobilize HSCs to spleen (Figure 5.3C-D), thus no extramedullary expansion of HSCs was observed in this organ.

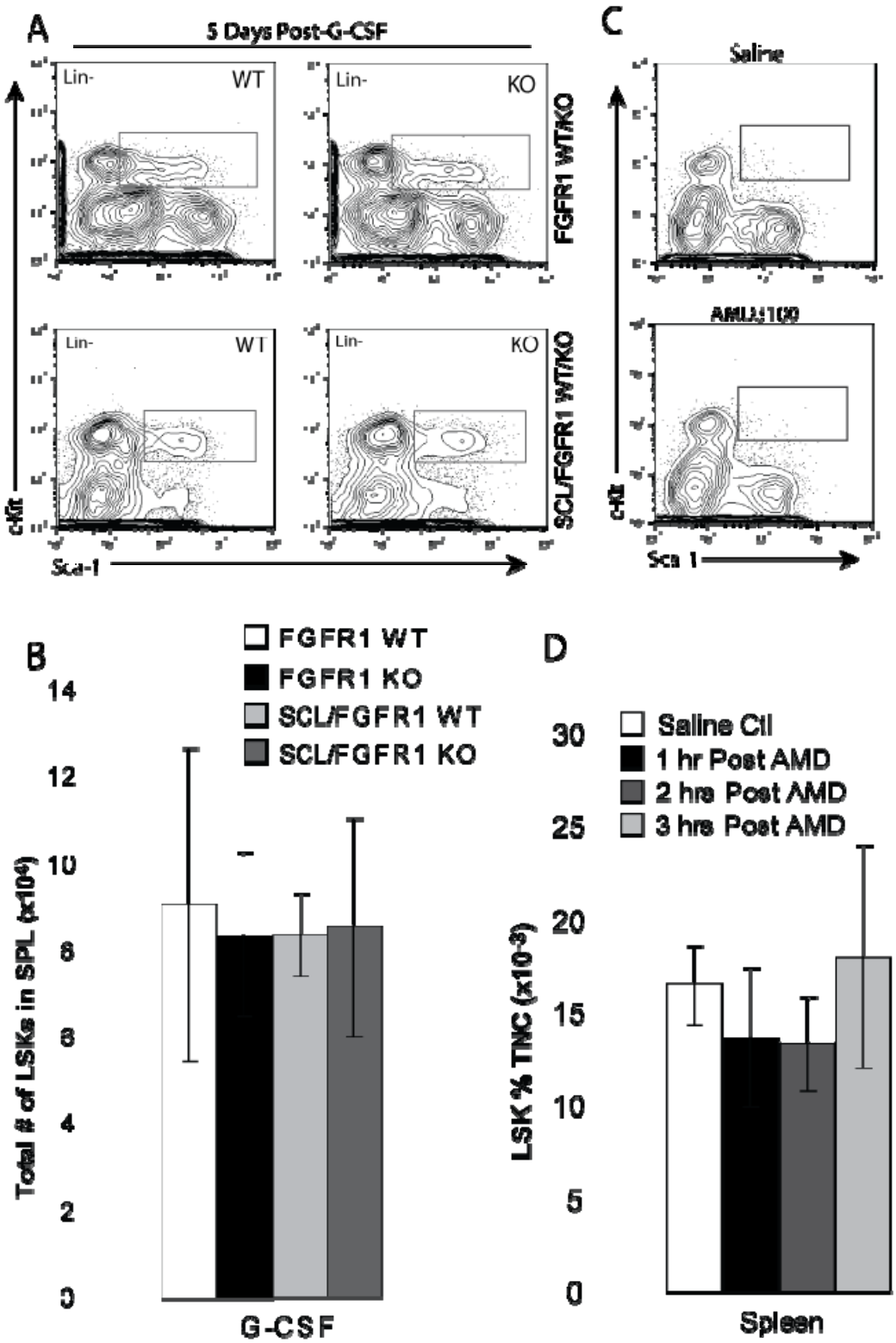

Figure 5.3. G-CSF and AMD3100 Stimulate Little to no Extramedullary Expansion of HSCs
(A) Representative flow cytometric analyses of the LSK population (pre-gated on live, lineage negative cells) in FGFR1 and SCL/FGFR1 WT and KO spleen after G-CSF. (**B)** Comparison of absolute numbers of LSK cells in spleens of FGFR1 WT and KO mice and SCL/FGFR1 WT and KO mice after G-CSF (n=3). (**C)** Flow cytometric analysis of the LSK population (pre-gated on lineage negative cells) in spleens of FGFR1 WT mice 1 hour after AMD3100 and saline injection. (**D)** Comparison of the LSK % TNC in spleen of FGFR1 WT mice 1, 2, and 3 hours after treatment with AMD3100 and saline as a control (n=3). Error bars indicate SD.

5.2.2 FGFR1 is required for HSC expansion *in vitro*

Given these *in vivo* data, I tested the ability FGFR1 KO HSCs to proliferate *in vitro*. I adapted a previously reported *ex vivo* HSC expansion technique (Zhang and Lodish 2005), as described in Materials and Methods. Initially, I wanted to gain experience with this culture technique and test different culture conditions on LSK/Flk2$^-$ cells sorted from BM of FGFR1 WT mice. As previously reported (Zhang and Lodish 2005), STIF media could support robust expansion of HSCs as compared to media without growth factors (Figure 5.4A, 5.4B). Moreover, our lab has determined that IGF-2 and FGF1 are dispensable for HSC expansion (Perry, J., unpublished observation); indeed, ST media appear to expand HSCs similarly to STIF media (Figure 5.4B, 5.4C). Interestingly, addition of the FGFR inhibitor SU5402 appeared detrimental to cell survival and expansion (Figure 5.4D). Given this data, I moved forward with testing the expansion of HSCs from FGFR1 KO mice.

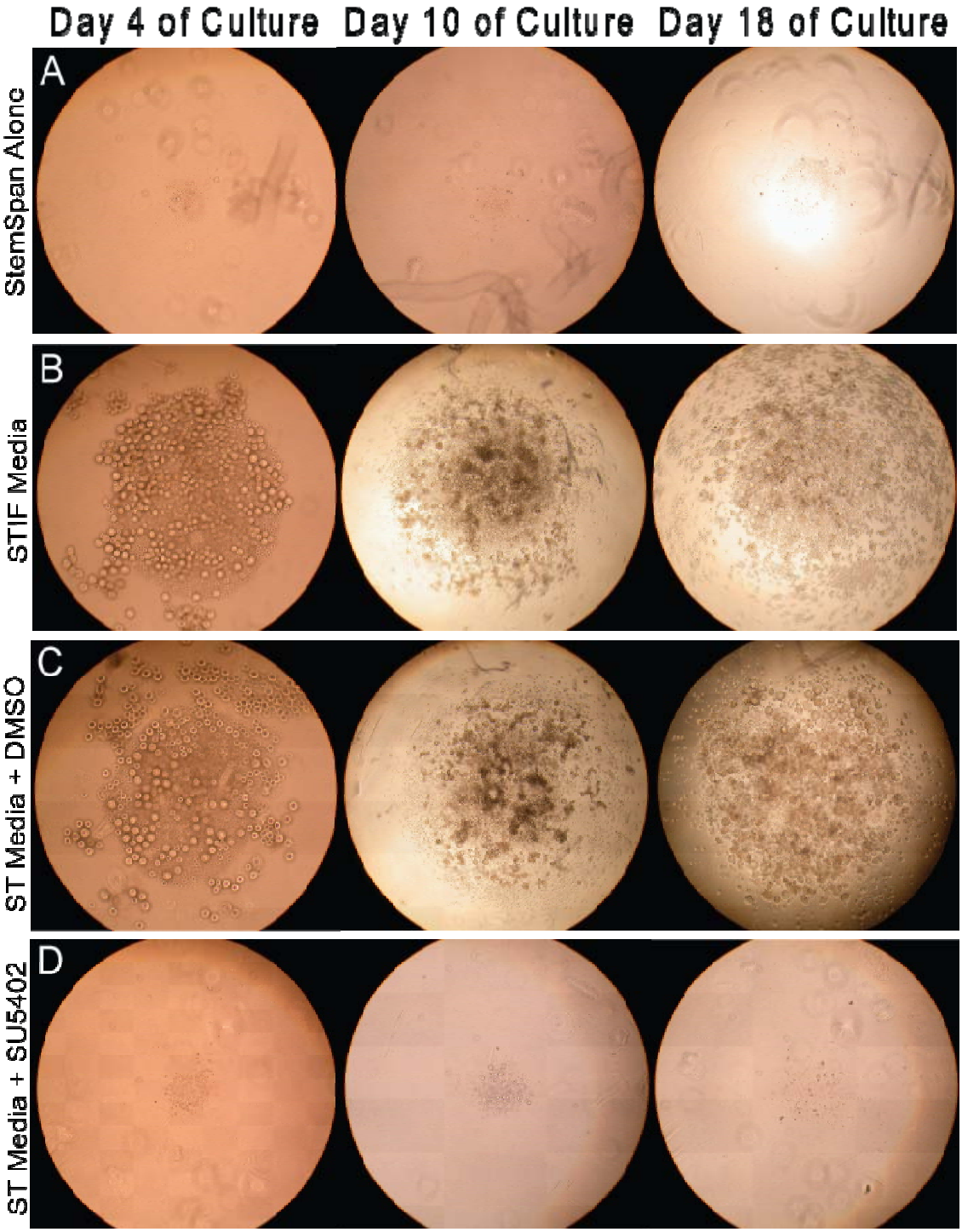

Figure 5.4. Expansion of HSCs *in vitro* Requires FGFR1 Even in Absence of Supplemented FGF Ligand

(A-D) Images of LSK/Flk2$^-$ cultures on days 4, 10, and 18 with StemSpan media alone (**A**), STIF media (**B**), ST Media + DMSO (**C**), and ST media + SU5402 (**D**).

Cultured BM HSCs from FGFR1 WT mice appeared via microscopy to expand much more robustly than FGFR1 KO cells (Figure 5.5A). Flow cytometry analysis revealed a significant loss of the LSK/Flk2$^-$ (or LT-HSC) population in the FGFR1 KO cultures compared to WT cultures (Figure 5.5B). Indeed, FGFR1 WT HSCs expanded in culture 10.1 fold more than FGFR1 KO HSCs (p-value = 0.0005, Figure 5.5C). Furthermore, the FGFR inhibitor SU5402 blocked expansion of WT HSCs *in vitro* 3.2 fold at 1μM (p-value = 0.0002) and 11.0 fold at 5 μM (p-value = 0.0001) (Figure 5.5C). Together these data indicate the importance of functional FGFR1 in the expansion of HSCs, an integral part of successful stress-induced mobilization.

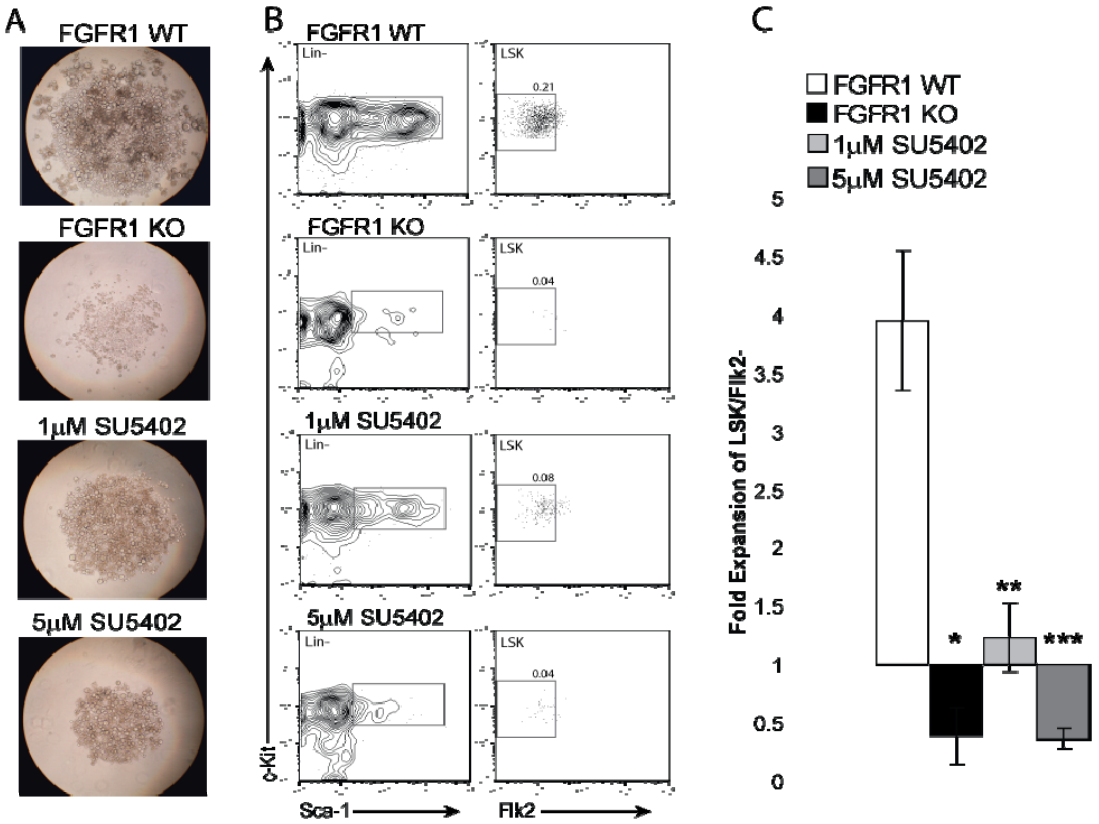

Figure 5.5. FGFR1 is Required for Expansion of HSCs in vitro
(A) Microscopic images of FGFR1 WT and KO (upper panels) and FGFR1 WT plus SU5402 (lower panels) cultures at 14 days. (**B)** Flow cytometric analysis of LSK/Flk2- phenotype (pre-gated on live, lineage negative cells) after 14 days of culture. Numbers indicate the frequency of TNC of the gated population. (**C)** Comparison of the fold increase of LSK/Flk2- cells from FGFR1 WT and KO mice (n=6, *p-value = 0.0005) and FGFR1 WT plus SU5402 at 1 (n=3, **p-value = 0.0002) and 5 (n=3, ***p-value = 0.0001) μM after 14 days of culture. Error bars indicate SD.

5.3 Discussion

After demonstrating defective migration of FGFR1-null HSCs I analyzed the other major step in stress response, stem cell expansion. This step is vital to the reconstitution of a compromised hematopoietic system, and it is likely that mobilization simply serves as a means for HSCs to reach and temporarily reside in a microenvironment that is devoid of inhibitory endosteal niches and is more permissive to rapid, robust expansion (Heissig, Hattori et al. 2002; Yin and Li 2006). Indeed, after 5FU treatment HSCs expanded within spleens of FGFR1 and SCl/FGFR1 WT mice 3-4 times more than in BM. Even so, defects in expansion of HSCs in FGFR1 and SCL/FGFR1 KO mice manifested first within BM. It is interesting to note that FGFR1 WT and KO mice exhibited similar nadirs of HSCs within BM 5 days post-5FU, indicating that FGFR1-null HSCs do not bear survival defects or are more prone to undergo apoptosis than HSCs in WT controls. Rather, differences were seen in HSC numbers at later time points during HSC expansion in BM, wherein FGFR1 KO mice began to display defects. It is not entirely clear why expansion of FGFR1-null HSCs was not completely blocked within BM; though a likely explanation is that alternate signaling pathways that are able to help promote cell cycle progression and proliferation become activated. Indeed, it has been shown that matrix metalloproteinase-9 (MMP-9) is activated as part of hematopoietic stress response, allowing MMP-9 to act on secondary targets that affect HSCs(Heissig, Hattori et al. 2002). Additionally, IFNa, produced by immune cells in response to challenge, can activate HSCs and promote their proliferation (Essers, Offner et al. 2009).

As I have mentioned, once mobilized into the peripheral circulation HSCs can lodge within organs such as the spleen and undergo significant extramedullary expansion. While

one obvious explanation for the apparent lack of splenic expansion in FGFR1 and SCL/FGFR1 KO animals is that they are severely impaired in the mobilization step requisite for HSCs to reach the spleen, it should be noted that there is not a complete block of mobilization within these mice. HSC numbers both in PB and spleen do increase above homeostatic levels, i.e. FGFR1-null HSCs are reaching the spleen during stress response, albeit in small numbers. However, it is clear that any FGFR1-null HSCs that reach the spleen are unable to respond to signals driving stem cell expansion.

My results with testing the expansion of FGFR1-null HSCs *in vitro* are also of interest. Though FGFR1-null HSCs only showed slightly diminished expansion in BM during stress response, LSK/FLK2$^-$ cells sorted from BM of FGFR1 KO mice were severely impaired in their ability to expand *in vitro*. As discussed, there are likely additional signals *in vivo* that are capable of supporting some expansion of FGFR1-null HSCs, yet putting these cells in defined culture medium with limited cytokines reveals the severe defect in expansion in the absence of functional FGFR1. Intriguingly, I observed this in the absence of added FGF ligands, however, work from our lab has shown that the major non-HSC component of these cultures is megakaryocytes (Perry, J., unpublished observation). Given the recent finding that megakaryocytes are capable of producing bFGF to support expansion of niche cells (Dominici, Rasini et al. 2009), it is likely that "naturally produced" FGF ligands are still playing an integral role in expansion of HSCs by this culture method, and are simply not required to be artificially supplemented. Furthermore, this data indicates that signaling of SCF through the c-Kit receptor alone is not sufficient to support expansion of HSCs, as FGFR1-null HSCs are unable to expand, even with the addition of SCF in the defined culture media.

Chapter 6

Differential effects on bFGF levels in bone marrow and spleen by 5FU and G-CSF

6.1 Introduction

After demonstrating that FGFR1 signaling is required in HSCs to facilitate migration and expansion of HSCs during stress response, I became interested in understanding which ligand or ligands might be stimulating this receptor *in vivo* to drive these processes. However, as discussed in Chapter 1, the FGF signaling pathway is large and complex, with at least 8 of the 23 known ligands capable of activating FGFR1 (Ornitz, Xu et al. 1996; Zhang, Ibrahimi et al. 2006). My investigation became much more focused with a published report of megakaryocyte-produced bFGF capable of stimulating niche expansion. As discussed in Chapter 1, in this study mice receiving a lethal dose of total body irradiation exhibited a radioresistant population of megakaryocytes in BM that relocalized to the bone surface. There they produced growth factors including bFGF that stimulated expansion of niche cells and supported engraftment of donor HSCs (Dominici, Rasini et al. 2009). This coupled with findings that bFGF expands HSCs in culture (Yeoh, van Os et al. 2006), as well as observations made by our lab in the *in vitro* expansion of HSCs (discussed in the previous chapter), led me to analyze megakaryocytes and bFGF levels in FGFR1 WT and KO mice at homeostasis and after treatment with 5FU, G-CSF, and AMD3100, in an effort to determine if bFGF could be a stress-induced factor that activated the FGF signaling pathway in HSCs to promote stress response.

6.2 Results

6.2.1 Enumeration of megakaryocytes in BM and spleen

Using CD41 as a marker to enumerate megakaryocytes in BM and spleen by flow cytometry, I found that at homeostasis there was no difference in megakaryocytes as determined by $CD41^{+}$ % TNC between FGFR1 WT and KO mice either in BM or spleen (Figure 6.1A, 6.1B). 5FU and G-CSF induced slight increases in megakaryocytes in FGFR1 WT mice in BM, and these increases were also seen in FGFR1 KO animals (Figure 6.1A). As expected, given its negligible effect on HSC proliferation (Figure 5.1D), AMD3100 had no effect on megakaryocyte numbers in BM of FGFR1 WT or FGFR1 KO mice (Figure 6.1A).

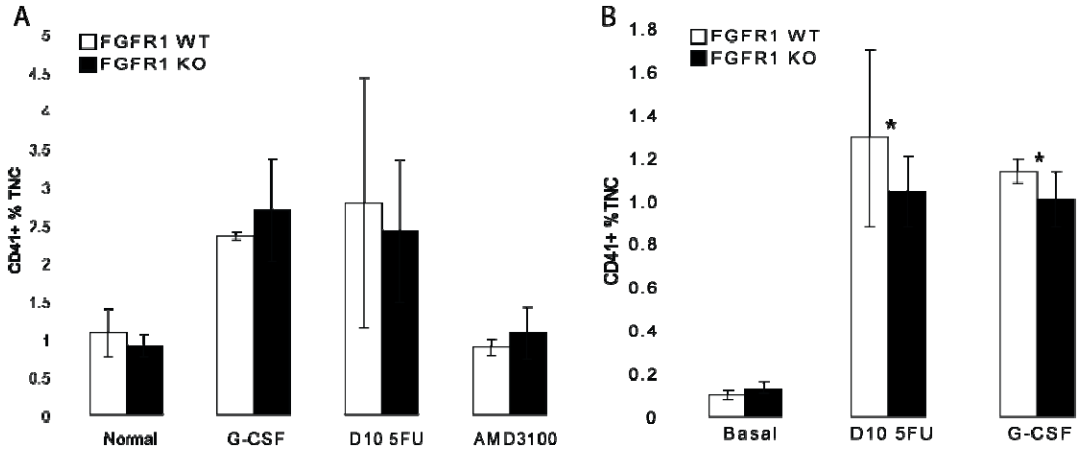

Figure 6.1. 5FU and G-CSF Induce Changes in Megakaryocyte Number in BM and Spleen
(A) Comparison of megakaryocyte numbers (based on expression of CD41) in BM of FGFR1 WT and KO at homeostasis and after treatment with 5FU, G-CSF, and AMD3100. (**B)** Comparison of megakaryocyte numbers (based on expression of CD41) in spleens of FGFR1 WT and KO at homeostasis and after treatment with 5FU and G-CSF (*p-value = 0.0001). Error bars indicate SD.

Interestingly, the number of megakaryocytes in spleens of FGFR1 WT and KO mice increased more than 9 fold after 5FU as wells as after G-CSF treatment, as compared to homeostasis (p-value = 0.0001, Figure 6.1B).

6.2.2 Analysis of bFGF levels in BM and spleen

Analysis of BM sections for bFGF protein expression by immunohistochemistry revealed that in FGFR1 WT and KO mice at steady state bFGF was diffusely expressed throughout the marrow. Specifically, megakaryocytes, identified as large, multinucleated cells that stained positive for vWF (Schick, Walker et al. 1997) in separate sections (Figure 6.2), were observed to stain positive for bFGF (Figure 6.3A, 6.3B). At 10 days after 5FU treatment, a time when HSCs as well as megakaryocytes are proliferating, numerous bFGF-positive megakaryocytes were observed scattered throughout the marrow in both FGFR1 WT and KO animals (Figure 6.3C, 6.3D). Interestingly, bFGF staining of BM sections from G-CSF treated FGFR1 WT and KO mice appeared less prevalent than in steady state sections, and in particular megakaryocytes lacked expression of bFGF (Figure 6.3E, 6.3F). AMD3100 treatment did not appear to dramatically affect bFGF expression in BM, as sections from FGFR1 WT and KO mice appeared similar to that of steady state with bFGF-positive megakaryocytes present (Figure 6.3G, 6.3H).

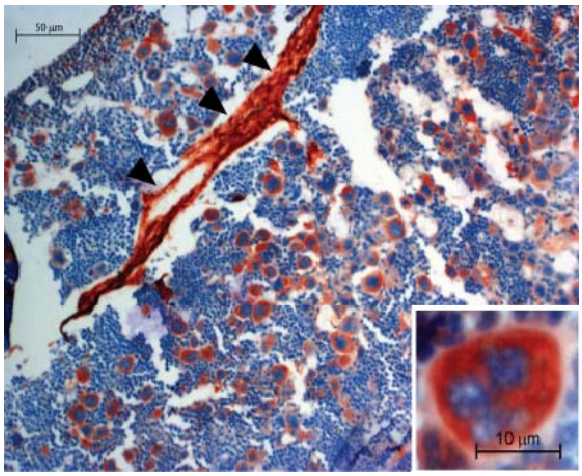

Figure 6.2. vWF Stains Megakaryocytes
BM from an FGFR1 WT mouse 10 days post-5FU stained with an antibody against von Willebrand Factor (vWF, red). vWF stains megakaryocytes (inset) as well as blood vessels (arrowheads).

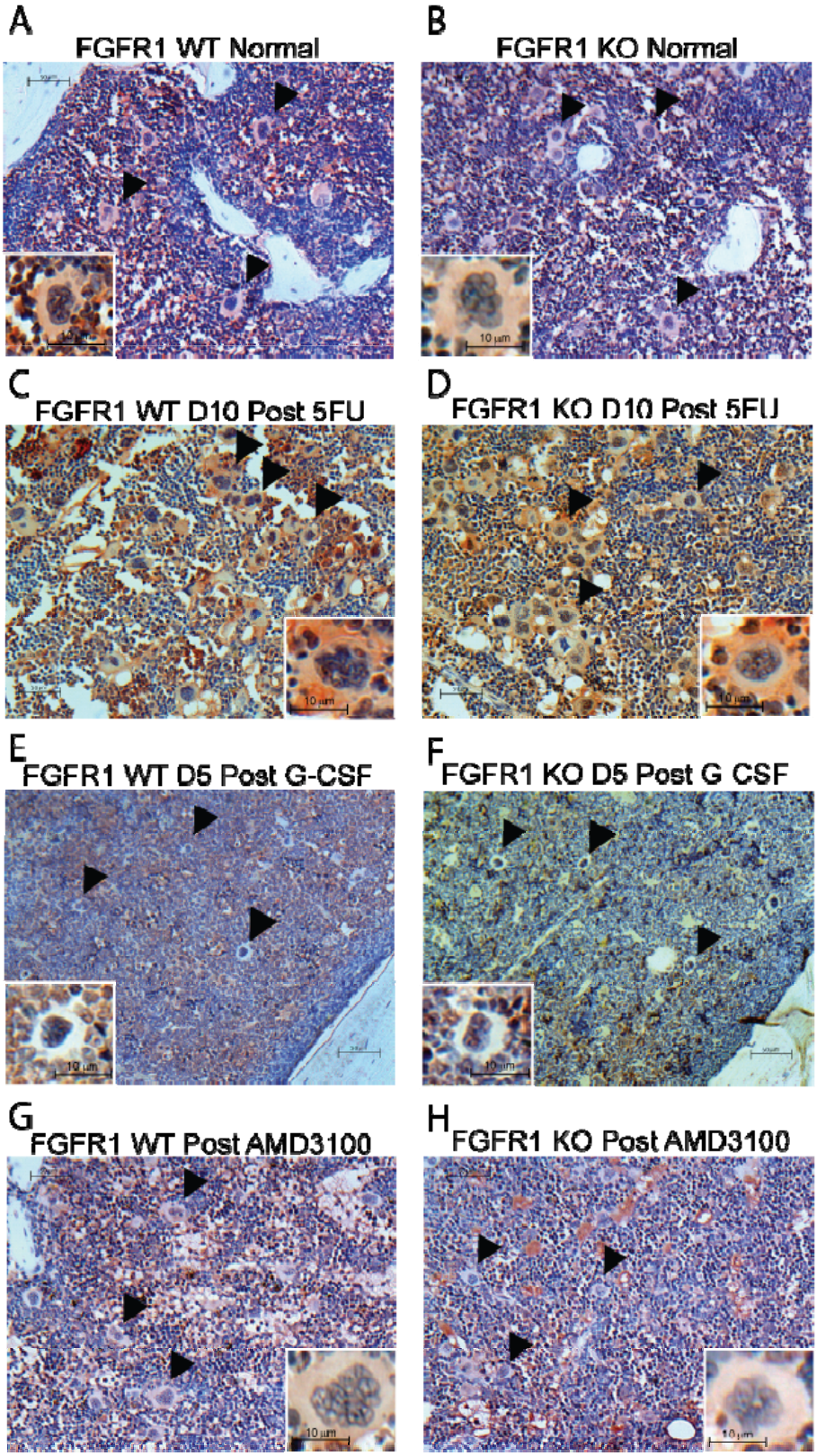

Figure 6.3. bFGF Levels in BM are Affected Differently by 5FU and G-CSF
(A-H) Representative bFGF staining (red) on BM sections from FGFR1 WT and KO mice under normal conditions (A, B), after 5FU (C, D), G-CSF (E, F), and AMD3100 (G, H).

In collaboration with Tomer Itkin and Tsvee Lapidot at the Weizmann Insitute of Science, we quantified the amount of bFGF protein present by enzyme-linked immunosorbent assays (ELISAs) on BM supernatants collected from FGFR1 WT animals at homeostasis, and after 5FU, G-CSF, and AMD3100 treatment as described Materials nad Methods. The bFGF protein level in BM increased 1.5 fold 10 days post 5FU (p-value = 0.02, Figure 6.4). In contrast G-CSF treatment reduced the amount of bFGF protein present in BM 2.0 fold (p-value = 0.0004, Figure 6.4). Treatment with AMD3100 resulted in a slight but not statistically significant reduction in bFGF protein levels (Figure 6.4).

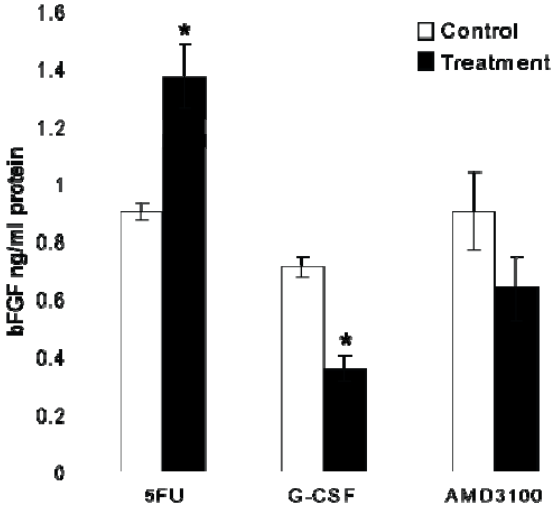

Figure 6.4. Changes in bFGF Protein Detected by ELISA
bFGF protein levels as determined by ELISA of BM supernatants of FGFR1 WT mice under normal conditions, after 5FU (*p-value = 0.02), G-CSF (*p-value = 0.0004), and AMD3100. Error bars indicate S.E.; n=3.

Because this ELISA data supported my initial observations in BM by immunohistochemistry, I investigated spleen sections for bFGF expression. As in BM, bFGF expression in spleen appeared to increase significantly 10 days post-5FU (Figure 6.5C, 6.5D) and to decrease after G-CSF (Figure 6.5E, 6.5F). Splenic bFGF expression after AMD3100 (Figure 6.5G, 6.5H) appeared similar to homeostasis (Figure 6.5A, 6.5B). Collectively these data indicate that production of bFGF is increased after stress, providing FGFR1 stimulation to support HSC expansion. These data also provide further insights into the mechanism of G-CSF mobilization in that G-CSF operates in a FGFR1 independent manner.

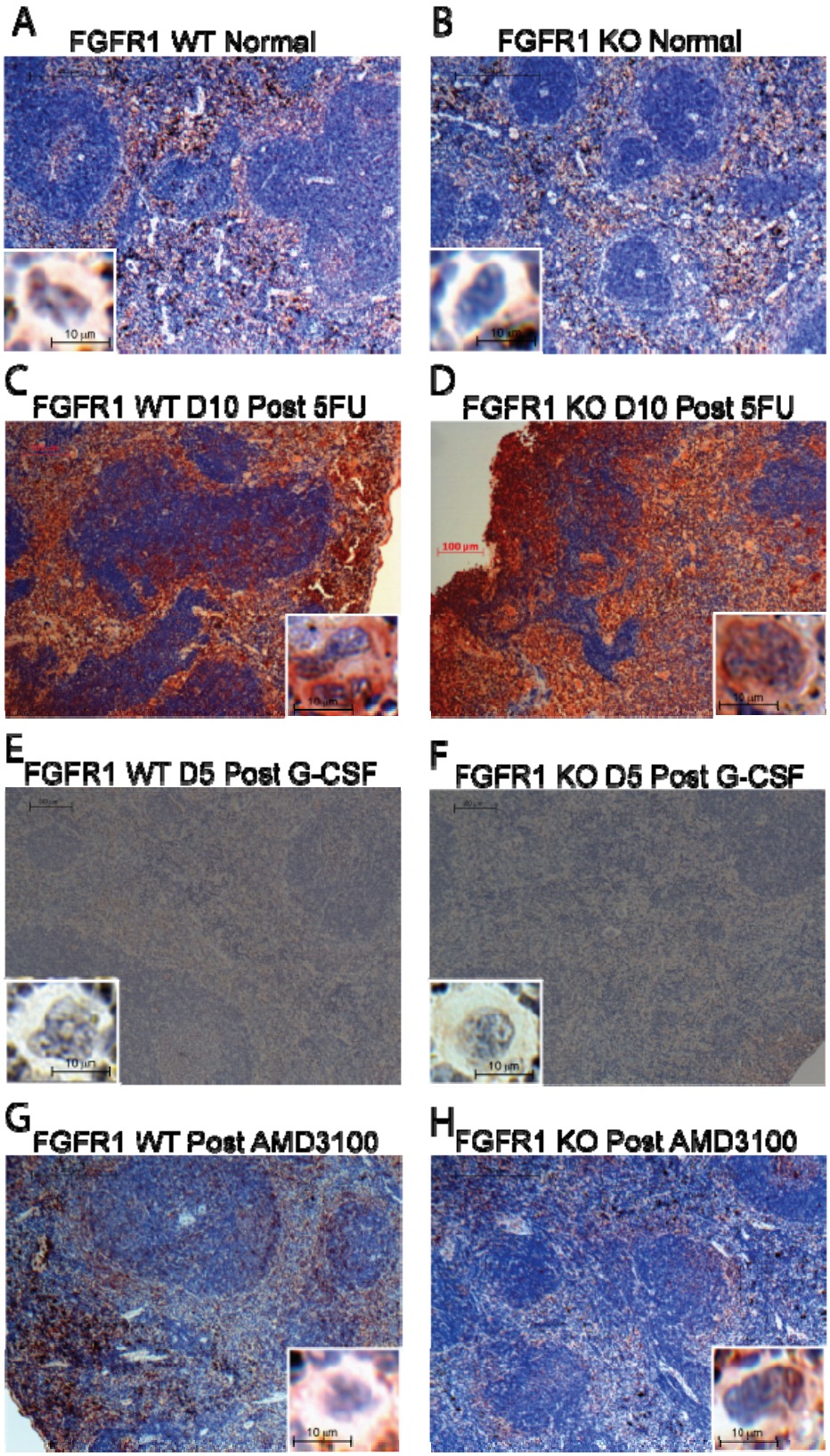

Figure 6.5. bFGF Levels in Spleen are Affected Differently by 5FU and G-CSF
(A-H) Representative bFGF staining (red) on spleen sections from FGFR1 WT and KO mice under normal conditions (A, B), after 5FU (C, D), G-CSF (E, F), and AMD3100 (G, H).

6.3 Discussion

While the involvement of other FGF ligands has not been determined, these results indicate a role for bFGF as a stimulator of FGFR1 and its subsequent functions in HSCs *in vivo*. bFGF levels increased in BM and spleen after 5FU treatment, as seen both by immunohistochemistry on tissue sections and ELISA on BM supernatants. These data indicate that the FGF pathway, and more specifically bFGF/FGFR1 signaling, is activated as part of the hematopoietic stress response. While it is difficult to make quantitative judgments based on immunohistochemistry data, it is interesting to note that it appears that bFGF levels in spleen increase more dramatically after 5FU than in BM. Furthermore, tt is interesting to note that megakaryocytes increased in both BM and spleen after stress, yet the increase in spleen was nearly 10 fold above homeostatic levels, while the increase seen in BM was only ~3 fold. These findings correlate with observations made in the expansion of HSCs after stress, specifically that expansion within the spleen is greater than within BM.

Additionally, our results further elucidate that G-CSF induced mobilization acts through a FGFR1-independent mode, as bFGF levels were found to decrease in BM and spleen after G-CSF treatment. Indeed, bFGF was recently identified as a target of neutrophil elastase (Ai, Cheng et al. 2007). Neutrophil elastase is one of several proteases (including MMP-9) that are released by mature hematopoietic cells in response to G-CSF and remodel the BM microenvironment and stimulate HSC mobilization (Thomas, Liu et al. 2002). These data indicate that, like c-Kit (Heissig, Hattori et al. 2002) and SDF-1 (Christopher, Liu et al. 2009), bFGF produced by cells and deposited in the extracellular matrix of the BM and spleen is degraded during G-CSF-induced mobilization. It remains unknown what purpose this down-regulation serves, though it is interesting to speculate that

exogenous G-CSF does not activate the same cellular response as stress/BM damage. The bFGF/FGFR1 signaling axis may represent an “emergency” program only activated in times of extreme need.

Chapter 7

General Discussion

In this dissertation, I set out to understand the potential role of FGFR1 in the regulation of HSCs. Previous work by our lab and others found that *Fgfr1* is expressed predominantly within HSCs (Akashi, He et al. 2003; de Haan, Weersing et al. 2003), and published reports have built upon this finding, primarily by demonstrating that FGF ligands can maintain and expand HSCs in culture (de Haan, Weersing et al. 2003; Zhang and Lodish 2005; Yeoh, van Os et al. 2006). However, it remained unknown to what extent, if any FGFR1 impacted the function of HSCs *in vivo*. Therefore, I bred conditional knockout mouse lines to obtain models of *Fgfr1* gene deletion within the adult hematopoietic system and carried out loss-of-function studies. I characterized the primary phenotype of FGFR1 KO mice as having normal hematopoiesis during homeostasis, but exhibiting defective stress-responsive mobilization of HSCs (Chapter 3).

Furthermore, I elucidated the mechanisms of this defect and demonstrated that FGFR1-null HSCs do not maintain appropriate expression of CXCR4 and are unable to respond to the CXCR4 ligand SDF-1 in migration assays (Chapter 4). I also showed that FGFR1-null HSCs display impaired expansion both *in vivo* during stress response as well as *in vitro* (Chapter 5). Additionally, I found evidence that bFGF, produced in part by megakaryocytes, may serve a major role during stress response as an activator of FGFR1 on HSCs, promoting their migration and expansion (Chapter 6). Concurrently, during these analyses I generated data that adds insight to the mechanisms of HSC mobilization by the clinically used agents AMD3100 and G-CSF (Chapters 3-6).

7.1 Insights into SDF-1/CXCR4 signaling

7.1.1 SDF-1 gradients during stress response

It is well documented that, during hematopoietic stress response, SDF-1 gradients change dramatically. SDF-1 levels within BM decrease, while levels within vasculature and the peripheral circulation are elevated, as seen by increased protein concentration within blood plasma (Heissig, Hattori et al. 2002). Recent work has demonstrated that bFGF is able to down-regulate SDF-1 production by BM stromal cells. This regulation was shown by *in vitro* studies on BM stromal cell lines to be at the protein, rather than gene expression level (Nakayama, Mutsuga et al. 2007; Nakayama, Mutsuga et al. 2007). It is intriguing to consider these findings in light of my observations of increased bFGF levels in BM after 5FU treatment. It may be that in addition to effects on HSCs, bFGF may also be acting on cells of the BM stroma by decreasing their secretion of SDF-1, thereby playing a direct role in changing SDF-1 gradients *in vivo*. With concomitant regulation of CXCR4 expression on HSCs, bFGF could be promoting migration of HSCs in a multi-faceted manner during stress response. Furthermore, it has been shown that SDF-1 and FGF4 synergize to promote association of megakaryocyte precursors with BM sinusoids, which promotes their survival, maturation, and expansion (Avecilla, Hattori et al. 2004). Indeed, such activity within the vascular niche of the BM could support expansion of megakaryocytes that could, in turn support stress response of HSCs via bFGF. It is interesting to consider the possibility of "waves" of production of different FGF ligands, as has been documented during somitogenesis of the developing embryo (Dale, Malapert et al. 2006; Wahl, Deng et al. 2007). Alternatively, FGF ligands and SDF-1 could directly promote association of HSCs with BM endothelium, leading to their mobilization.

7.1.2 CXCR4 and HSC retention

Via binding of its ligand SDF-1, CXCR4 has varied roles throughout development as well as in the adult, particularly within the hematopoietic system. Inactivation of CXCR4 or SDF-1 results in an embryonic lethal phenotype, with more pronounced defects in the BM than the fetal liver, indicating a potential role for CXCR4 in colonization of the BM in the latter stages of fetal development (Nagasawa, Hirota et al. 1996; Ma, Jones et al. 1998; Tachibana, Hirota et al. 1998; Zou, Kottmann et al. 1998). Indeed, the role of CXCR4 in directing the chemotaxis of HSCs through embryonic tissues has been well characterized (Christensen, Wright et al. 2004). However, CXCR4 has also been implicated in the retention of HSCs within BM. Transplantation of CXCR4-/- fetal liver cells to adult irradiated recipient mice results in impaired reconstitution of the hematopoietic system. Investigation demonstrated that these cells, while unaffected in their ability to home to the marrow, were defective in their retention, as CXCR4-/- HSCs were constitutively mobilized to the peripheral circulation (Foudi, Jarrier et al. 2006). Additional support came from the finding that AMD3100 mobilizes HSCs from BM by interrupting interaction of CXCR4 with SDF-1 (Liles, Broxmeyer et al. 2003; Devine, Flomenberg et al. 2004). However, conflicting results were obtained by conditional deletion of CXCR4 in adult mice. While HSCs were found to be reduced within BM of knockout mice, no concomitant increase in circulating HSCs was observed, indicating that reduced retention was not the cause of HSC loss in BM (Sugiyama, Kohara et al. 2006). Moreover, in my studies though FGFR1-null HSCs displayed reduced CXC4 expression, I did not observe defects in their retention.

It is difficult to ascertain precisely what roles CXCR4 and FGF signaling may be playing in the retention of HSCs, though a recent study on the metastasis of tumor cells

may offer some insight. It was found that N-cadherin prevented ligand-induced internalization of FGFR1 by bFGF, increasing stability of FGFR1 at the cell surface, and prolonging downstream signaling of this complex via the MAPK/ERK cascade. This resulted in increased migration and cellular invasion of tumor cells (Suyama, Shapiro et al. 2002). Considering these findings in light of work by our lab on the role of N-cadherin in HSCs, specifically that N-cadherin mediates adhesion of HSCs to endosteal niche cells (Zhang, Niu et al. 2003) and promotes stem cell quiescence (Haug, He et al. 2008), it may be that in the absence of FGFR1, despite reduced CXCR4 expression that might otherwise lessen the retention of HSCs through SDF-1, N-cadherin functions primarily as an adhesion molecule, unable to promote migration of HSCs in the presence of bFGF.

7.2 Insights into HSC Expansion

Expansion of HSCs in response to BM damage is vital to recovery of the hematopoietic system, and by extension survival of the organism. Yet such need conflicts with the imperative to preserve HSCs in a quiescent state to maintain the stem cell pool during homeostasis, which is accomplished through tight regulation of HSCs by cells of the endosteal niche (Perry and Li 2007). As discussed in Chapter 5, the purpose of mobilization may be to facilitate escape of HSCs from inhibitory signals of BM niches and allow them to temporarily reside in more stimulatory microenvironments that promote HSC expansion. Data from this dissertation and other studies indicate that the spleen, which normally acts as a filter of aged RBCs and foreign matter from the blood as well as a reservoir of RBCs and immune cells (Mebius and Kraal 2005) can serve as an ideal site for rapid, robust expansion of HSCs that might not be possible with the BM. Indeed, the spleen has been found to serve as a minor hematopoietic organ during ontogeny, as HSCs have been detected within

the spleen during the time of BM colonization by fetal liver HSCs as well as at early time points after birth (Wolber, Leonard et al. 2002; Christensen, Wright et al. 2004). It may be that the spleen reaquires HSC supportive function normally restricted to fetal stages during stress response in the adult.

In fact, the spleen can serve as a site of limited hematopoiesis when normal BM niches are disrupted. For example, targeted deletion of the retinoblastoma (Rb) gene in hematopoietic tissues results in eventual degradation of osteoblastic niche cells by bone absorbing osteoclasts, mobilization of HSCs, and extramedullary hematopoiesis in the spleen. There HSCs undergo extensive proliferation and myeloid differentiation, setting the stage for development of preleukemic myeloproliferative disorders (Walkley, Shea et al. 2007). Furthermore, disruption of retinoic acid receptor γ (RARγ) within hematopoietic tissues results in a similar phenotype, with mice exhibiting loss of endosteal niche, extramedullary hematopoiesis in the spleen, and myeloproliferative disorder (Walkley, Olsen et al. 2007). These data indicate that the spleen may be capable of supporting expansion of HSCs, but can only do so for limited periods of time, such as during stress response, else risk stem cell disorders.

7.3 Model of Role of FGFR1 in HSC Function

Based on the data generated during the course of this dissertation, I propose a model in which during adult homeostasis, FGFR1, although expressed on HSCs, is largely inactive and not required for HSC self-renewal and differentiation in BM, functions regulated by other signaling pathways (Figure 6A). However, in the event of hematopoietic tissue damage, the FGFR1 signaling pathway is activated and promotes limited HSC expansion within and migration from BM, as well as subsequent extramedullary expansion of HSCs during

stress response (Figure 6C-D). Such a model explains why deletion of FGFR1 in adult hematopoietic tissues does not result in any HSC defect, as FGFR1 signaling would not be routinely activated in HSCs during homeostasis. It may be that this FGF signaling program is activated only during extraordinary times of need for rapid, significant HSC expansion, such as during recovery from BM damage, as unchecked or promiscuous activation could lead to HSC exhaustion as well as replicative mutations that promote tumorigenesis.

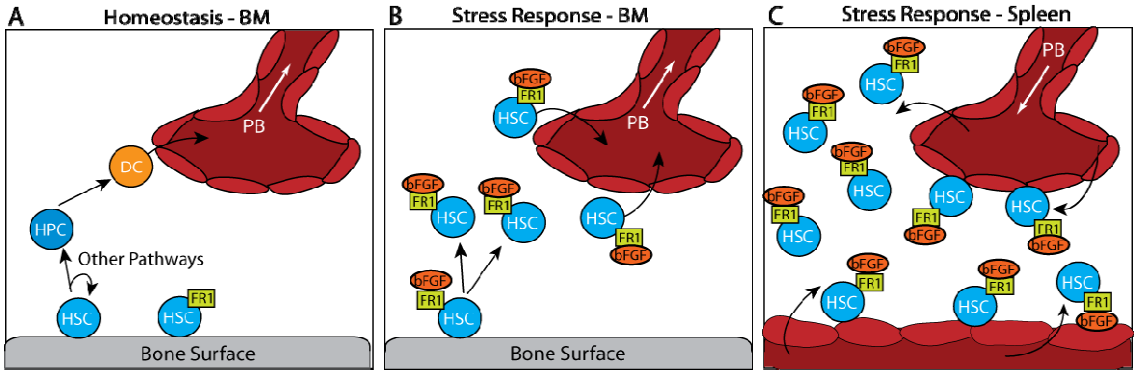

Figure 7.1. Model of Role of FGFR1 in HSC Function
(A) In the adult FGFR1 (FR1) is not required for normal hematopoietic stem cell (HSC) maintenance of the hematopoietic system in BM, as other signaling pathways promote self-renewal of HSCs and commitment to progenitors (HPC) and differentiated cells (DC). **(B)** bFGF/FGFR1 signaling is activated and promotes limited HSC expansion in and emigration from bone marrow (BM) to the peripheral blood (PB), as well as dramatic extramedullary expansion in the spleen **(C)** during stress response.

7.3.1 Implications of Model

7.3.1.1 bFGF as a radioprotective factor

The observations made in this dissertation implicate bFGF/FGFR1 signaling in HSCs as a critical part of hematopoietic stress response. Though I have focused on using the chemotherapeutic drug 5FU to initiate stress response in animal models, another well studied and clinically used inducer of BM damage is exposure to ionizing radiation. Exposure of mice to 8.5-10 grays of ^{137}Cesium constitutes a lethal dose primarily due to ablation

of the BM (Floersheim 1992). Considering the studies in my dissertation, it follows that FGF ligands may have radioprotective qualities, i.e. they may be capable of promoting survival and hematopoietic recovery of irradiated subjects, be they mouse or human. Indeed, studies have shown that administration of FGF1 or FGF2 (bFGF) bestows a radioprotective effect on mice receiving lethal doses of ionizing radiation (>8.5 grays) as measured by survival curves out to 30 days post-irradiation; furthermore, in these studies it was determined that bFGF was more effective than FGF1 (Ding, Huang et al. 1997). Moreover, as has been discussed in this dissertation, it has been found that a radioresistant population of BM megakaryocytes secrete bFGF to support recovery of niche cells (Dominici, Rasini et al. 2009). Also, bFGF expression has been detected in cells of human brain tumors that respond poorly to radiation therapy including gliomas and meningiomas (Takahashi, Mori et al. 1990). While bFGF's potent mitogenic activity is one possible mechanism for its radioprotective qualities, studies have found that bFGF is capable of increasing the activity of DNA dependent protein kinase (DNA-PK), a key factor in the process of non-homologous end joining (NHEJ), the primary mechanism for human cells to repair DNA double strand breaks (DSB) induced by ionizing radiation (Ader, Muller et al. 2002). Considering these various results as well as my own work, it is clear that bFGF (and perhaps other FGF ligands) are capable of impacting multiple cellular processes to promote survival and growth in the face of cytotoxic stress, and these functions are likely carried out within HSCs as well as other cell types.

7.3.1.2 FGFR1 in hematopoietic malignancy

Considering the model I have proposed for the function of FGFR1 in HSCs, an obvious question that may arise is, why is this signaling program so rarely activated? As

mentioned, as a growth factor receptor unregulated activation could lead to replicative mutations that could promote malignancy. Indeed, in addition to FGFR1 being expressed in certain human leukemia cells (Moroni, Dell'Era et al. 2002), mutations in *Fgfr1* itself have been implicated in progression of various proliferative disorders and cancers of the hematopoietic system. In particular, *8p11 myeloproliferative syndrome* (EMS) describes a group of aggressive neoplasms that are believed to occur in HSCs. EMS is characterized by chromosomal translocations involving *Fgfr1* and one of 10 known gene partners that generate novel fusion genes and chimeric proteins with constitutive activation of the FGFR1 tyrosine kinase. Often EMS progresses to acute myeloid leukemia, and despite aggressive chemotherapy EMS has proven difficult to treat by conventional leukemia regimens with patients receiving poor prognoses for long-term survival, with the exception of those treated by complete BM ablation and stem cell transplantation (Jackson, Medeiros et al. 2010).

Studies on the most common translocation involving *Fgfr1* and *ZNF198* have characterized multiple signaling events downstream of the mutated ZNF198-FGFR1 fusion protein. Both AKT and MAPK have been found to be activated and to promote prosurvival pathways (Dong, Kang et al. 2007). Also, the ZNF198-FGFR1 fusion protein has been found to phosphorylate and activate STAT5, which proceeds to elevate levels of the anti-apoptotic protein BclXL, promote cell cycle progression, and upregulate the DNA DSB repair protein Rad51 (Heath and Cross 2004). Additionally, the ZNF198-FGFR1 fusion protein has been found to result in overexpression of plasminogen activator inhibitor 2, a proteinase inhibitor that, among other functions, blocks tumor necrosis factor α-induced apoptosis (Kasyapa, Kunapuli et al. 2006). Such an accumulation of evidence highlights the danger of unrestricted signaling via FGFR1 and the need for tight regulation of this signal-

ing pathway.

7.3.1.3 A role for FGFR1 in fetal HSCs?

Given my findings that FGFR1 promotes expansion and migration of adult HSCs during stress response and that these same processes are vital during fetal-stage hematopoiesis (Christensen, Wright et al. 2004), it is possible that FGFR1 may be regulating HSCs of the developing fetus in a similar fashion as during stress response. Published microarray data sets report detection of *Fgfr1* in Sca-1^{+} fetal liver cells; though not HSCs the cell population investigated does approach a stem cell immunophenotype (Ivanova, Dimos et al. 2002). Moreover, Flk1^{+} hematopoietic precursors have been found to express *Fgfr1*, and in related studies *Fgfr1*$^{-/-}$ ES cells poorly differentiate into hematopoietic lineages, and *Fgfr1*$^{-/-}$ embryoid bodies display impaired hematopoietic development, as measured in part by their ability to generate Flk1^{+} cells. Additionally, addition of bFGF to differentiation protocols of wildtype embryoid bodies increased their hematopoietic differentiation (Faloon, Arentson et al. 2000). While these data indicate a role for FGFR1 directly in the generation of early hematopoietic precursors, expression of *Fgfr1* in immature hematopoietic cells of the fetal liver raise the possibility that it may function in the expansion of HSCs in the liver and in their migration ultimately to the BM. Further research is required to explore this potential role of FGFR1.

7.4 Future directions

7.4.1 FGF signaling in stress response

Although I have characterized a requirement for FGFR1 in the expansion and mobilization of HSCs in response to stress, there is still much to be understood. Additional ex-

periments would further elucidate the mechanism of FGFR1 in this process. While data indicates a role for bFGF in mediating HSC stress response, the involvement of other FGF ligands has not been determined. Tissues from mice treated with 5FU would be assayed for expression of various FGF ligands by immunohistochemistry and ELISA and compared to control tissues taken from mice at homeostasis. Furthermore, a more detailed analysis of bFGF levels in a time course after 5FU treatment would reveal more dynamic changes in this factor during stress response. Moreover, observed increases in certain FGF levels would indicate they also mediate activation of FGFR1 to promote stress response of HSCs, or as discussed in preceding sections in this chapter, act earlier in the stress response process to mediate recruitment of megakaryocytes. Alternatively, these experiments may show specific increases in bFGF levels in the absence of other FGF ligands being upregulated. This would be interesting as it would support findings that indicate an almost unique radioprotective quality of bFGF, which would indicate significant potential of bFGF to be a therapeutic target for promoting hematopoietic recovery in affected human patients.

Additionally, in this dissertation I have not fully elucidated the molecular mechanism of how FGFR1 regulates CXCR4 expression on HSCs. To begin, expression of prospective factors in HSCs would be identified by checking published microarray datasets followed by confirmation by RT-PCR. RNA interference experiments using specific short hairpin RNAs (shRNAs) would be done to test if reducing expression of a prospective factor in HSCs impairs the expression of CXCR4 and accompanying responsiveness to SDF-1. Alternatively, experiments that involve treatment of HSCs with specific chemical inhibitors to prospective downstream mediators of FGFR1 and subsequent assays of CXCR4 expression by flow cytometry and/or chemotactic response to SDF-1 would provide additional

evidence as to which factor (s) may be involved.

7.4.2 Transplantation studies

As discussed, 5FU-induced cytotoxicity is not the only method of inducing BM damage and subsequent stress response. Reconstitution of lethally irradiated recipient mice would provide a strenuous test of the requirement of FGFR1 in this process, particularly when FGFR1-null HSCs are placed in competition with wildtype stem and progenitor cells. Unexpectedly, attempts to test HSC function by transplantation proved troublesome. Early attempts showed minimal to no engraftment of irradiated recipients by either FGFR1-null HSCs or wildtype control HSCs (data not shown). The reason for this difficulty was determined to be the breeding of FGFR1 WT and KO mice with a *Z/EG* reporter line that was not on a C57/B6 background. While the resulting mixed strain nature of FGFR1 WT and KO mice did not affect phenotypic studies, it did affect the expression of the CD45.2 haplotype of the pan-hematopoietic marker CD45 in these mice. Transplantation experiments rely on strain-specific expression of CD45 haplotypes to distinguish donor from recipient (or "rescue" cells). If FGFR1-null HSCs are to be tested by the functional transplantation assay, FGFR1 WT and KO mice will need to be backcrossed to restore the C57/B6 background of these transgenic lines. Once it can be demonstrated that hematopoietic cells from these mice express the CD45.2 marker, HSCs from these mice will be flow sorted and transplanted to lethally irradiated recipients. If defects in the engraftment of these recipient mice by FGFR1-null HSCs are observed, subsequent homing assays that more directly test the ability of HSCs to migrate (or "home") from the peripheral circulation to the BM and associate with BM niches may be performed. Such experiments would offer further insight into the functions of FGFR1 in HSCs *in vivo*.

7.4.3 FGF signaling in fetal hematopoiesis

If the potential role of FGFR1 in fetal stage HSCs is to be explored, the first step will be to cross *Fgfr1*$^{fx/fx}$ mice to a reliable Cre line that can successfully target HSCs in the developing embryo. Though existing SCL/FGFR1 KO mice, based upon reports of the *HSC-SCL-CRE-ER*^{T} mouse line, could potentially be put to this purpose, the efficiency of targeting fetal liver HSCs was reported to be only ~10% (Gothert, Gustin et al. 2005), which would likely prove insufficient. Alternatively, transgenic mice such as the Tie2 (Tek)-Cre line has been reported to target embryonic HSCs at high efficiencies and is capable of eliciting hematopoietic phenotypes when combined with floxed alleles of target genes (Mikkola, Klintman et al. 2003; Kim, Saunders et al. 2007). Breeding of these mouse lines would produce *Tie2-Cre*$^{+}$ *Fgfr1*$^{fx/fx}$ embryos, which would be allowed to develop to term to check for generation of viable pups, as well as harvested at pre-natal time points for harvest of fetal tissues such as the liver, spleen, peripheral blood, and BM for analysis of HSC populations as compared to wildtype littermate controls. If FGFR1 truly is required for expansion and/or migration of HSCs during ontogeny, one would expect to observe significant differences in the numbers of HSCs present in these tissues in knockout embryos as compared to controls, as measured by direct enumeration of phenotypic HSCs by flow cytometry. Furthermore, fetal HSCs would be assayed for CXCR4 expression as well as placed in transwell migration assays *in vitro*, to test if they display the same defects in chemotactic response to SDF-1 as has been observed in adult HSCs. Investigation of fetal tissues such as the liver during times of peak HSCs expansion (E14.5-15.5) for expression of bFGF by immunohistochemistry and ELISA would determine if this ligand is responsible for activating FGFR1 on HSCs to promote these processes. The experiments outlined

here may lead to a re-interpretation of my model of FGFR1 function in HSCs to reflect that the FGF signaling pathway is required for embryonic HSCs, largely shut down during homeostasis, but is reactivated during stress response. This line of investigation would prove valuable, as this would be the first evidence showing adult reactivation, in response to severe tissue damage, of the same signaling program required for HSC expansion and migration during fetal development.

7.5 Concluding remarks

In this dissertation I have characterized a role for FGFR1 in the regulation of HSCs in vivo. Conditional deletion of FGFR1 demonstrated that this growth factor receptor is not required for homeostatic hematopoiesis, but it is vital to the response of HSCs to facilitate recovery of the hematopoietic system to induced BM damage. This work indicates that the FGF signaling pathway is selectively activated in HSCs in times of extreme need, but is otherwise unengaged as alternate pathways mediate day-to-day HSC functions. Indeed, FGFR1 can be dangerous, as unregulated activation can lead to proliferative disorders and tumorigenesis. Further investigation into the FGF signaling pathway within the hematopoietic system may well lead to yet more insights into important functions in the biology of HSCs.

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Appendix

Curriculum Vitae

Personal

Home Address:	8014 Summit St Lenexa, KS 66215
Phone:	(913) 908-0344
Email:	jtr@stowers.org jason.ross000@gmail.com

Education

B.S.	Fort Hays State University Hays, KS May 2001 Honors Biology, Chemistry Minor
B.S.	University of Kansas Medical Center Kansas City, KS May 2002 Clinical Laboratory Science
Ph.D.	University of Kansas Medical Center Kansas City, KS August 2010 Pathology & Clinical Laboratory Science

Research Experience

1999-2001	Undergraduate Research Assistant Dr. Dwight Wray Dept. of Biology Fort Hays State University
2002	Summer Internship Dr. Linheng Li Stowers Institute for Medical Research
2002-2004	Research Technician Linheng Li Stowers Institute for Medical Research

2004-2010	Predoctoral Researcher Linheng Li Laboratory Dept. of Pathology and Laboratory Medicine University of Kansas Medical Center Stowers Institute for Medical Research

Publications

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W. G., Ross, J., Haug, J., Johnson, T., Feng, J. Q., Harris, S., Wiedemann, L. M., Mishina, Y., and Li, Linheng. (2003**). Identification of the haematopoietic stem cell niche and control of the niche size**. *Nature 425*, 836-841.

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Oral Presentations

Jason T. Ross and Linheng Li
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"Exploring a Role for FGF Signaling in Hematopoietic Stem Cell Stress Response" (2007)

Jason T. Ross and Linheng Li
Student Research Forum – University of Kansas Medical Center, Kansas City, KS, U.S.A.
"Novel Function of FGF Signaling in Stress Response of Hematopoietic Stem Cells" (2008)

Poster Presentations

Jason T. Ross, Jeffrey S. Haug, Xi He, and Linheng Li
American Society of Hematology 49th Annual Meeting and Exposition
"Novel Function of FGFR1 in Hematopoietic Stem Cell Stress Response" (2007)

Jason T. Ross, Xi He, Jeffrey S. Haug, and Linheng Li
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