

ROLE OF STAT3 AND SDF-1/CXCL12 IN
MITOCHONDRIAL FUNCTION IN HEMATOPOIETIC STEM
AND PROGENITOR CELLS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Microbiology and Immunology,
Indiana University

October 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my wife Becky and my daughter Maya. Becky has been a pillar of strength for me during some very difficult personal and professional times during my time in graduate school. Her love, encouragement, and support have kept me going during times of frustration and self-doubt. My wonderful and beautiful daughter Maya has been the brightest spot in my life. Her love and happiness has given me the motivation and inspiration to continue on to achieve my Ph.D. I would like to also thank my parents. My mother and father, Ruby and Jim Graham, and my father and mother Victor and Deborah Messina, who never stopped believing in me and continually gave me love and support during my studies. My extended family and close friends and classmates have also been an incredible support system that I have counted on numerous times and I am forever grateful for their support and friendship.

I would also like to thank my committee members, Dr. Ken Cornetta, Dr. Eddie Srour, Dr. Maureen Harrington, and Dr. Lou Pelus for giving me an immense amount of support over the course of my studies. I would also like to thank the past and present members of the Broxmeyer lab. Their help and support both scientifically and personally is greatly appreciated. I would like to thank Charlie Mantel formerly of the Broxmeyer lab for being a good friend and for his support and encouragement during my studies.

Last but not least, I would like to thank Dr. Hal Broxmeyer for never giving up on me. Dr. Broxmeyer continued to encourage and push me to be a better scientist. He has been there for me through thick and thin and the experience in his lab has left an indelible mark on me personally and scientifically. I am truly honored and thankful to have had the opportunity to be a part of his lab.

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Mitochondria are the major ATP producing source within cells. There is increasing data supporting a direct involvement of mitochondria and mitochondrial function in regulating stem cell pluripotency. Mitochondria have also been shown to be important for hematopoietic stem and progenitor cell function. Hematopoietic stem cells have lower numbers of mitochondria (mass), lower mitochondrial membrane potential, and lower ATP levels as compared to other blood cell types. Mitochondria play an important role in hematopoietic stem and progenitor cells, thus we investigated the role of the chemokine, SDF-1/CXCL12, in mitochondrial function in hematopoietic stem and progenitor cells using an SDF-1/CXCL12 transgenic mouse model. We found increased mitochondrial mass is linked to CD34 surface expression in hematopoietic stem and progenitor cells, suggesting that mitochondrial biogenesis is linked to loss of pluripotency. Interestingly these hematopoietic progenitor cells have low mitochondrial membrane potential and these mitochondria become active prior to leaving the progenitor cell compartment. We also tested the ability of SDF-1/CXCL12 to modulate mitochondrial function in vitro by treating the human leukemia cell line, HL-60, and primary mouse lineage⁻ bone marrow cells with SDF-1/CXCL12. We found significantly reduced mitochondrial function at two hours while mitochondrial function was significantly increased at 24 hours. This suggests that SDF-1/CXCL12 regulates mitochondrial function in a biphasic manner in a model of

hematopoietic progenitors and immature blood cells. This suggests SDF-1/CXCL12 may play a role in regulating mitochondrial function in hematopoiesis. We also investigated STAT3 in hematopoietic stem and progenitor cells. Mitochondrial STAT3 plays an essential role in regulating mitochondrial function. By using a knockout (*Stat3^{-/-}*) mouse model we found that *Stat3^{-/-}* hematopoietic progenitor cells had reduced colony forming ability, slower cell cycling status, and loss of proliferation in response to multi-cytokine synergy. We also found mitochondrial dysfunction in *Stat3^{-/-}* hematopoietic stem and progenitor cells. Our results suggest an essential role for mitochondria in HSC function and a novel role for SDF-1/CXCL12 and STAT3 in regulating mitochondrial function in hematopoietic stem and progenitor cells.

Hal E. Broxmeyer, Ph.D.-Chair

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ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial membrane potential
7TM	7 transmembrane
BFU-E	Burst forming unit erythroid
CAR	CXCL12 abundant reticulocytes
CFU-GEMM	Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony forming unit-granulocyte, macrophage
CFU-M	Colony forming unit-macrophage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRU	Competitive repopulating unit
ECAR	Extracellular acidification rate
EPO	Erythropoietin
ETC	Mitochondrial electron transport chain
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GM-CSF	Granulocyte, macrophage colony stimulating factor
GMP	Granulocyte, macrophage progenitors
GPCR	G protein coupled receptor

HIV	Human immunodeficiency virus
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IL-3	Interleukin 3
IMDM	Iscove's modified Dulbecco medium
IMM	Inner mitochondrial membrane
Lin ⁻	Lineage negative
LK	Lineage ⁻ c-kit ⁺
LSK	Lineage ⁻ sca-1 ⁺ c-kit ⁺
LTC-IC	Long-term culture initiating cell
LT-HSC	Long-term repopulating hematopoietic stem cell
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte, erythrocyte progenitors
MPN	Myeloproliferative neoplasms
mPTP	mitochondrial permeability transition pore
NAC	N-Acetyl cysteine
OCR	Oxygen consumption rate
Oligo A	Oligomycin A
OMM	Outer mitochondrial membrane
OXPPOS	Mitochondrial oxidative phosphorylation
PBS	Phosphate-buffered saline
PE	Phycoerythrin
plpC	polyI-polyC

PTPMT1	Protein tyrosine phosphatase mitochondrial 1
qRT-PCR	Quantitative reverse transcription PCR (polymerase chain reaction)
RNAseq	RNA sequencing
ROS	Reactive oxygen species
SCF	Stem cell factor
SD	Standard deviation
SDF-1	Stromal cell-derived factor-1; also known as CXCL12
SEM	Standard error of the mean
siRNA	small interfering RNA
ST-HSC	Short-term repopulating hematopoietic stem cell
TG	Transgenic

INTRODUCTION

Chemokines

Chemokines are small, secreted proteins that are part of the cytokine family which are so named for their chemoattractant properties. Many chemokines induce cell migration, chemotaxis, and directed cell movement (Murphy PM, 1994). Nomenclature for chemokines is based on the arrangement of amino acids around the first two of four conserved cysteine residues near the N-terminus. Four different motifs distinguish the chemokine subfamily members (CXC, CC, XC, and CX3 motifs) (Broxmeyer HE, 2008; Allen SJ et al., 2007). Chemokines play a role in a multitude of biological functions. For example, pro-inflammatory chemokines are produced by cells as a means to recruit immune cells to the site of injury or infection (Constantin G, et al., 2000). Chemotaxis is the main feature of chemokines, however, their physiological role is more complex. Many chemokines have additional functions such as immune surveillance, organ development, organ homeostasis and angiogenesis (Allen SJ et al., 2007; Wang J and Knaut H, 2014). The chemokine SDF-1/CXCL12 plays an essential role in the homing, engraftment, and survival of hematopoietic stem cells (Lapidot T and Kollet O, 2002; Christopherson KW 2nd et al., 2004; Broxmeyer HE et al., 2007; Fukuda, S et al., 2007), whereas the chemokine GRO β mobilizes early hematopoietic stem cells to peripheral blood (King AG et al., 2001; Lapidot T and Kollet O, 2002;

Christopherson KW 2nd et al., 2004; Broxmeyer HE et al., 2007; Fukuda, S et al., 2007).

Most chemokines are soluble, and the effect they elicit is determined by a concentration gradient, but some chemokines, such as CX3CL1, interact with its surface bound receptor, CX3CR1 to mediate leukocyte-endothelial cell adhesion and rolling (Allen SJ et al., 2007). Some chemokines are sequestered in the extracellular matrix and this milieu of extracellular matrix and chemokines provides important combinatorial signals that influence cell behavior (Vaday G et al., 2001). There are at least 50 chemokine ligands and 22 G-protein coupled chemokine receptors, of which some are exclusively expressed in humans or mice (Wang J and Knaut H, 2014) (Table 1). The chemokine receptor, CXCR1, and the chemokines CLCL8, CXCL11, CCL13-15, CCL18, CCL23 and CCL24/26 are only expressed in humans, while the chemokines CCL6, CCL9, CXCL15 and CCL12 are only expressed in mice (Wang J and Knaut H, 2014). Some chemokine receptors bind more than one chemokine. The chemokine, Stromal Cell-Derived Factor-1 (SDF-1, also known as CXCL12) binds to the chemokine receptor CXCR4, but SDF-1/CXCL12 can also bind CXCR7 (Balabanian K et al., 2005; Burns JM et al, 2006). With the discovery of Interleukin-8 (IL-8) in 1987 and shortly after, macrophage inflammatory proteins 1- α and 1- β (MIP-1 α/β) it became clear to researchers that differences in chemokine function related to their structure (Wolpe SD and Cerami A, 1989). Four invariant cysteine residues form

Table 1.

	Systematic name (common name)	Receptor
CC chemokine/receptor family	CCL1(I-309)	CCR8, R11
	CCL2 (MCP-1, MCAF)	CCR2
	CCL3 (MIP-1 α /LD78 α)	CCR1,R5
	CCL3L1 (LD78 β)	CCR5
	CCL4 (MIP-1 β)	CCR5
	CCL4L1	CCR5
	CCL4L2	CCR5
	CCL5 (RANTES)	CCR1, R3, R4, R5
	CCL6 (C-10)	CCR1, R2, R3
	CCL7 (MCP-3)	CCR1, R2, R3
	CCL8 (MCP-2)	CCR1, R2, R5, R11
	CCL9 (MRP-2/MIP-1 γ)	CCR1
	CCL10 (MRP-2/MIP-1 γ)	CCR1
	CCL11 (Eotaxin)	CCR3
	CCL12 (MCP-5)	CCR2
	CCL13 (MCP-4)	CCR1, R2, R3, R11
	CCL14 (HCC-1)	CCR1
	CCL15 (HCC-2, Lkn-1)	CCR1, R3
	CCL16 (HCC-4, LEC)	CCR1
	CCL17 (TARC)	CCR4
	CCL18 (DC-CK1, PARC)	Unknown
	CCL19 (MIP-3 β , ELC)	CCR7, R11
	CCL20 (MIP-3 α , LARC)	CCR6
	CCL21 (6Ckine, SLC)	CCR7, R11
	CCL22 (MDC, STCP-1)	CCR4
	CCL23 (MPIF-1)	CCR1
	CCL24 (MPIF-2, Eotaxin- 2)	CCR3
	CCL25 (TECK)	CCR9, R11
	CCL26 (Eotaxin-3)	CCR3
	CCL27 (CTACK, ILC)	CCR2, R3, R10
	CCL28 (MEC)	CCR3, R10

Table 1. Continued

	Systematic name (common name)	Receptor
C chemokine/receptor family	XCL1 (Lymphotactin)	XCR1
	XCL2 (SCM1-b)	XCR1
CXC chemokine/receptor family	CXCL1 (GRO α , MGSA- α)	CXCR2N R1
	CXCL2 (GRO β , MGSA β)	CXCR2
	CXCL3 (GRO γ , MGSA γ)	CXCR2
	CXCL4 (PF4)	CXCR3
	CXCL4L1 (PF4V1)	CRCR3
	CXCL5 (ENA-78)	CXCR1, R2
	CXCL6	(GCP-2) CXCR1, R2
	CXCL7 (NAP-2)	CXCR2
	CXCL8 (IL-8)	CXCR1, R2
	CXCL9 (Mig)	CXCR3
	CXCL10 (IP-10)	CXCR3
	CXCL11 (I-TAC)	CXCR3
	CXCL12 (SDF-1 α/β)	CXCR4, R7
	CXCL13 (BLC, BCA-1)	CXCR3, R5
	CXCL14 (BRAK, bolekine)	Unknown
	CXCL15	Unknown
	CXCL16 (SR-PSOX)	CXCR6
	CXCL17 (VCC1, DMC)	Unknown
CX3C chemokine/receptor family	CX3CL1 (Fractalkine)	CX3CR1

Table 1. Chemokines and chemokine receptors

List of chemokine families, chemokines and their receptors. Systemic and common names are included for the known human chemokines (Adapted from Turner MD et al., 2014)

disulfide bonds and define the primary structure of chemokines. Subfamilies of chemokines are classified by the sequence location of the first two cysteine residues near the N-terminus (Allen SJ et al., 2007). Thus, chemokines were classified into two groups based on conserved cysteine residues at their N-termini. They were classified into two families known as the α and β chemokines (Baggiolini M et al., 1994). The α chemokines consisted of two conserved cysteine residues at the N-terminus with an amino acid between them. The first cysteine residue forms a covalent bond with the third cysteine residue, while the second forms a covalent bond with the fourth cysteine residue (Allen SJ et al., 2007). These chemokines are known as the CXC chemokines and include SDF-1/CXCL12. On the other hand, the β chemokines consist of two conserved amino acids near the N-terminus that are next to each other and are thus termed CC chemokines. CC chemokines include MIP-1 α /CCL3. Along with the CXC and CC chemokines, two other groups have been identified that are much smaller than the CXC and CC chemokine groups. The γ -chemokine, CX3C, has only one member and is defined by three intervening amino acid residues between the first two cysteine moieties. Fractalkine (CX3CL1) is the only member of the CX3C chemokine subfamily (Allen SJ et al., 2007). The last subfamily of chemokines is δ -chemokines also known as the C chemokines. The C chemokine subfamily contains two members, which are both encoded by the same gene and are splice variants that differ in only two amino acid residues. C chemokines only contain two of the four cysteine residues. This subfamily contains the chemokines XCL1 and XCL2 C (lymphotactine) chemokines (Fernandez E and Lolis E, 2002). CX3CL1 (fractalkine) is an unusual

chemokine in the sense that it forms the N-terminus of the membrane bound receptor neurotactin (Pan Y et al., 1997). When CX3CL1 binds to its receptor CX3CR1, the interaction functions in cellular adhesion (Imai T et al., 1997) and recombinant fractalkine missing the receptor residues is chemotactic (Bazan JF et al., 1997).

The secondary structure of chemokines consists of an elongated N-terminus that precedes the first cysteine residue. Interestingly, the extended N-terminus has no particular structural features and at times is not resolved in high-resolution structural studies. After the first two cysteine residues is a loop of approximately ten residues that is at times followed by one strand of a 3_{10} helix (Fernandez E and Lolis E, 2002). The amino acids in a 3_{10} helix are arranged in a right-handed helical structure and each amino acid corresponds to a 120° turn in the helix, which equates to the helix having three amino acid residues per turn and 10 atoms in the ring formed by hydrogen bonding. A structure called an N-loop is formed between the second cysteine residue and the 3_{10} helix. Following the single-turn 3_{10} helix are three β -strands and a C-terminal α -helix. Each unit of secondary structure is connected by turns known as the 30s, 40s, and 50s loops, which are indicative of the number of amino acid residues of the mature protein (Fernandez E and Lolis E, 2002). The 30s, 40s, and 50s loops contain the third and fourth cysteine residues characteristic of the family of chemokines. The flexibility of the N-terminus is limited by the first two cysteine residues following the N-terminal region, owing to the disulfides with the third cysteine on the 30s loop

and the fourth cysteine in the 50s loop, respectively (Fernandez E and Lolis E, 2002). NMR studies have shown that the flexibility of the N-loop is greater than that of other regions of the protein (excluding the N- and C-termini) and this flexibility plays an important role in the mechanism of chemokine/chemokine receptor binding and/or activation by allowing the chemokine to overcome steric hindrance (Crump MP et al., 1999; Ye J et al., 2000).

The structures of many chemokines have been solved by techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Such studies have revealed that despite low sequence homology between chemokines (ranging from less than 20% to greater than 90%), chemokines share remarkably similar and conserved tertiary structure (Kufareva I et al., 2015). The β -strands that follow the N-loop and 3_{10} helix are positioned anti-parallel to each other and form a β -plated sheet. Each β -strand is linked to the next by flexible 30s and 40s loops. In particular, the 30s loop is very important to the activity of a number of chemokines. The third β -strand is connected by a 50s loop to the C-terminal α -helix. The chemokine core structure is stabilized mainly by the two-disulfide bonds and by the hydrophobic interactions from one side of the C-terminal α -helix and a portion of the β -sheet (Fernandez E and Lolis E, 2002).

Many Chemokines form dimers or oligomers alone or in solution in vitro upon binding of glycosaminoglycans (GAGs) (Johnson Z et al., 2005; Handel TM et al., 2005; Lau EK et al., 2004; (Kufareva I et al., 2015). Formation of dimers falls

into two categories. CC type chemokines associate primarily through the formation of an anti-parallel β -sheet through amino acid residues near the N-terminus, including the first two cysteine residues, giving considerable flexibility of the subunits in comparison to one another. For CXC chemokines, such as SDF-1/CXCL12, amino acid residues from the first strand of the β -sheet of one subunit form a hydrogen bond with the same strand of the second subunit. This interaction forms a single extended six-stranded sheet. Further stability is provided by interactions of the C-terminal α -helices with the β -sheet of the opposite subunit. Some chemokines form tetramers. For example, the structure of CCL2 was solved as a dimer in solution, but the X-ray crystallographic studies revealed it could form dimers and tetramers (Lubkowski J et al., 1997). Some chemokines such as CCL5/RANTES, CCL3/MIP-1 α , and CCL4/MIP-1 β can form even higher order oligomers (Czaplewski LG et al., 1999). It is widely accepted that despite oligomerization of chemokines, they interact with chemokine receptors as monomers at least with respect to cellular migration (Rajaratnam K et al., 1994). It is the monomer form of MIP-1 α that acts as a suppressor of hematopoietic progenitor cell proliferation (Mantel C et al., 1993; Cooper S et al., 1994).

Despite data showing that many chemokines bind their receptors as monomers, there is also data that supports an aspect of in vivo chemokine signaling that seems to be independent of direct chemokine receptor binding and appears to be associated with glycosaminoglycan binding, which is essential for some cytokines (Proudfoot AEI et al., 2003). There is some data that suggests

chemokines can also hetero-oligomerize. This is not surprising since all chemokines have a similar fold and use a limited number of dimer interface motifs. The binding affinity of most chemokines for their receptors is quite high and they have dissociation constants in the low nanomolar range. Chemokines of the same family with high sequence similarity around the dimer interface are prone to forming heterodimeric or higher order hetero-oligomeric structures. Also, structural studies have revealed the potential of CC/CXC hetero-oligomer formation (Swaminathan GJ et al., 2003; (Lubkowski J et al., 1997).

Chemokine Receptors

Chemokine receptors are members of a subset of proteins known as G protein-coupled receptors (GPCRs). Chemokine receptors are embedded in the lipid bilayer of the cell surface and possess seven-transmembrane (7TM) domains. Despite structural similarities between most GPCRs, there are specific structural determinants that are found most frequently on chemokine receptors (Murphy PM et al, 2000). For example, chemokine receptors contain a conserved DRY motif, an acidic N-terminus, and lengths of 340 to 370 amino acids (Allen SJ et al., 2007). Structurally, chemokine receptors contain variation in their second intracellular loop, a short and basic third intracellular loop and a cysteine residue in each of their four extracellular loops.

Like chemokines, chemokine receptors were given a new nomenclature in 1998 (Murphy PM et al, 2000). These receptors have been designated CXCR1 through 6, CCR1 through 11, XCR1, and CX3CR1. This designation is based on whether they bind chemokines from the CXC, CC, C, or CX3C subfamilies of chemokines, respectively (Murphy PM et al, 2000). All GPCR structures, including chemokine receptors, are based on that of rhodopsin, (Palczewski et al., 2000), The crystal structures of almost all classes of GPCRs have been determined (Katritch V et al., 2013; Venkatakrishnan AJ et al., 2013; Tehan BJ et al., 2014). A wealth of information about GPCR structure and function has been gleaned from the field of GPCR research. GPCR signaling involves coupling to the heterotrimeric G-proteins ($\alpha\beta\gamma$) bound to the intracellular loops of the 7TM GPCR (Allen SJ et al., 2007; Krumm BE and Grisshammer R, 2015). In this manner, the $G\alpha$ subunit binds directly to the intracellular loop of the GPCR while binding to the $G\beta$ subunit, which is in tight association with the $G\gamma$ subunit. The $G\alpha$ subunit has an intrinsic GTPase activity that is involved in the binding and hydrolysis of GTP. When inactive, the $G\alpha$ subunit is bound to GDP. Upon ligand binding, for example, when the chemokine SDF-1/CXCL12 binds its receptor, CXCR4, the GPCR is stabilized and a conformational change takes place in the GPCR that activates the heterotrimeric G-protein inside the cell. This causes the dissociation of GDP from the $G\alpha$ subunit and GTP replaces GDP. The GTP bound $G\alpha$ subunit then dissociates from both the receptor and the $G\beta\gamma$ heterodimer and both complexes activate downstream effectors that lead to physiological responses such as chemotaxis or cell survival. GPCRs desensitization occurs with continued stimulation leading to internalization

that is mediated by the phosphorylation of the c-terminal tail of the GPCR. Phosphorylation also allows the binding of arrestins, which block any further interaction of the GPCR with G proteins, and mediates endocytosis via clathrin-coated pits and calveoli (Allen SJ et al., 2007; Krumm BE and Grisshammer R, 2015). Mutational analysis of chemokine receptors has revealed the specific regions that interact with chemokine ligands. These studies have provided strong evidence that the chemokine recognition site and receptor activation site are distinct, and mutagenesis studies of chemokine receptors suggest that binding sites are spread throughout the protein (Allen SJ et al., 2007; Krumm BE and Grisshammer R, 2015). HIV uses CXCR4 as one of the obligate co-receptors for HIV entry into host cells (Berson JF et al., 1996; Feng YX et al., 1996; Horuk, R 1999; Moore JP et al., 1997).

SDF-1/CXCL12 Biology

SDF-1/CXCL12 α and β were first identified by a cDNA cloning strategy that enriched for cDNA encoding proteins that contain hydrophobic signal sequences at their N-termini (Nishikawa et al., 1988). SDF-1 α and β were the first chemokines to be identified by this strategy and were isolated from a mouse bone marrow stromal cell line and were first characterized to stimulate the growth of a B cell precursor clone (Nagasawa T et al, 1994). Secretion of SDF-1 from bone marrow stromal cells led to the original name of the chemokine (Tashiro K et al, 1993; Nagasawa T et al, 1994). The amino acid sequences for both SDF-1 α and β are

identical in their 89 N-terminal amino acids but differ by an addition of 4 amino acids, RFKM, at the C-terminus of SDF-1 β . SDF-1/CXCL12 gene splicing results in two human isoforms named SDF-1 α /CXCL12 α and SDF-1 β /CXCL12 β (Shirozu M et al, 1995). Based on their amino acid sequences, SDF-1 α and β are members of the CXC chemokine subfamily and SDF-1 was renamed CXCL12 (Murphy PM et al., 2000). Unlike most chemokines, the nucleotide and amino acid sequence of human and mouse SDF-1 are highly conserved. Furthermore, human SDF-1 α is highly conserved, with >95% amino acid sequence identity to its known mammalian counterparts, including feline and murine SDF-1 α (Shirozu M et al., 1995; Tashiro K et al., 1993; Nagasawa T et al., 1994). Lower vertebrates such as *Xenopus* and zebrafish express SDF-1/CXCL12 orthologues (Doitsidou M et al., 2002).

The gene encoding murine SDF-1/CXCL12 resides on chromosome 6 and the human SDF-1/CXCL12 gene has been mapped to chromosome 10. This differs from other CXC subfamily chemokines for mice and humans, which have been respectively mapped closely together on chromosomes 5 and 4, (Shirozu M et al., 1995). The distinct chromosomal location of the SDF-1/CXCL12 gene suggests that there may be biological functions that differ from other members of the CXC chemokine subfamily members. SDF-1/CXCL12 has six isoforms, named α , β , γ , δ , ϵ , and ϕ (Shirozu M et al., 1995), but the focus of this writing is SDF-1 α , which is called SDF-1/CXCL12 from now on. SDF-1/CXCL12 plays a role in the retention of, and homing and engraftment of, hematopoietic stem cells (HSC) and

progenitor cells (HPC). SDF-1/CXCL12 also functions in growth, differentiation and survival of hematopoietic stem and progenitor cells HSCs and HPCs (Lapidot T and Kollet O, 2002; Broxmeyer HE et al., 2003; Christopherson KW 2nd et al., 2004; Guo Y et al., 2005; Fukuda S and Pelus LM, 200; Broxmeyer HE et al., 2007).

Signal transduction of SDF-1/CXCL12 is mediated through the chemokine receptor CXCR4 (Bleu CC et al., 1996 and Oberlin E, et al., 1996). Studies have revealed that knockout of SDF-1/CXCL12 is perinatal lethal and mice lacking SDF-1/CXCL12 have severe defects in gastrointestinal vascularization, cerebral development, and hematopoietic defects (Tachibana k et al., 1998; Zou YR et al., 1998; Nagasawa T et al., 1994). Furthermore, CXCR4 knockout studies revealed a strikingly similar phenotype to that of SDF-1/CXCL12 knockout mice (Ma Q et al., 1998). These results have established that the SDF-1/CXCL12 and CXCR4 signaling axis is non-promiscuous. Recently, however, it has been reported that SDF-1/CXCL12 binds to and signals through a second receptor known as RDC-1/CXCR7 (Balabanian et al, 2005; Burns et al, 2006); the function of this receptor in hematopoietic stem and progenitor cells has yet to be well established, though it is thought that CXCR7 may play a role in the context of leukemia (Melo RCC et al., 2014; Kim HY et al., 2015).

In both SDF-1/CXCL12 deficient and CXCR4 deficient mice, it was shown that SDF-1/CXCL12-CXCR4 signaling is essential for the colonization of the bone marrow by neutrophils during development (Nagasawa T et al, 1996; Ma Q et al.,

1998 Tachibana K et al., 1998; Zou YR et al., 1998; Ara T et al., 2003). It was also shown that the development of B-cells, from the earliest progenitors to developed B-cells and plasma cells, as well as their homing to the bone marrow is dependent on the SDF-1/CXCL12-CXCR4 axis (Hargreaves DC et al., 2001; Tokoyoda K, et al., 2004). Interestingly, in SDF-1/CXCL12 or CXCR4 knock out mice, the earliest T-cell progenitors in the adult thymus were not dependent on SDF-1/CXCL12-CXCR4 signaling, while the earliest T-cell precursors in the embryos of mice lacking three chemokine receptors, CXCR4, CCR7, and CCR9, were severely reduced, suggesting that these three receptors are essential for the homing of primitive T-cell precursors to the postnatal thymus (Calderon L et al., 2011; Noda M et al., 2011). SDF-1/CXCL12 is a potent chemotactic factor for HSCs and HPCs cells (Aiuti et al., 1997; Kim CH and Broxmeyer HE, 1998). It plays an essential role in the maintenance of HSCs, including homing, engraftment and repopulating activity, as well as HSC quiescence and retention in the bone marrow (Kawabata K et al., 1999; Peled A et al., 1999; Bonig H et al., 2004; Nie Y et al., 2008). It enhances the survival of HSCs and HPCs, an effect increased in synergy with other cytokines (Lee Y et al., 2002; Broxmeyer HE et al., 2003; Tzeng YS et al., 2011). Treatment of mouse bone marrow cells and human cord blood HPCs with soluble SDF-1/CXCL12 enhanced their replating efficiency, and bone marrow cells from mice expressing a human SDF-1/CXCL12 transgene exhibited increased replating capacity of single macrophage- and multipotent progenitor-derived colonies (Broxmeyer HE et al., 2007).

The chemotactic activity of SDF-1/CXCL12 is regulated in vivo through post-translational enzymatic cleavage by a number of different enzymes such as matrix metalloproteinase (McQuibban GA, et al, 2001), cathepsin G (Delgado MB et al, 2001), elastase (Valenzuela-Fernandez A et al, 2002), and dipeptidyl peptidase IV (DPPIV)/CD26 (Ohtsuki T et al., 1998; Proost P et al., 1998; Shioda T et al., 1998). DPPIV/CD26 mediated proteolytic cleavage of the two N-terminal amino acid residues at the penultimate proline or alanine results in a truncated form of SDF-1/CXCL12 that has lost chemotactic activity (Proost P et al., 1998; Christopherson KW 2nd et al., 2002). Truncation of SDF-1/CXCL12 modifies its ability to bind to CXCR4. The truncated molecule has been shown to block the effect of full-length SDF-1/CXCL12 (Crump MP et al, 1997, Christopherson KW 2nd et al., 2002). It is unknown whether truncated SDF-1/CXCL12 is able to induce signaling through CXCR4 and if so to what extent. CXCR4 is also cleaved by neutrophil proteases at the N-terminus and this cleavage reduces SDF-1 chemotaxis (Levesque JP et al., 2003). Genetic deletion of CD26 or pharmacological inhibition of CD26 enzyme activity significantly enhances the efficiency of hematopoietic stem cell transplantation, suggesting a role for CD26 in vivo processing of SDF-1/CXCL12 (Christopherson KW 2nd et al, 2004; Campbell TB et al., 2007; Farag SS et al., 2013). Also, the enzyme Carboxypeptidase N has been shown to play a role in regulating the activity of SDF-1/CXCL12 by cleavage of the carboxy-terminal lysine (Davis DA et al., 2005). The other splice variants of SDF-1/CXCL12 have the same N-terminus, suggesting they can be modified by CD26 as well, but it may be the differences in the amino acids sequences at the

C-termini that dictate the functions of each splice variant. Evidence for this comes from the fact that removal of the C-terminal lysine from SDF-1 α /CXCL12 α oblates its chemokine activity (De La Luz Sierra M et al., 2004; Davis DA et al., 2005), and also from experiments in which the CXCL12 α N-terminus is fused with the carboxy terminus α -helix of SDF-1 β /CXCL12 β and chemokine activity is restored (Luo J et al., 1999; Tudan C et al., 2002). There is also evidence that suggests that complement proteins play a role in proteolytic cleavage of SDF-1/CXCL12 and this affect mobilization and engraftment of hematopoietic stem and progenitor cells (Ratajczak MZ et al., 2006; Ratajczak MZ et al., 2008; Wysoczynski M et al., 2009; Lee HM et al., 2009; Ratajczak MZ and Kim C, 2011). Interestingly, heparin sulfate/heparin oligosaccharides bind to the lysine at amino acid position 1 of SDF-1/CXCL12 and protects the N-terminus from cleavage by CD26 (Sadir R et al., 2004).

SDF-1/CXCL12 and Hematopoietic Stem Cell Maintenance

Hematopoiesis is the process by which the myriad types of mature blood cells are produced. The vast majority of hematopoiesis occurs in the bone marrow microenvironment from a limited number of multipotent hematopoietic stem cells (HSCs) and the HSC niche provides signals regulating their functions, including quiescence, self-renewal and long term repopulating capability, as well as the ability to undergo multi-lineage differentiation. Along with HSCs, the bone marrow contains cells that support and regulate HSCs and the process of hematopoiesis.

Mesenchymal stem cells (MSCs), endothelial cells, adipocytes, and neurons play a critical role in the maintenance of HSCs and osteocytes, osteoblasts, osteoclasts and osteoprogenitor cells play a role in the maintenance of bone (Anthony BA and Link DC, 2014) There are several different niches in the bone marrow and depending on which niche cells HSCs interact with, helps to define the specific “sub-niche” in which HSCs may reside. HSCs have been shown to localize with nestin⁺ perivascular mesenchymal stem cells that are also in close association with nerve fibers of the sympathetic nervous system that innervate the bone marrow. in the bone marrow (Mendez-Ferrer S et al., 2010). Another report suggests that HSCs are in close contact with mesenchymal cells and CAR cells expressing high levels of Foxc1 (Omatsu Y et al., 2010). Interestingly, it was shown that HSCs occupy a perivascular niche while early lymphoid progenitors occupy an endosteal niche (Ding L and Morrison SJ, 2013; Greenbaum A et al., 2013). The bone marrow microenvironment is generally known to be hypoxic and HSCs are thought to reside in the hypoxic zone of the endosteal region, however, a recent report has shown that the hypoxic state of HSCs is regulated, at least in part by cell intrinsic mechanisms, regardless of their localization and oxygen percentage in the bone marrow (Nombela-Arieta C et al., 2013). Deletion of SDF-1/CXCL12 from different types of niche cells leads to the reduction in HSC numbers, competitive repopulation, and increases in splenic HSCs, all of which indicate an essential role for SDF-1/CXCL12 in HSC function in the bone marrow microenvironment (Mendez-Ferrer S et al., 2008; Mendez-Ferrer S et al., 2010; Ding L et al., 2012;

Ugarte F and Forsberg EC, 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014).

SDF-1/CXCL12 is a key regulator of HSCs in the bone marrow microenvironment (Sugiyama T et al., 2006) and processes in the bone marrow such as B-lymphopoiesis are dependent on SDF-1/CXCL12 (Tokoyoda K et al., 2004). Sugiyama et al. showed that the SDF-1/CXCL12-CXCR4 signaling axis is essential for the maintenance of HSCs in the bone marrow of adult mice (2006). By using a conditional CXCR4 knockout mouse model (MxCRE-CXCR4^{fl/fl}) researchers found HSCs (CD34⁻ Lin⁻ Sca1⁺ C-Kit⁺ Side Population^{low}) in the bone marrow were significantly reduced compared to WT controls (Sugiyama T et al., 2006). Genes highly expressed and involved in the regulation of HSCs, Tek, Junb, and Vegfa, were significantly reduced in knockout mouse HSCs versus control mice. This suggests that SDF-1/CXCL12-CXCR4 signaling is important for and may be involved in the regulation of genes involved in HSC maintenance. Also, long-term culture initiating cells (LTC-IC), were assayed by limiting dilution in vitro culture on primary bone marrow stromal cells and there was a marked reduction in LTC-ICs from bone marrow of CXCR4 knockout mice. They also performed limiting-dilution analysis in vivo with competitive repopulating unit (CRU) analysis to determine the frequency of cells capable of long-term bone marrow repopulation. There was a drastic reduction in the CRUs in knockout mice versus control. CXCL12 was tagged with GFP and used to study the location of SDF-1/CXCL12 expression in the bone marrow of GFP knock-in mice. A population of

reticular cells expressing high levels of SDF-1/CXCL12 and long cellular structures/processes were scattered throughout adult bone marrow. Histological analysis of these cells showed that they were not in association with the surface of the bone in the endosteal region, but rather scattered throughout the intertrabecular architecture with negligible GFP expression detected in bone (Sugiyama T et al., 2006). These high SDF-1/CXCL12 expressing cells scattered throughout the bone marrow were called CXCL12-abundant reticular (CAR) cells and are major producers of CXCL12 in the bone marrow (Sugiyama T et al., 2006). By using aged mice, which have increased numbers of Lineage⁻ Sca-1⁺ c-Kit⁺ (LSK) cells (enriched for hematopoietic stem and progenitor cells), researchers were able to assess the number of HSCs that were in contact with CAR cells. Ninety-seven % of HSCs were associated with CAR cells and almost all HSCs that were near the endosteal region were also in association with CAR cells (Sugiyama T et al., 2006). Furthermore, 85% of HSCs associated with sinusoidal endothelium were in contact with CAR cells surrounding endothelial cells. These results were some of the first to illustrate an essential role for SDF-1/CXCL12 in HSC maintenance in the bone marrow. In adults, CAR cells do not express Sca-1, but express platelet-derived growth factor receptor beta (PDGFR β), as well as adipogenic and osteogenic transcription factors that including Osterix (Osx), Runx2 and peroxisome proliferator-activated gamma (PPAR γ) and have the potential to differentiate into adipocytes and osteocytes in vitro (Omatsu Y et al., 2010). These results suggest that CAR cells might provide the requirements for an HSC niche.

Tie2⁺ sinusoidal endothelial cells in the bone marrow have also been reported to be associated with HSCs, and SDF-1/CXCL12 and stem cell factor (SCF) produced by these cells are necessary for HSC maintenance (Ding L et al., 2012; Ding L and Morrison SJ, 2013; Greenbaum A et al., 2013). During fetal development the first definitive HSCs arise from hemogenic endothelium in the dorsal aorta at embryonic day 10.5 (E10.5) (Boisset JC et al., 2010). Like CAR cells, endothelial cells in the bone marrow express several genes involved in HSC maintenance such as angiopoietin, SCF and CXCL12 (Chute JP et al., 2006). Expression of these genes enables these bone marrow endothelial cells to support HSCs and hematopoietic progenitor cells (HPCs) in culture (Chute JP et al., 2006).

SDF-1/CXCL12 is a potent chemotactic (directed cell movement) factor for HSCs and HPCs cells (Aiuti et al., 1997; Kim CH and Broxmeyer HE, 1998). It plays an essential role in the maintenance of HSCs, including homing, engraftment and repopulating activity, as well as HSC quiescence and retention in the bone marrow (Kawabata K et al., 1999; Peled A et al., 1999; Bonig H et al., 2004; Nie Y et al., 2008). It enhances the survival of HSCs and HPCs, an effect increased in synergy with other cytokines (Lee Y et al., 2002; Broxmeyer HE et al., 2003; Tzeng YS et al., 2011). Treatment of mouse bone marrow cells and human cord blood HPCs with soluble SDF-1/CXCL12 enhanced their replating efficiency, and bone marrow cells from mice expressing a human SDF-1/CXCL12 transgene exhibited increased replating capacity of single macrophage- and multipotent progenitor-derived colonies (Broxmeyer HE et al., 2007).

Despite work from several groups describing the role of SDF-1/CXCL12 in the maintenance of HSCs and HPCs in the various niches in the bone marrow (Mendez-Ferrer S et al., 2010; Ding L et al., 2012; Ugarte F and Forsberg EC, 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014), there is limited information on the mechanism by which SDF-1/CXCL12 functions at the molecular level for immature blood cell function in the bone marrow (Lee Y et al., 2002). Regulation and restriction of mitochondrial metabolism has been shown to be critical in maintaining the quiescent state of HSCs in the bone marrow by preventing mitochondrial produced reactive oxygen species (ROS), which can promote differentiation and HSC attrition and potential dysfunction (Yu YM et al., 2013; Qian p et al., 2015; Mantel C et al., 2012; Yalcin S et al., 2010; Mantel CR et al., 2015; Broxmeyer HE et al., 2015). Recent work from our group has shown that SDF-1/CXCL12 can modulate mitochondrial activity and mitochondrial mass in murine bone marrow cells expressing a mouse SDF-1/CXCL12 transgene (Mantel C et al., 2010). We therefore hypothesized that SDF-1/CXCL12 regulates mitochondrial respiration in early hematopoietic cells.

Mitochondria and Hematopoietic Stem Cell Maintenance

Mitochondria are the main source of ATP production within cells. It is thought that mitochondria arose around two billion years ago from the engulfment of a α -proteobacterium by a eukaryotic ancestor cell type (Lane N and Martin W, 2010; Freidman JR and Nunnari J, 2014). The human mitochondrial

genome contains genetic material encoding 13 proteins which are core constituents of the mitochondrial electron transport complexes I-IV, that are embedded in the inner mitochondrial membrane. Together with the Krebs cycle in the mitochondrial matrix, the electron transport chain creates an electrochemical gradient through the coupled transfer of electrons to oxygen and the transfer of protons from the matrix to the intermembrane space. The electrochemical gradient, coupled with oxygen consumption drives complex V of the chain, also known as ATP synthase, which catalyzes the production of most of the cellular ATP. Changes in the electrochemical gradient of the mitochondria have become readouts of mitochondrial functional status. Along with being the major energy producing organelle within cells, mitochondria also play critical roles in amino acid, fatty acid, and steroid metabolism as well as production of and cell signaling by reactive oxygen species (ROS), calcium homeostasis and apoptosis (Hock MB and Kralli A, 2009).

Stem cells are characterized by two key properties: self-renewal, and pluripotency. For HSCs another key feature is metabolic quiescence in the bone marrow microenvironment and careful regulation of these three properties is fundamental to ensure proper HSC maintenance and function. The blood system is a highly dynamic tissue in mammals and has a high rate of cellular turnover on a daily basis. Most mature cells of the blood system have short lifespans and the task of maintaining blood cell homeostasis rests almost entirely on the self-renewal and differentiation ability of the long-term but rare population of HSCs. These long-

term HSCs maintain themselves and generate all types of mature blood cells by producing increasingly committed progenitor cells (Orkin SH and Zon LI, 2008).

In adults, HSCs are found in specialized niches in the bone marrow, and these niches are thought to help regulate their metabolic quiescence while allowing them to stay poised for rapid and wide spread production of blood cells under emergency or stress conditions, while at the same time limiting HSC proliferation to homeostatic blood cell production. Under steady state/homeostatic conditions, adult HSCs divide rarely in order to maintain the HSC pool and to produce a low number of committed progenitor cells. In mice, most adult HSCs divide once every 30 days, but there is a small population of very deeply quiescent HSCs that divide on average once every 145-193 days (Passegue E et al., 2005; Wilson A et al., 2008; Foudi A et al., 2009). Despite such low cycling of quiescent HSCs, they are capable of rapid response to stress or damage. As HSCs age, their numbers increase, but at the expense of their functionality (Beerman I et al., 2010; Geiger H et al., 2013, Snoeck HW, 2013; Geiger H et al., 2014; Mendelson A and Frenette, 2014). HSC functional attrition can lead to impaired blood cell production, with characteristic anemia, immunosenescence and increased age related blood disorders such as bone marrow failure, myeloproliferative neoplasms and leukemia (Rossi DJ et al., 2008; Beerman I et al., 2010; Geiger H et al., 2013, Snoeck HW, 2013; Geiger H et al., 2014; Mendelson A and Frenette, 2014). HSC quiescence, proliferation and differentiation demand a unique set of bioenergetics demands, respectively (Shyh-Chang N et al., 2013). HSCs have a high degree of

metabolic plasticity, which allows them to transition effectively from quiescence to activity.

There is increasing data that supports a direct involvement of mitochondria and mitochondrial respiration (OXPHOS) in the regulation of stem cell pluripotency (Teslaa t et al., 2015; Zhang H et al., 2016). HSC quiescence functions to maintain cellular integrity by limiting cellular damage from mitochondrial respiration and cytotoxic agents and at the same time prevents HSC exhaustion through uncontrolled cell cycle entry and proliferation (Orford KW and Scadden DT, 2008; Bakker ST and Passegue E 2013; Yu WM et al., 2013). There is increasing evidence that HSCs have lower numbers of mitochondria (mitochondrial mass) and lower mitochondrial membrane potential ($\Delta\Psi_m$) and ATP levels as compared to other blood cell types (Romero-Moya D et al., 2013; Simsek T et al., 2010; Norddahl GL et al., 2011; Takubo K et al. 2013; Mantel C et al., 2012; Maryanovich M et al., 2016; Oburoglu L et al., 2016; Mohrin M and Chen D, 2016). These results have begun to suggest a role for mitochondria metabolism in the regulation of HSC quiescence, proliferation and differentiation. Furthermore, these mitochondria^{low}, $\Delta\Psi_m$ ^{low} populations of HSCs have a greater reliance on anaerobic glycolysis as compared to mitochondrial OXPHOS and the TCA cycle as evidenced by increased glycolytic intermediates and a near absence of TCA metabolites (Simsek T et al., 2010; Norddahl GL et al., 2011; Takubo K et al., 2013). There is also evidence suggesting that mitochondrial fission and fusion plays a roles in lineage specification (Luchsinger LL et al., 2016). Glycolysis is an

inefficient energy producing process, producing 2 ATP per molecule of glucose as opposed to 36 ATP via mitochondrial OXPHOS (Vander Heiden MG et al., 2009). However, enforced glycolysis and low ATP production may be sufficient for the low energy demands of quiescent HSCs and also is a protective measure against mitochondrial associated damage (Folmes CD et al., 2012; Mantel C et al., 2010; Mantel C et al., 2012). Furthermore, metabolic quiescence seems to be supported by recent evidence that HSCs reside in hypoxic niches within the bone marrow microenvironment and reliance on glycolytic metabolism in hypoxia is conducive to metabolic quiescence (Parmar K et al., 2007; Eliasson P and Jönsson JI 2010; Suda T et al., 2011). Several lines of evidence support this.

First, HSCs have stable expression of the transcription factor hypoxia-inducible factor 1 α (HIF-1 α), which is stabilized under hypoxia and undergoes proteasomal degradation when oxygen levels are above 5%. Under hypoxia, HIF-1 α directly targets the microRNA (miRNA) mir-210, which is upregulated by HIF-1 α . Mir-210 directly targets and inhibits the expression of the Fe-S clustering scaffold proteins ISCU1/2, thus inhibiting the assembly of Fe-S clusters of complex I of the electron transport chain and the enzymatic activity of aconitase, an enzyme that catalyzes the isomerization citrate to isocitrate, which fuels the TCA cycle (Chan YC et al., 2012). HIF-1 α and mir-210 function to block mitochondrial metabolism in HSCs in hypoxia (Mantel C et al., 2015). Next, researchers studied the location of HSCs in the bone marrow by using pimonidazole, a compound that is incorporated into cells under hypoxia and forms adducts with cellular proteins.

HSCs in hypoxia exhibited increased incorporation of pimonidazole suggesting that they reside in hypoxic regions of the bone marrow (Simsek T et al., 2010; Parmar K et al., 2007; Takubo K et al., 2010). Lastly, HSC loss was seen when researchers injected tirapazamine, a toxin that is selective for cells in hypoxia (Parmar K et al., 2007). Taken together, these results suggest that HSCs reside in low oxygen regions in the bone marrow, but a recent report using laser scanning cytometry to study the spatial distribution of HSCs based on pimonidazole incorporation as well as HIF-1 α levels has revealed that HSCs may not reside in regions of minimal oxygen tension, suggesting a hypoxia-independent, cell intrinsic mechanism of HIF-1 α stabilization (Nombela-Arrieta C et al., 2013). However, it may be possible that HIF-1 α is more stable under increased levels of oxygen in the bone marrow as previously thought allowing HSCs to reside in areas of the bone marrow with increased levels of oxygen.

It is becoming increasingly clear that mitochondria play an important role in HSC metabolism and lineage fate decisions (Mantel C et al., 2010; Romero-Moya D et al., 2013; Simsek T et al., 2010; Norddahl GL et al., 2011; Takubo K et al., 2013; Mantel C et al., 2012; Maryanovich M et al., 2016; Oburoglu L et al., 2016; Mohrin M and Chen D, 2016). Mitochondrial dysregulation is a potential cause of hematopoietic stem and progenitor cell dysfunction. Differentiation from a quiescent HSC to a committed progenitor involves increased proliferation and imposes a unique set of metabolic demands on HSCs. Proliferating cells must generate energy while at the same time inducing many biosynthetic pathways

involved in replication. For HSCs, differentiation is a metabolic switch, and differentiation demands higher energy inputs which are required to maintain the differentiated progeny derived from the parent HSC (Folmes CD et al., 2012). To adapt to the greatly increased energy demands, cells rely on changes in the mitochondrial network. Mitochondria are highly dynamic organelles that undergo biogenesis and degradation as well as fission and fusion (Xu X et al. 2013). As mitochondrial dynamics change in response to greater energy demands, increases in mitochondrial DNA copy number as well as increases in electron transport chain subunits and decreases in glycolytic enzymes accompany these changes.

A recent report from our lab suggest that as HSCs differentiate, LSK cells increase mitochondrial mass but still have low $\Delta\Psi_m$. This suggests that HSCs begin to upregulate mitochondrial biogenesis but keep $\Delta\Psi_m$ as a means to protect themselves from mitochondrial associated oxidative stress/ reactive oxygen species production, while at the same time being poised to increase mitochondrial OXPHOS in committed progenitors. Furthermore, increases in mitochondrial biogenesis are paralleled by increased in CD34 surface expression and the potential loss of pluripotency (Mantel C et al., 2010). Mice with conditional knockout of the PTEN-like mitochondrial phosphatase PTPMT1 (protein tyrosine phosphatase mitochondrial 1), revealed that HSCs strictly rely on their ability to activate mitochondrial OXPHOS in order to differentiate (Yu WM et al., 2013). To investigate the role that PTPMT1 plays in hematopoiesis, investigators generated PTPMT1^{fl/fl} Mx1-Cre mice. In this system expression of the Cre recombinase is

placed under the control of the Mx1 promoter, whose activity is inducible by interferon α or β , as well as by pl-pC (an interferon inducer). Cre recombination was observed in vivo after pl-pC administration (Kuhn R et al., 1995). Four week old PTPMT1^{fl/fl} Mx1-Cre mice were treated with polyI-polyC (plpC) to induce Cre expression and PTPMT1 deletion in pan-hematopoietic cells. Once deleted, 80% of PTPMT1^{-/-} mice died 2-3 weeks after plpC treatment due to pancytopenia and severe anemia (Yu WM et al., 2013). 20% of plpC treated mice survived owing to incomplete deletion of PTPMT1. In colony assays, myeloid and lymphoid progenitors were markedly decreased in PTPMT1^{-/-} mice as compared to controls, and interestingly, HSCs (Lineage⁻Sca-1⁺ c-Kit⁺ CD150⁺ CD48⁻ Flk2⁻) in PTPMT1^{-/-} knock out mice were ~40 fold greater than control mice (Yu, W.M. et al., 2013). In a PTPMT1^{fl/fl} Vav1-Cre⁺ hematopoietic specific knockout mouse model, pups failed to survive past 5-9 days, but knockout pups transplanted with WT bone marrow were rescued. Total bone marrow cellularity of knockout pups was decreased by ~75% at postnatal day 5 and hematopoietic progenitor cells were undetectable, suggesting a failure of postnatal hematopoiesis (Yu WM et al., 2013). However, the numbers of HSCs in knockout pups were increased ~30 fold. Despite PTPMT1 being a critical factor in proper mitochondrial function, bone marrow cells lacking PTPMT1 did not exhibit the apoptosis and ROS production normally seen with mitochondrial dysfunction (Yu WM et al., 2013). Purified, PTPMT1 depleted HSCs failed to produce colonies in CFU assays in response to cytokine stimulation and when co-cultured with OP9 stromal cells failed to produce Lineage⁺ progeny. Single cell knockout HSCs also failed to differentiate as

compared to WT cells under in vitro culture conditions. Together these results suggested that a block in differentiation in PTPMT1 knockout mice/cells mainly causes hematopoietic failure (Yu WM et al., 2013). Competitive and serial repopulation capabilities of PTPMT1 knockout HSCs are blocked as well. PTPMT1 knock out LSK cells had lower basal and maximal oxygen consumption rates (OCR) and higher extracellular acidification rates (ECAR, a measure of the glycolytic rate) than WT cells. These results suggest that knock out PTPMT1 cells have decreased mitochondrial function and enhanced glycolysis, which supports a role for PTPMT1 in HSC expansion. In this study the authors revealed an essential role for PTPMT1 in the metabolic regulation of HSC differentiation and a requirement for mitochondria in HSC differentiation.

Mitochondria are the main producers of ROS. ROS are highly reactive forms of molecular oxygen such as the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide, H_2O_2 . Under homeostatic conditions, ROS are produced naturally by the electron transport during OXPHOS (Murphy MP 2009). When over produced, ROS can induce oxidative stress and DNA damage of both nuclear and mtDNA. ROS is important for mouse HSC differentiation. Mouse HSCs lacking AKT1 and AKT2 have lower levels of ROS, and have a deficiency in their differentiation potential and this can be rescued by increasing ROS levels (Juntilla MM et al., 2010). These results show low levels of ROS are fundamental to maintaining HSC quiescence and that increased ROS levels are necessary for HSC differentiation. ROS can also function as a second messenger that can drive fate decisions in a dose

dependent manner. HSCs with low intracellular ROS are more quiescent and exhibit increased self-renewal potential, as compared to HSCs with high ROS levels, which exhibit increased exhaustion (Jang YY and Sharkis SJ, 2007). The negative effects of ROS signaling on HSC self-renewal can be attributed to activation of p38 mitogen activated protein kinase (p38 MAPK) and the mammalian target of rapamycin (mTOR) pathways that can function to cause HSC exhaustion. Inhibition of either pathway or ROS scavenging can restore the long-term bone marrow reconstitution capability of HSCs with high levels of ROS (Jang YY and Sharkis SJ 2007; Ito K et al., 2006; Mantel CR et al., 2012). Inhibiting ROS production by inhibiting mTOR with rapamycin can enhance the ex-vivo expansion of HSCs (Rohrbaugh SL et al., 2011). Interestingly Inoue et al., found that increased mitochondrial respiration was more important for the commitment of HSCs to lineage-committed progenitors than for their differentiation to mature cell types and these results seem to be independent of ROS levels. (Inoue S et al., 2010).

Observations from our lab using a tissue specific hematopoietic *Stat3* knockout (*Stat3^{-/-}*) mouse model revealed a noncanonical role for STAT3 in mitochondrial function through the regulation of mitochondrial mass, $\Delta\Psi_m$, and ROS production (Mantel C et al 2012). *Stat3^{-/-}* bone marrow cells had severely depleted CD34⁻ LSK HSCs but greatly increased CD34⁺ LSK and total LSK cells as compared to WT. In primary competitive repopulating assays, recipients transplanted with *Stat3^{-/-}* bone marrow had significantly reduced % chimerism.

Decreased engraftment and repopulating capacity were also seen in secondary noncompetitive transplants. When HPCs were functionally assessed by bone marrow and spleen CFU assays, *Stat3*^{-/-} cells had severely impaired colony-forming ability deriving from colony forming unit granulocyte, macrophage (CFU-GM; granulocyte macrophage progenitors), blast forming units erythroid (BFU-E; erythroid progenitors), and colony forming unit's granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM; multipotent progenitors). Also, the cycling status of bone marrow and spleen HPCs were greatly reduced, supporting a decreased functional capacity of bone marrow and spleen HPCs. Mitochondrial dysfunction was also seen in *Stat3*^{-/-} bone marrow mice. Both mitochondrial mass and $\Delta\Psi_m$ were significantly increased in *Stat3*^{-/-} bone marrow HSCs versus wild-type (WT). Similar to humans, aged mice display shifts in ratios of lymphoid and myeloid blood cells as well as changes in erythroid cell morphology/function, and changes in HPCs. There was a pronounced lymphoid to myeloid cell shift in the peripheral blood of the *Stat3*^{-/-} mice as compared to WT. The *Stat3*^{-/-} mice also had a significant reduction in total hemoglobin content, as well as significantly lower erythrocyte counts, but were still in the normal range compared with WT. The red cell distribution width was significantly increased in the *Stat3*^{-/-} mice, which indicates anemia in the *Stat3*^{-/-} mice (Mantel C et al., 2012). These results suggest that *Stat3* deletion causes mitochondrial changes/dysfunction in HSCs and HPCs that is potentially related to the noncanonical, mitochondrial function of STAT3 in the regulation of the mitochondrial permeability transition pore (mPTP) and the electron transport chain (Wegrzyn J et al., 2009; Reich NC, 2009; Mantel C et al.,

2012) which have significant effects on HSC and HPC function and peripheral blood indices.

Hypothesis

With the hypothesis that mitochondria are critical to HSC function we sought to elucidate the role that mitochondrial function plays in HSCs. We hypothesized that mitochondria are essential to HSC self-renewal, quiescence, maintenance and fate determination. To begin testing our hypothesis, we analyzed bone marrow cells from SDF-1/CXCL12 transgenic (TG) mice to determine the effect that SDF-1/CXCL12 transgene expression has on mitochondrial function. We found that SDF-1/CXCL12 transgene expression potentially functions to upregulate mitochondrial biogenesis and mitochondrial function. We also analyzed the effect of SDF-1/CXCL12 treatment, , on mitochondrial function in the human leukemia cell line HL-60, and in primary mouse lineage negative bone marrow cells and found that SDF-1/CXCL12 treatment regulates mitochondrial respiration of these cells in a biphasic manner. We also analyzed bone marrow cells from a *Stat3*^{-/-} mouse model based on reports of the function of STAT3 in mitochondrial function. We found that *Stat3* gene deletion produces mitochondrial dysfunction in HSCs and HPCs.

MATERIALS AND METHODS

Animals

WT and *Stat3*^{-/-} mice were on a C57Bl/6 background and have previously been described (Welte T et al., 2003). Both male and female mice, approximately four to six months of age were used for these studies and WT littermates were used as controls. SDF-1/CXCL12 TG mice, expressing the murine SDF-1/CXCL12 gene under the control of a CMV promoter, on a C3H/HeJ-FEB background were previously described (Broxmeyer H et al., 2003). WT female C3H/HeJ-FEB mice were from Jackson Laboratories (Bar Harbor, Maine) and female TG mice were used for these studies. Female WT C57Bl/6 mice were from Charles Rivers, and were approximately six-eight weeks old. All animal studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC).

Hematopoietic progenitor functional assays

To assess the functional potential of hematopoietic stem and progenitors in vitro we harvested bone marrow from the femurs of WT and *Stat3*^{-/-} mice. Mouse bone marrow cells were plated at 5×10^4 cells in 1% methylcellulose containing 0.1mM hemin, 30% FBS (Hyclone, Logan, UT), 2 mM glutamine, and 2-mercaptoethanol. The following growth factors were used: 1 U/ml of recombinant human

erythropoietin (EPO) (Amgen, Thousand Oaks, CA), 50 ng/ml of recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF), interleukin 3 (IL-3), SCF, and Flt-3 ligand (R&D, Minneapolis, MN), and 5% vol/vol pokeweed mitogen mouse spleen cell conditioned media with fetal bovine serum (FBS). Cytokines were at a final concentration of 50 ng/ml. To estimate the percentage of HPCs in the S-phase of the cell cycle, we performed the thymidine kill technique using high specific-activity tritiated thymidine and was done as previously described (Becker AJ et al., 1965; Ponchio L et al., 1995). Cells, cytokines and methylcellulose were mixed together and 1 ml of methylcellulose/cell mixture was plated out onto 35 mm tissue culture dishes in triplicate and incubated for 7 days at 5% CO₂ and lowered (5%) O₂ in a humidified tissue culture incubator chamber. Colonies were scored after 7 days of incubation and colony forming unit granulocyte- macrophage (CFU-GM), burst forming unit erythroid (BFU-E), and colony forming unit (CFU-GEMM) were distinguished by colony morphology (Carow CE et al., 1993). Differences in colony formation were assessed by Student's t-test and a P≤0.05 was considered significant.

Surface marker phenotyping of WT and *Stat3*^{-/-} hematopoietic progenitors

To assess the lineage cell surface marker phenotypes of WT and *Stat3*^{-/-} mouse bone marrow cells, bone marrow was harvested from the femurs of WT and *Stat3*^{-/-} mice. Bone marrow cells were incubated for 2-5 minutes in red cell lysis buffer (eBioscience, San Diego, CA). Cells were then washed two times and

suspended in 500µl of staining buffer containing phosphate buffered saline (PBS) (Lonza, Walkersville, MD) and 5% FBS and incubated with a cocktail of fluorochrome conjugated antibodies specific for cell surface markers. All antibodies were from BD Bioscience (San Jose, CA). Anti-mouse antibodies used were lineage cocktail (lin), CD34, IL-7 receptor-alpha (IL-7Ra), sca-1, and c-kit. a combination of lin, sca-1, CD34, and Fc-receptor^{l/III} gamma (FcRII/IIIg) antibodies were used to analyzed megakaryocyte-erythroid progenitors (MEP), granulocyte-macrophage progenitors (GMP), and common myeloid progenitors (CMP). LSK cells were considered to be Sca-1⁺c-kit⁺ Lin⁻ IL-7Ra⁺, CMP were lin⁻ sca-1⁻ c-kit⁺ CD34⁺ FcRII/IIIg^{lo}, GMP were lin⁻ sca-1⁻ c-kit⁺ CD34⁺ FcRII/IIIg^{hi} and MEP are lin⁻ sca-1⁻ c-kit⁺ CD34⁻ FcRII/IIIg^{lo}. Cells were incubated for 15 minutes at room temperature then washed two times and suspended in PBS and analyzed by flow cytometry. Flow cytometry was performed with a BD Bioscience LSR II flow cytometer. Flow cytometry data were analyzed using Cyflogic (CyFlo Ltd., Turku, Finland). Data was analyzed by Student's t-test using SigmaPlot 11.0 software (Systat Software, San Jose, CA) and plotted using the same software.

Phenotypic analysis of lineage negative bone marrow cells

To analyze the percentage of hematopoietic stem and progenitor cells in the LSK compartment of WT, *Stat3*^{-/-}, and SDF-1/CXCL12 TG mouse bone marrow cells, respectively, bone marrow was harvested from the femurs of these mice, washed in PBS and resuspended in 5ml of red cell lysis buffer for 2-5 minutes,

then washed 2X and resuspended in 500 μ l of staining buffer (PBS + 2% FBS) and incubated with fluorochrome conjugated anti-mouse CD34, anti-mouse Sca-1, anti-mouse c-kit, and anti-mouse lineage cocktail antibodies for 15 minutes at room temperature. Cells were washed two more times and resuspended in PBS, and analyzed by flow cytometry.

Analysis of mitochondrial mass, membrane potential and ROS production

To analyze mitochondrial mass, membrane potential and ROS production in hematopoietic stem and progenitor cells in the LSK compartment of WT and *Stat3*^{-/-} mouse bone marrow cells, bone marrow was harvested from the femurs of WT, *Stat3*^{-/-} and SDF-1/CXCL12 TG mice, respectively. Bone marrow cells were washed in PBS and resuspended in 5ml of red cell lysis buffer (eBioscience, San Diego, CA) for 2-5 minutes. Cells were washed 2X with PBS and resuspended in RPMI without serum. MT Green FM, JC-1, MT Orange CM-H₂TM and MT Red CMXRos were from (Molecular Probes/Invitrogen, Carlsbad, CA). MT Green FM (50 nM) staining to quantitate mitochondria was done at room temperature for 30 minutes in RPMI 1640 (without FBS) and then washed twice with RPMI, then resuspended in cold RPMI before flow analysis. JC-1 (2 μ M) staining to quantitate mitochondrial membrane potential was done at 37°C for 30 min. in RPMI Cells were washed twice with cold RPMI and suspended in cold RPMI before flow analysis. Flow analysis was done as soon as possible (usually within 10 min) after staining/incubation with MT Green FM and JC-1 as these probes are not effectively

amenable to formaldehyde fixation, as indicated by the manufacturer, and they are subject to slow leakage from mitochondria, even in cold RPMI. We found that another critical consideration for accurate measurement of mitochondrial activities is to incubate and wash cells in RPMI 1640 with glucose and pyruvate (Gibco/Invitrogen; Carlsbad, CA) instead of PBS, because RPMI provides a source of substrates for glycolysis and mitochondrial metabolism. On the other hand, MT Red CMXRos (50 nM) for membrane potential and MT Orange CM-H₂TM (50 nM) for ROS are readily fixable in formaldehyde and can be stained and washed in PBS and fixed in Cytfix, (BD Bioscience, San Jose, California), and then analyzed by flow cytometry. Bone marrow cells were stained with surface marker antibodies (CD34, c-kit, sca-1, and lineage cocktail) first, and then promptly stained with mitochondrial probes. Flow cytometry was performed with a FACS Calibre or LSR II flow cytometer from BD (Bioscience, San Jose, California). Flow cytometry data were analyzed using WinList Software (Verity Software House, Topsham, MD), Cyflogic (CyFlo Ltd., Turku, Finland) and FlowJo (Ashland, Oregon).

Cell Culture and lineage negative mouse bone marrow cell isolation

Human HL-60 cells (ATCC CCL-240) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Iscove's Modified Dulbecco's Medium (IMDM) with 20% FBS. HL-60 cells were incubated in IMDM +20% FBS with and without 50 ng/ml SDF-1/CXCL12 (R&D, Minneapolis, MN) for two and 24 hours, respectively. This concentration of SDF-1/CXCL12 has been

shown to elicit optimal responses in several of our chemotaxis assays (Kim CH and Broxmeyer HE 1998; Broxmeyer HE et al., 2003; Broxmeyer HE et al., 2003 Broxmeyer HE et al., 2007; Capitano ML et al., 2015). Lin⁻ negative bone marrow cells were isolated from C57BL/6 mice using the Miltenyi Biotech (Auburn, CA) Mouse Lineage Cell Depletion Kit. After lineage depletion, Lin⁻ cells were incubated in IMDM +10% FBS and stimulated with or without 50 ng/ml SDF-1/CXCL12 (R&D) for two and 24 hours, respectively. HL-60 cells were pretreated for 30 min with 100ng/ml of AMD3100 (a SDF-1/CXCL12 antagonist that can block SDF-1/CXCL12 binding and signaling through CXCR4 (Broxmeyer H et al., 2005)) and then stimulated for 2 and 24 h, respectively, with 50 ng/ml of SDF-1/CXCL12.

Analysis of OCR and ECAR

HL-60 cells and Lin⁻ mouse bone marrow cells were cultured and treated as described above (for *Stat3*^{-/-} experiments, 10⁵ splenocytes per well were used, isolated from WT or *Stat3*^{-/-} mice). Seahorse Bioscience cell culture plates were coated with 50µl of Cell-Tak per well (23.1 µg/ml in 0.1 M NaCHO₃) for one hour at room temperature and then washed with dionized H₂O and allowed to air-dry completely. HL-60 and Lin⁻ cells were then seed onto plates in Seahorse Bioscience XF Assay Media containing 1mM pyruvate, 2 mM glutamine, and 10mM glucose for OCR measurements or containing 2 mM glutamine for ECAR measurements. Both HL-60 and Lin⁻ cells were plated at a density of 10⁵ cells/100 µl/ well. Cells from each group, for each experiment, were plated in minimum

replicates of three. Plated cells were spun down at 500 RPM for 5 minutes to allow cells to attach to the Cell-Tak coated wells prior to beginning the Seahorse analysis. Basal oxygen consumption rates (OCR), mitochondrial-linked ATP production, and the extracellular acidification rate (ECAR), a measure of the glycolytic rate, were obtained using the Seahorse Bioscience XF96 Extracellular Flux Analyzer from Seahorse Bioscience, and measurements were performed according to the manufacturer's instructions and as described previously (Mantel C et al., 2012; Gerencser AA et al., 2009; Bernier M et al., 2011). The XF Mito Stress test measures key parameters of mitochondrial function by directly measuring the OCR of cells by using a pharmacological approach, which utilized compounds that target the electron transport chain of the mitochondria to reveal important parameters of mitochondrial function. The assay uses oligomycin A (oligo A), FCCP, and rotenone. These compounds are loaded into special ports in the assay cartridge and sequentially injected into cell culture wells. This allows the assay to measure mitochondrial ATP production, maximal respiration, and non-mitochondrial respiration and proton leak. Oligo A targets ATP Synthase (complex V). The reduction in OCR due to oligo A correlates with mitochondrial-linked ATP production (Zhang E et al., 2012; Messina-Graham and Broxmeyer HE, 2016), FCCP is an ionophore that uncouples oxygen consumption from ATP production by collapsing the proton gradient and $\Delta\Psi_m$. FCCP allows the uncontrolled flow of protons from the intermembrane space of the mitochondria into the matrix, thus increasing oxygen consumption to its maximal rate. The third compound used is rotenone. We have optimized the concentration of rotenone and have found that

this concentration works very well in our system. Rotenone is a complex I inhibitor and shuts down oxygen consumption at the start of the ETC thus allowing us to determine non-mitochondrial respiration and proton leak. Figure 1 shows an illustration of the compounds and their ETC targets. Figure 2 shows a representation of the functional readout of a XF Mito Stress Test. Data were statistically analyzed and plotted using GraphPad Prism 6 and 7 (San Diego, CA). Differences were assessed with a Student's t-test or one-way ANOVA with Tukey's post-hoc correction. $P \leq 0.05$ was considered significant.

ECAR is a measure of the glycolytic rate of cells. Glucose is converted to lactate by glycolysis and in the process protons are produced and extruded from the cell into the extracellular media. As glycolysis produced lactate and thus protons, the media becomes acidic and the acidification is measured directly by the XF96 Extracellular Flux analyzer machine and is reported as ECAR. The Glycolysis Stress Test Kit allows the measurement of glycolysis, glycolytic capacity, and glycolytic reserve and non-glycolytic acidification. Like the Mito Stress Test, the Glycolytic Stress Test uses three sequentially injected compounds. Initially cells are incubated in the XF assay media without glucose and ECAR is measured. The first injection is 10mM glucose and cells begin to metabolize glucose through glycolysis, producing protons that acidify the assay media and the ECAR is measured which is the basal glycolytic rate. The second injection is oligomycin A which shuts down mitochondrial ATP production thus shifting cells fully to glycolysis to reveal the maximal glycolytic rate. The last

injection is 2-deoxyglucose, an analog of glucose, inhibits glycolysis by competitively binding to hexokinase of the glycolytic pathway, thus producing a decrease in ECAR which further confirms that the ECAR produced was in fact due to glycolysis. ECAR prior to glucose injection is referred to non-glycolytic acidification which may arise from CO₂ generated by the TCA cycle of the mitochondria that is converted to carbonic acid. Figure 3 is diagram of the readout of the XF Glycolytic Stress Test.

Figure 1.

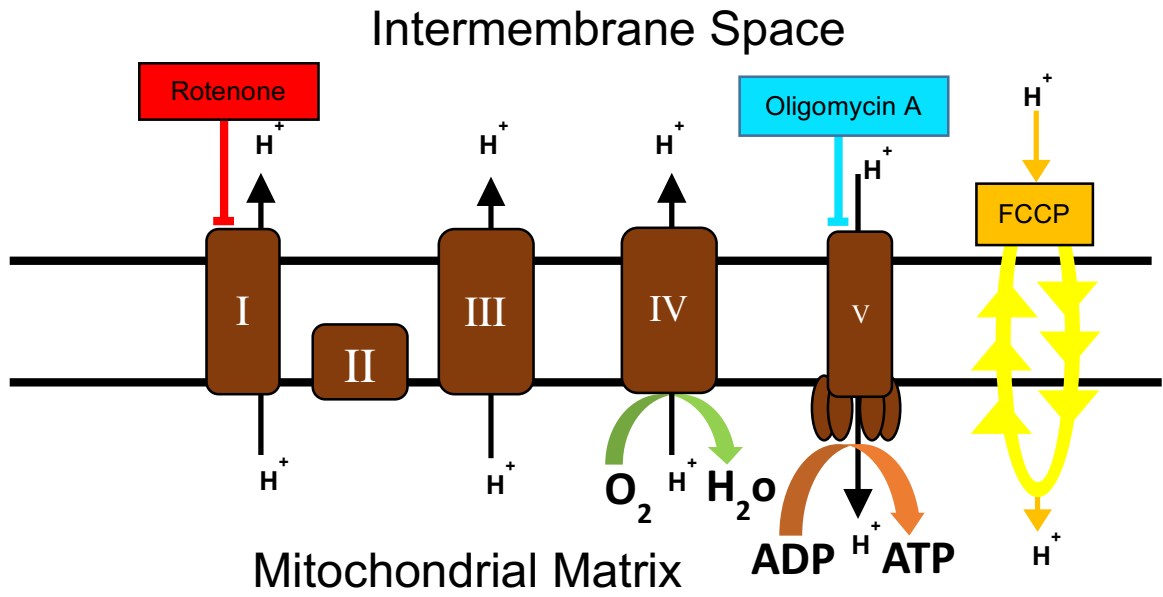


Figure 1. Modulators of the electron transport chain.

Illustration of the three compounds sequentially injected in the XF Mito Stress Test and their targets in the ETC. Oligomycin A inhibits complex V (ATP synthase), FCCP uncouples the mitochondria and rotenone inhibits complex I (www.seahorsebio.com).

Figure 2.

XF Cell Mito Stress Test Profile

Mitochondrial Respiration

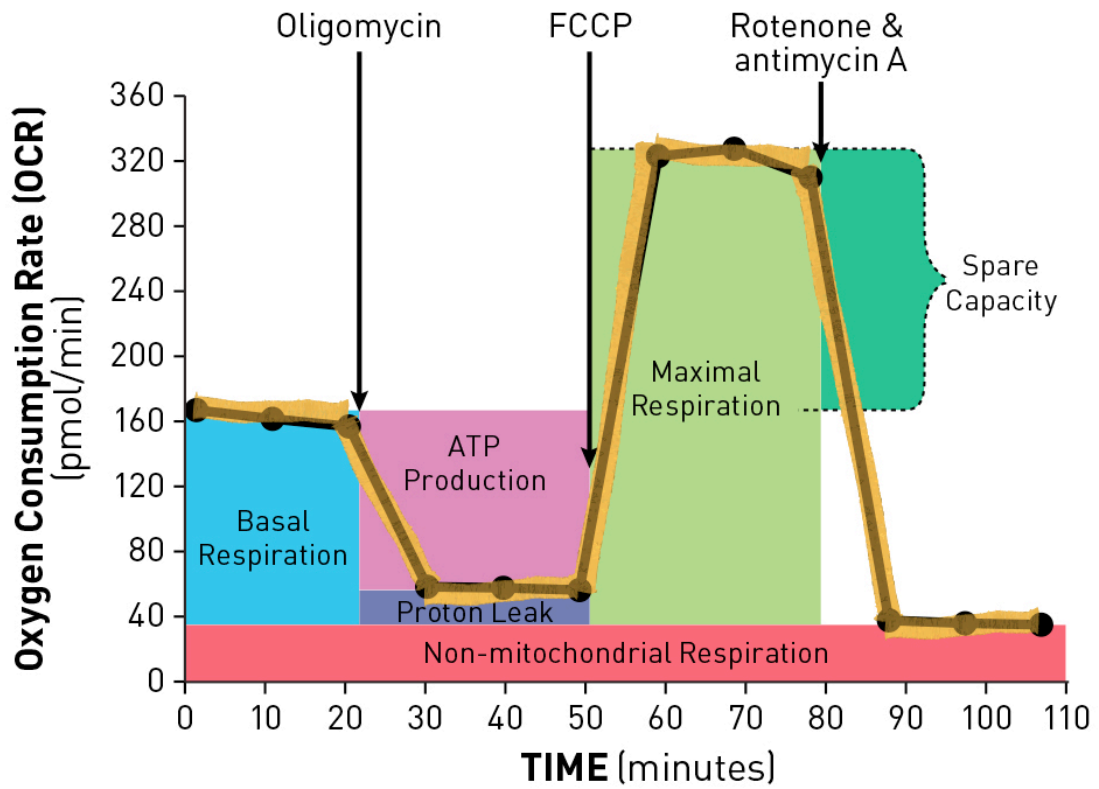


Figure 2. Profile of the XF Mito Stress Test Kit

Graphical representation of the data and different functional readouts of the XF Mito Stress Test Kit showing the sequential compound injections, the basal OCR (OCR), mitochondrial-linked ATP production, maximal respiration, spare respiratory capacity and non-mitochondrial respiration (www.seahorsebio.com).

Figure 3.

XF Glycolysis Stress Test Profile

Glycolytic Function

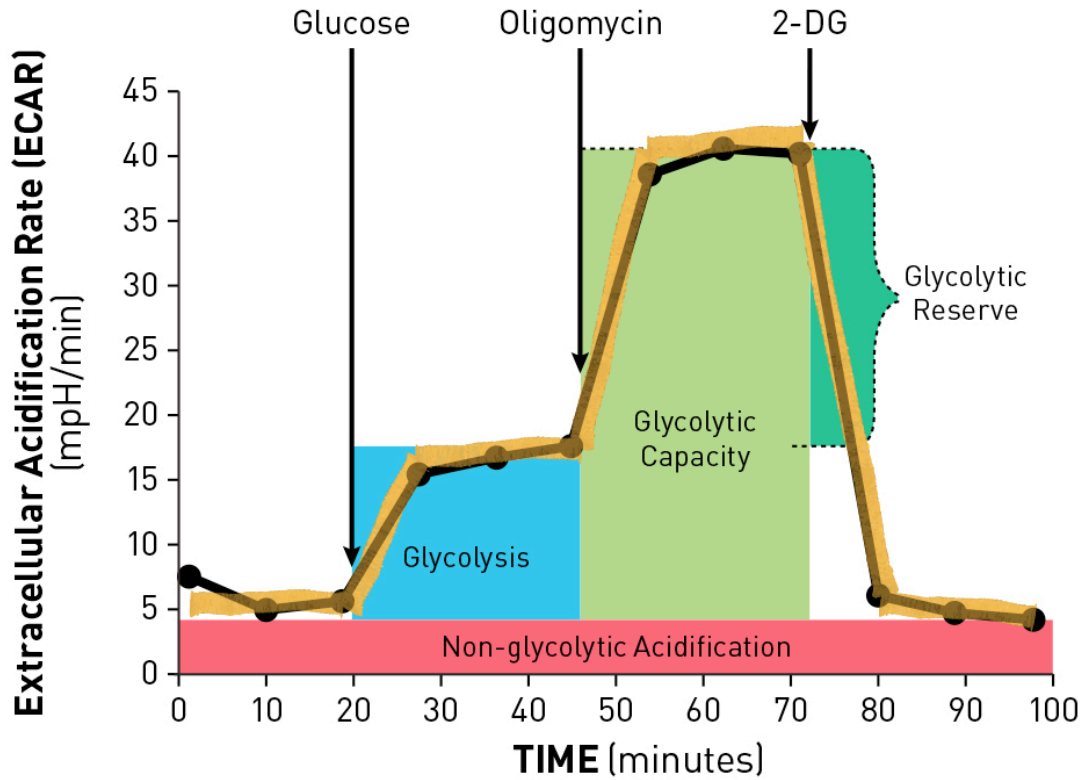


Figure 3. Profile of the XF Glycolytic Stress Test Kit

Graphical representation of the data and the different functional readouts of the XF Glycolytic Stress Test Kit showing the sequential compound injections and the basal glycolytic rate, maximal glycolytic rate, glycolytic reserve and non-glycolytic acidification (www.seahorsebio.com).

RESULTS

Role of SDF-1/CXCL12 in Mitochondrial Function in Hematopoietic Stem and Progenitor Cells

Understanding how SDF-1/CXCL12 functions in HSC maintenance, self-renewal, quiescence and differentiation could help to increase our understanding of how HSCs deal with or respond to increased oxidative risk and fate decisions during differentiation. This information that could be of broad use in studies of stem cells in general. To begin understanding the role that SDF-1/CXCL12 plays in the regulation of HSCs and HPCs we utilized a transgenic mouse model previously developed in the Broxmeyer lab, that globally expresses the murine SDF-1/CXCL12 transgene (Broxmeyer HE et al., 2003). Because SDF-1/CXCL12 is involved in HSC mobilization and homing to and from the bone marrow, and it been identified as a key factor in HSC maintenance in the bone marrow, and is also an important stress-induced HSC survival factor (Broxmeyer HE, 2008), we considered this is a good choice to use as a model to study perturbed steady-state hematopoiesis, and to begin to unravel the effect SDF-1/CXCL12 could potentially have on mitochondrial metabolism in HSCs and HPCs. CXCR4, a receptor for SDF-1/CXCL12, is expressed on HSCs and has been shown to inhibit glycolysis by suppressing the glycolytic enzyme, PGK-1, as well as being linked to mitochondrial biogenesis (Schioppa T et al., 2003; Wang J et al., 2007; Richard CL et al, 2008). The relationship of SDF-1/CXCL12 to metabolism in HSC and

HPC is not yet firmly established. Our study suggests a role for SDF-1/CXCL12 in the regulation of mitochondria in HSCs and a potential new role for both SDF-1/CXCL12 and mitochondria in fate determination.

Mouse bone marrow long term self-renewing HSC are enriched in a population of Lineage⁻, Sca-1⁺, c-Kit⁺ (LSK) cells (Blank U et al., 2008). LSK cells are composed of both long-term (LT) and short-term (ST) repopulating HSCs and HPCs. Expression of the surface-determinant CD34 can be used to distinguish between short and long-term repopulating HSCs (Blank U et al., 2008). Appearance of CD34 on the surface of LSK cells is closely linked to loss of long-term serial repopulating ability and pluripotency, and is an early marker to assess pluripotency and differentiation status of LSK cells. We noted two discrete populations of LSK cells based on CD34 surface expression and mitochondrial mass (Mt-mass; Figure 4). These populations in WT mouse bone marrow were either CD34^{low}/Mt-mass^{low} or CD34^{hi}/Mt-mass^{hi} (Figure 4A). On the other hand, SDF-1/CXCL12 TG mouse bone marrow contained one discrete population of CD34^{hi}/Mt-mass^{hi} cells (Figure 4B). This suggested that the expression of the murine SDF-1/CXCL12 transgene had a significant effect on the proportion of cells that were CD34^{lo}/Mt-mass^{lo}, compared to CD34^{hi}/Mt-mass^{hi} cells. Figure 5 is a quantitative comparison of the populations in Figure 4A and 4B. Furthermore, these data suggest that upregulation of mitochondrial biogenesis mediated by the SDF-1/CXCL12 transgene expression increases in HSCs early and is in close synchrony with HSC differentiation,

Figure 4.

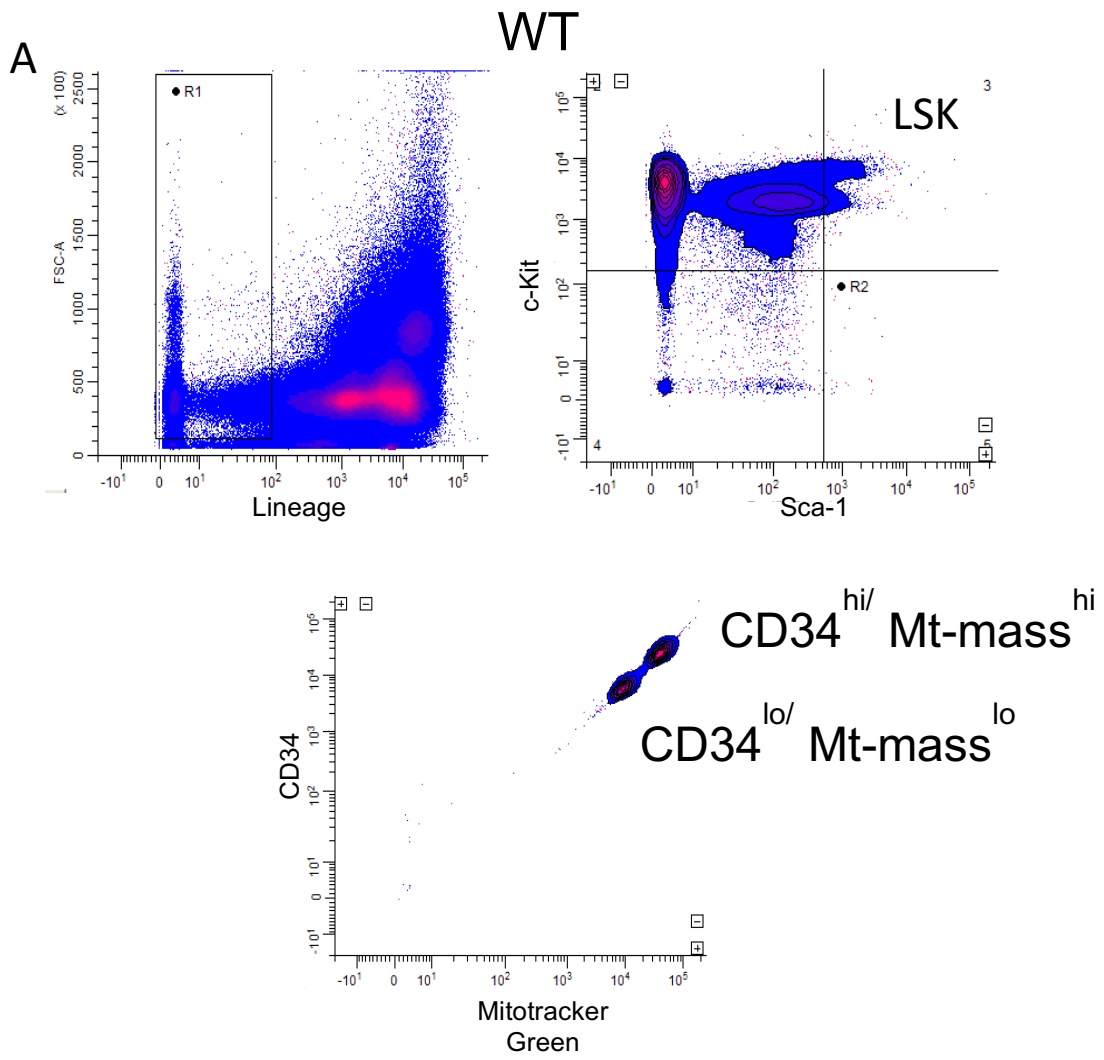


Figure 4. Continued

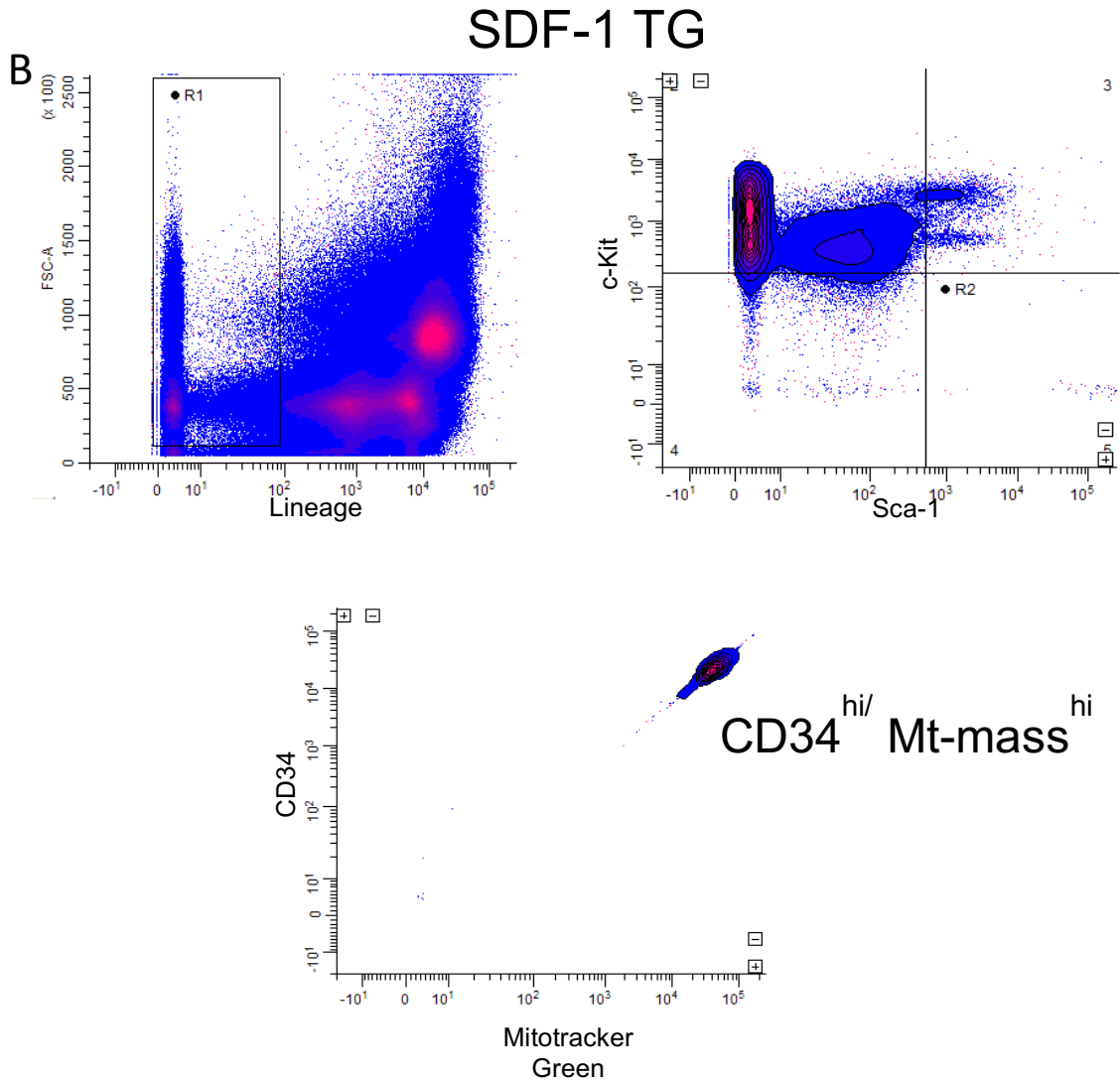


Figure 4. Mitochondrial mass is linked to CD34 expression in mouse HSCs

Flow cytometric analysis of mitochondrial mass in bone marrow LSK cells using MitoTracker Green FM is shown. WT mouse bone marrow (A) and SDF-1/CXCL12 TG expressing mouse bone marrow cells (B). The top left panels show lineage gating (LIN), the top right panels show LSK gating, and the bottom left panels show CD34 expression and MT Green FM fluorescence in gated LSK cells (Mantel C, Messina-Graham S, and Broxmeyer H, 2010)

Figure 5.

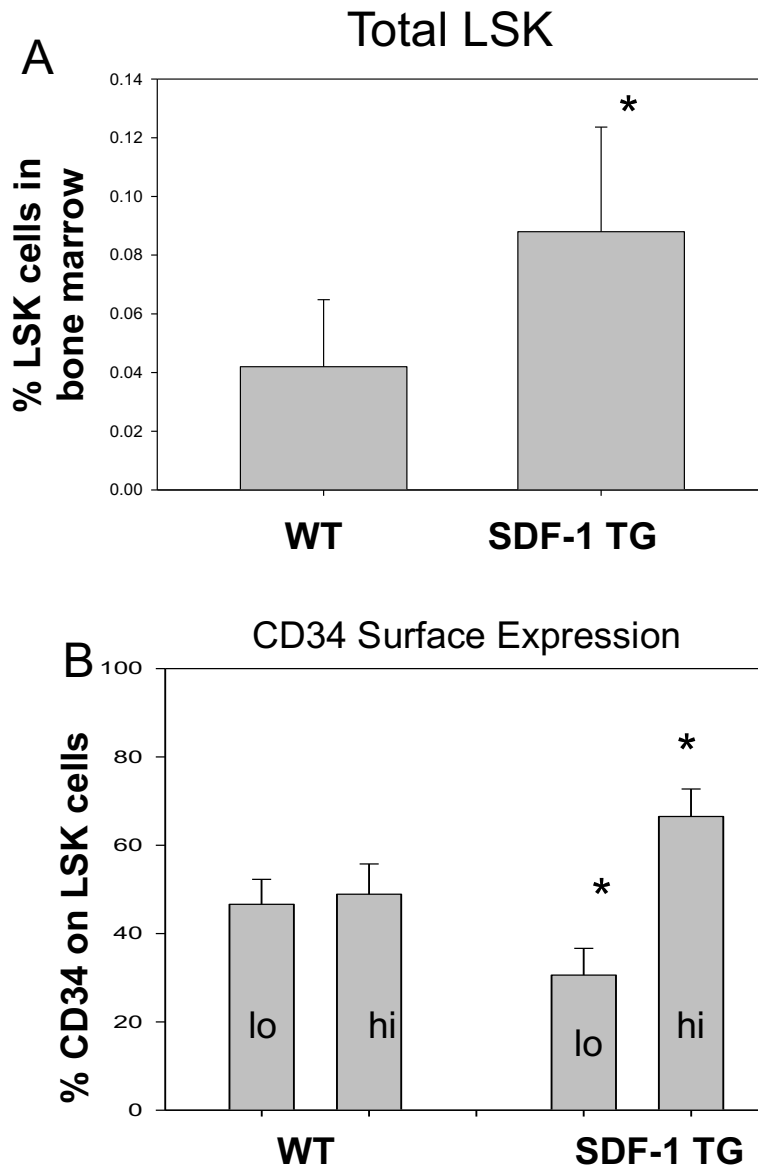


Figure 5. Total LSK and CD34^{hi/lo} LSK populations in mouse bone marrow

Quantitative analysis of % of total bone marrow cells from WT and SDF-1/CXCL12 TG LSK cells is shown (A). The mean \pm SEM from six independent experiments is shown. The P value indicates a significant increase of LSK cells in SDF-1/CXCL12 TG compared to WT bone marrow. Comparative analysis of the proportions of CD34^{lo/hi} LSK cells from WT and SDF-1/CXCL12 TG bone marrow are shown in (B). Data are expressed as mean % \pm SEM of LSK cells. Data from five independent experiments are shown and values of p indicate a significant difference in both population types from WT compared to SDF-1 TG LSK cells (*: $P < 0.05$; **: $P < 0.01$). (Mantel C, Messina-Graham S, and Broxmeyer H, 2010).

increased CD34 expression, and loss of pluripotency and a possible fate determinant during differentiation.

Our studies also reveal a relationship between the size of the LSK cells (indicated by forward scatter on a flow cytometry dot plot) and Mt-mass (Figure 6A). Three distinct populations of LSK cells were discernable in mouse bone marrow (Figure 6A and 6B). Population 1 was CD34^{lo} and Populations 2 and 3 were CD34^{hi} (Figure 6C). Figure 6D shows the percentage of each of the three mitochondrial distinct LSK population in the WT and SDF-1/CXCL12 TG mice bone marrow cells. We further characterized these three populations by measuring their mitochondrial activity by measuring the $\Delta\Psi_m$ using the mitochondrial membrane potential-dependent fluorochrome, JC-1 (Figure 6B). In WT mouse bone marrow LSK cells there are three populations based on size and $\Delta\Psi_m$ (Figure 6B) and the patterns observed in the SDF-1/CXCL12 TG bone marrows LSK cells are consistent with the presence of a smaller, Mt-mass^{hi} population of LSK cells having lower $\Delta\Psi_m$ than Population 3, but these have $\Delta\Psi_m$ similar to or slightly higher than that of Population 1. Figure 6C is a schematic of the Populations 1-3. The relative proportions of these populations in WT and SDF-1/CXCL12 TG mouse bone marrow are shown in Figure 6D. The influence of the SDF-1/CXCL12 transgene expression in HSC homeostasis appears to induce a quantitative proportional shift in the three populations rather than have a direct influence on Mt-mass or activity. This population shift is primarily toward LSK cells with increased Mt-mass and activity and is consistent with proportionally increased differentiation of CD34^{lo} LSK

Figure 6.

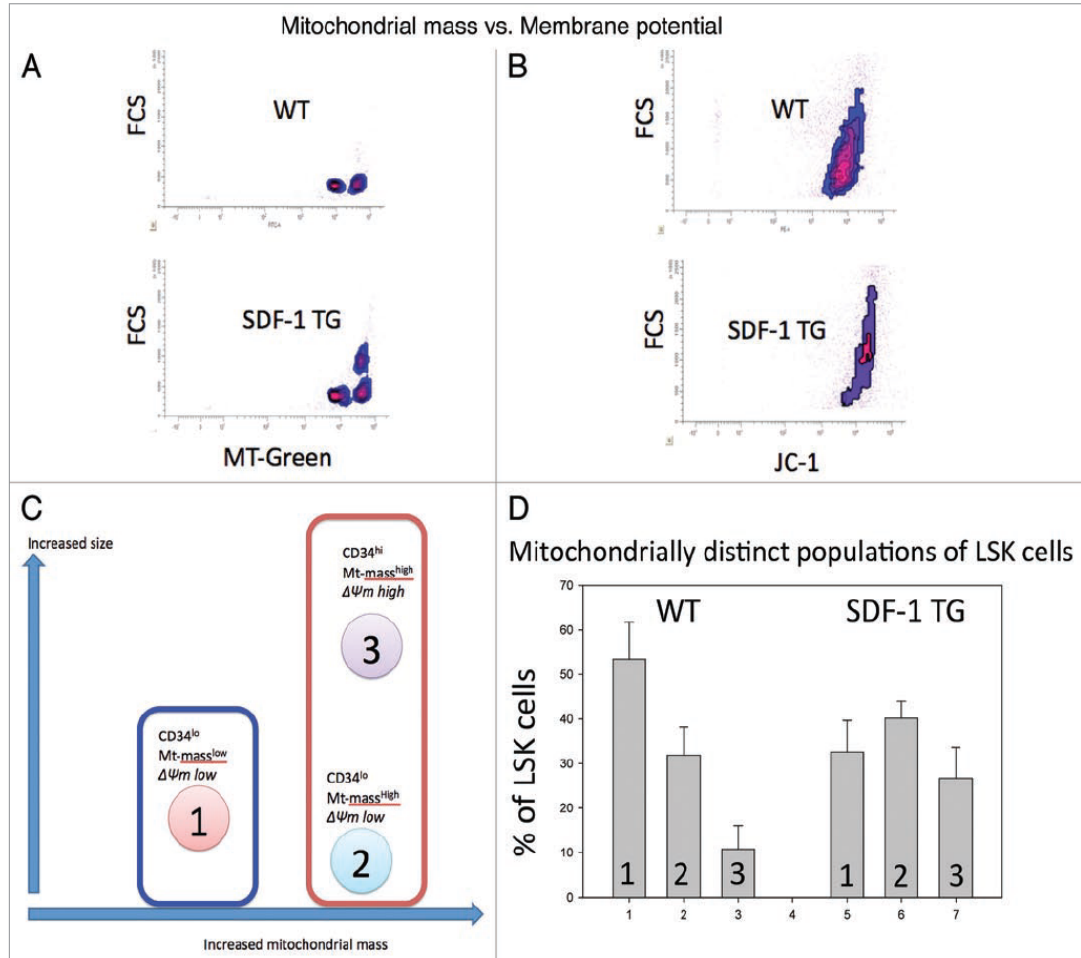


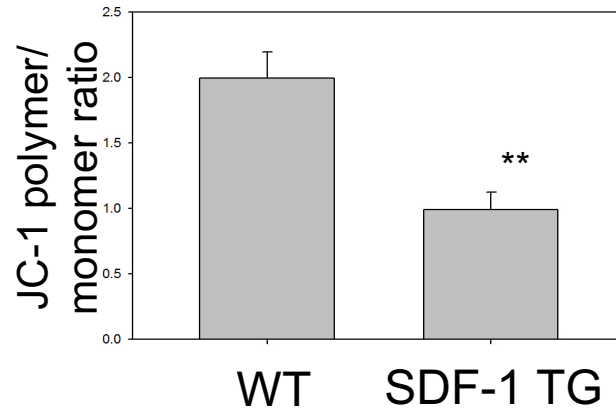
Figure 6. Three distinct populations of LSK cells are seen in bone marrow based on size and mitochondrial mass

Density plots of flow cytometric data comparing cell size and MitoTracker Green FM fluorescence or size and JC-1 polymeric (red) fluorescence intensity (A and B). Data from WT LSK cells and SDF-1/CXCL12 TG LSK cells are shown. Three distinct populations are discernable (A and B) and are indicated numerically (C). The percentage of mitochondrially distinct LSK cells is represented in (D). Data represent three independent experiments (Mantel C, Messina-Graham S, and Broxmeyer H, 2010).

cells to CD34^{hi} LSK cells (population 2 and 3). Interestingly we found a population of CD34^{hi} LSK cells with increased Mt-mass, but with low $\Delta\Psi_m$ (population 2) in the bone marrow. This further suggests that these cells in the SDF-1/CXCL12 TG mouse bone marrow have begun to differentiate and increase mitochondrial mass but have yet increase mitochondrial activity and are poised to become mitochondrially active as a means to prevent oxidative damage from increased ROS production. LSK cells can be categorized as two types based on their mitochondrial activity. LSK cells with high $\Delta\Psi_m$ and LSK cells with low $\Delta\Psi_m$ (Figure 7A). And the overall effect on LSK cells seems to reduce the overall mitochondrial activity in in LSK cells, but interestingly, there is a significant increase in LSK cells that have low $\Delta\Psi_m$ with a coordinate decrease in LSK cells with high $\Delta\Psi_m$ (Figure 7B). This is consistent with larger LSK cells with increased mitochondrial mass and reduced $\Delta\Psi_m$ in the SDF-1/CXCL12 TG mouse bone marrow. Taken together, these results suggest that SDF-1/CXCL12 transgene expression modulates mitochondrial activity potentially as means to overt oxidative damage during differentiation.

Figure 7.

A Overall $\Delta\psi_m$ of total LSK cells



B % of LSK cells that are high $\Delta\psi_m$ or low $\Delta\psi_m$

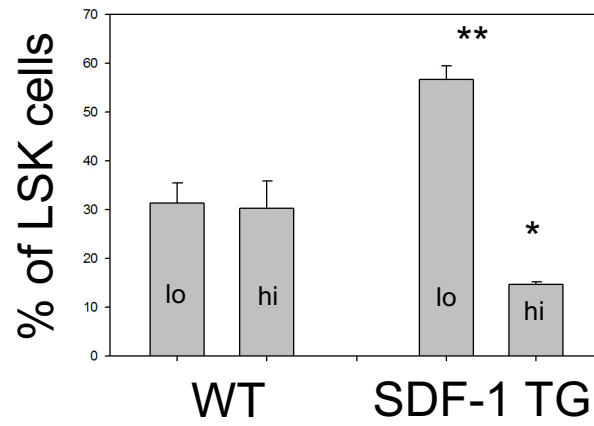


Figure 7. Two types of LSK cells with different mitochondrial membrane potential are in mouse bone marrow

Two populations of LSK cells are discernable based on JC-1 polymer/monomer ratios. JC-1 polymer/monomer ratio is a measure of $\Delta\Psi_m$. The overall $\Delta\Psi_m$ of total LSK cells (A) for WT or SDF-1/CXCL12 TG bone marrow cells are shown. Data are representative of five independent experiments. Mean JC-1 ratios \pm SEM for five independent experiments are shown. The P value indicates that $\Delta\Psi_m$ was significantly lower in SDF-1/CXCL12 TG LSK cells compared to WT LSK cells. Also shown are the relative proportions of the two populations with either low or high $\Delta\Psi_m$ (B) that were independently gated (and expressed as mean % of total LSK cells \pm SEM for WT or SDF-1/CXCL12 TG LSK cells. (*; P = 0.05; **; P = 0.01) (Mantel C, Messina-Graham S, and Broxmeyer H, 2010).

SDF-1/CXCL12 Modulates Mitochondrial Respiration of Immature Blood Cells in a Biphasic Manner

Effects on HL-60 cells, an established human cell line

To investigate the potential role that SDF-1/CXCL12 plays in controlling mitochondrial respiration, we employed the human promyelocytic leukemia cell line, HL-60, to model human immature subsets of hematopoietic cells, because HL-60 cells express high levels of CXCR4 and very little CXCR7. Furthermore, HL-60 cells are growth factor-independent, which allows the effect of SDF-1/CXCL12 treatment not to be masked by the effect of other cytokines needed for growth and cell maintenance. Along with HL-60 cells, we proceeded to confirm the results in Lin⁻ primary mouse bone marrow cells. We maintained HL-60 cells in culture with IMDM +20% fetal bovine serum under standard cell culture conditions, then cultured them for two and 24 hours, respectively, with or without 50 ng/ml of SDF-1/CXCL12 to assess rapid and more delayed effects, respectively. We assessed oxygen consumption rates (OCR), a measure of mitochondrial respiration, for each group on a Seahorse Bioscience XF96 Extracellular Flux Analyzer. Cells treated for two hours with SDF-1/CXCL12 had a significant reduction in OCR of $24.8 \pm 1.0\%$ (mean \pm SEM) as compared to two-hour unstimulated control cells (Figure 8A). In contrast, HL-60 cells treated for 24 hours with SDF-1/CXCL12 had a significantly increased OCR of $96.7 \pm 13.3\%$ over unstimulated 24-hour control cells (Figure 8B). This suggests that SDF-1/CXCL12 plays a potential role modulating mitochondrial

Figure 8.

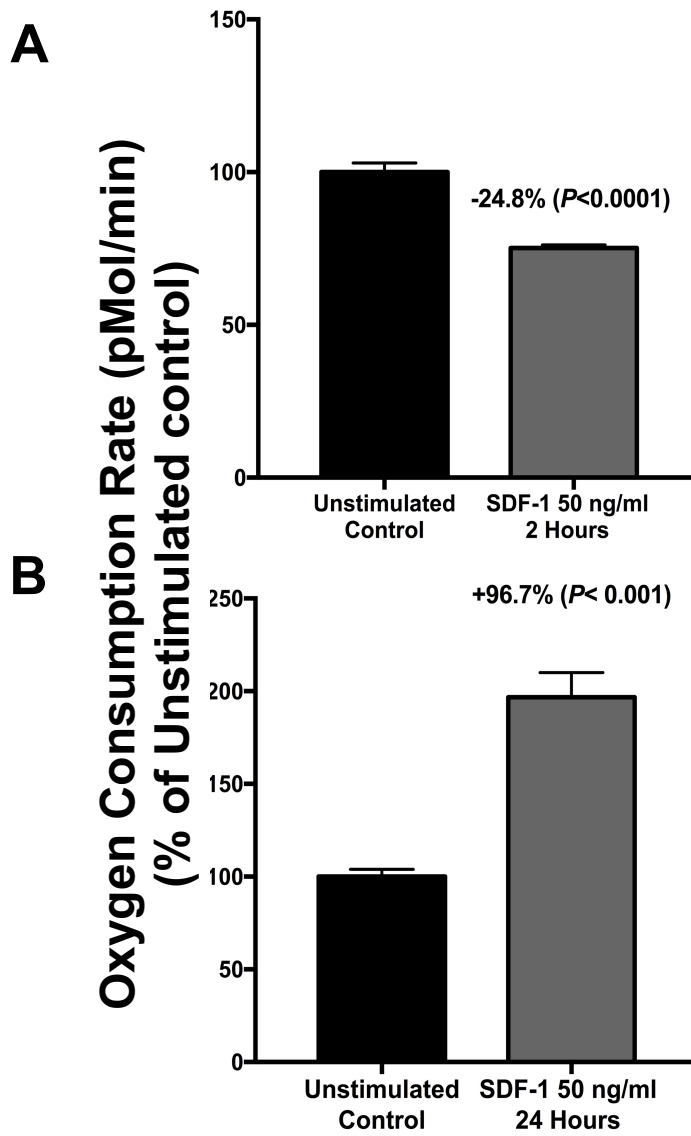


Figure 8. SDF-1/CXCL12 Regulates mitochondrial oxygen consumption rates of HL-60 cells in a biphasic manner

The human leukemia cell line, HL-60, was treated with 50ng/ml of SDF-1/CXCL12 for two (A) and 24 (B) hours respectively. After each time point, cells were collected and their oxygen consumption rates (OCR). Results are the mean \pm SEM of four independent experiments with significant differences as compared to unstimulated control shown in the figure (Messina-Graham S and Broxmeyer HE, 2016).

respiration in a bi-phasic manner as indicated by changes in mitochondrial OCR and the mediated SDF-1/CXCL12 effect is potentially time dependent and circadian.

We next analyzed mitochondrial-linked ATP production to determine if SDF-1/CXCL12 treatment changes ATP production in a similar manner to that seen in the OCR (Figure 9A and B). Mitochondrial-linked ATP production is the difference between the basal OCR and oligomycin-A (0.6 μ M) repressed OCR (Zhang JE et al., 2012). Mitochondrial-linked ATP production was significantly reduced in the two-hour SDF-1/CXCL12 treated group compared to the unstimulated control cells (-29.2 \pm 2.9%), while the 24-hour treated group was significantly increased versus control (+120.1 \pm 20.3%). These results show that as OCR is reciprocally decreased or increased over time by SDF-1/CXCL12 treatment of HL-60 cells, there are closely related changes in ATP production suggesting that these processes are tightly coupled and regulated in a biphasic manner in vitro. Furthermore, we investigated whether the SDF-1/CXCL12 mediated effects were mitochondrial specific or if there were any changes in glycolysis. We measured the extracellular acidification rate (ECAR), a measure of the cellular glycolytic rate. SDF-1/CXCL12 treated cells at two hours had a slight but significant increase in glycolysis (+6.7 \pm 2.5%) while the 24-hour group had a significant reduction in the glycolytic rate (-13.8 \pm 5.4%). These results suggest that as mitochondrial respiration changes, there is a glycolytic compensatory mechanism in HL-60 cells which may reflect the HL-60 cells' reliance on both ATP generating pathways, since these cells

Figure 9.

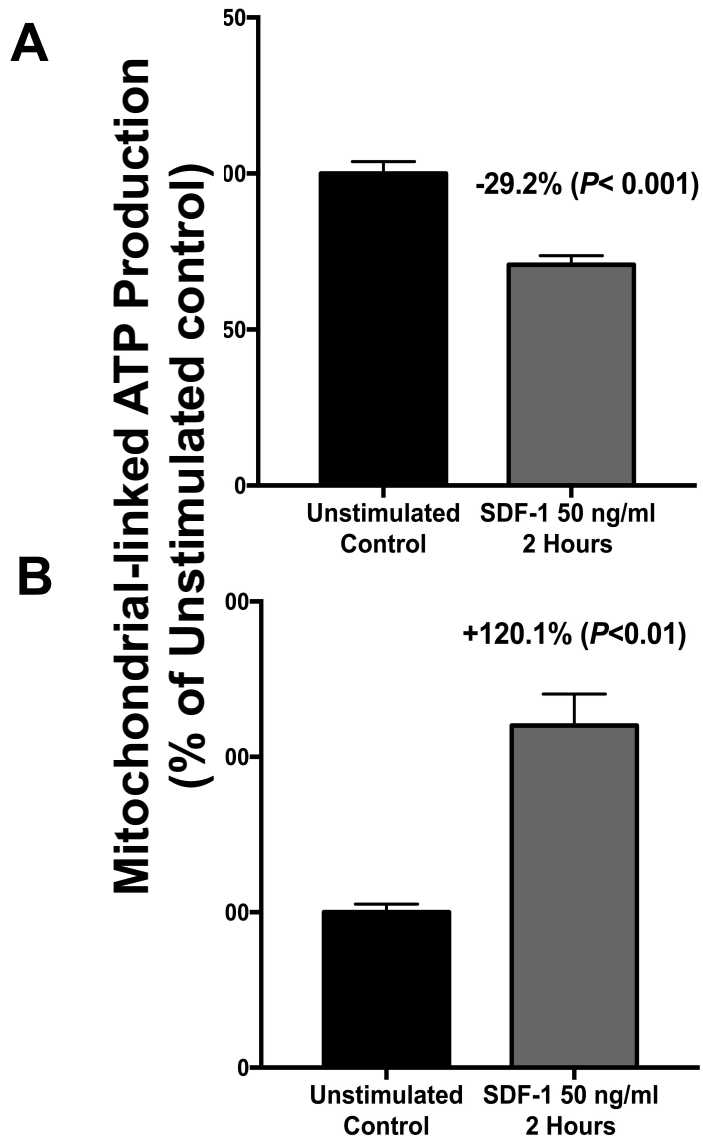


Figure 9. SDF-1/CXCL12 Regulates mitochondrial ATP production in HL-60 cells in a biphasic manner

The human leukemia cell line, HL-60, was treated with 50 ng/ml of SDF-1/CXCL12 for 2 (A) and 24 (B) hours respectively. Mitochondrial-linked ATP production of SDF-1/CXCL12 treated HL-60 cells was measured using the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SEM of four independent experiments with significant differences as compared to unstimulated control shown in the figure (Messina-Graham S and Broxmeyer HE, 2016).

are a leukemia cell line and their metabolism may not truly reflect that of primary bone marrow cells.

Next we sought to confirm that the changes in mitochondrial respiration and ATP production in HL-60 cells are mediated through the SDF-1/CXCL12-CXCR4 axis. We first measured surface expression of human CXCR4 and CXCR7, both receptors for SDF-1/CXCL12, using fluorophore-conjugated antibodies. (Figure 10). Once we determined that the HL-60 cells expressed CXCR4 with minimal or no expression of CXCR7, we used the CXCR4 antagonist AMD3100 to attempt to block the SDF-1/CXCL12-CXCR4 mediated changes in mitochondrial respiration. HL-60 cells were pretreated for 30 minutes with 100 ng/ml of AMD3100 and then stimulated for 2 and 24 hours, respectively, with 50ng/ml of SDF-1/CXCL12. HL-60 cells treated with SDF-1/CXCL12 for two hours have significantly reduced OCR as compared to untreated control cells, while both the AMD3100 alone group and the SDF-1/CXCL12 + AMD3100 group were not significantly different from untreated control (Figure 11A). Cells treated with SDF-1/CXCL12 for 24 hours had significantly increased OCR as compared to untreated control and cells pretreated with AMD3100 alone or pretreated with AMD3100 and then stimulated with SDF-1/CXCL12 for 24 hours had significantly reduced OCR as compared to untreated control cells (Figure 11B). These results show that blocking CXCR4 with AMD3100 ablates the SDF-1/CXCL12 mediated biphasic changes in OCR, suggesting that these metabolic effects of SDF-1/CXCL12 are mediated through the CXCR4 receptor.

Figure 10.

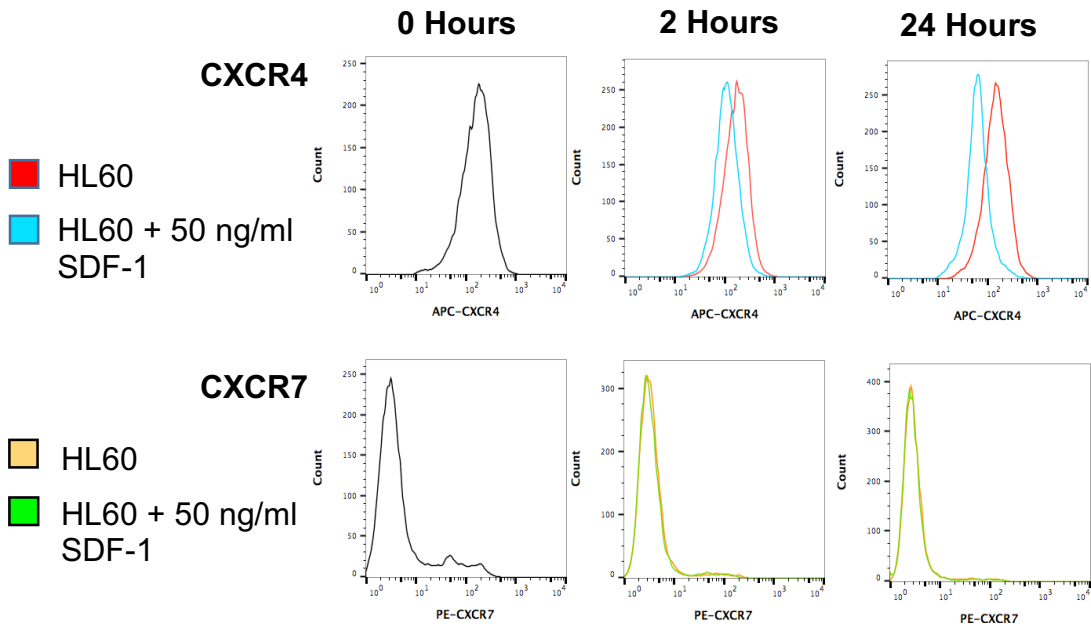


Figure 10. HL-60 express high surface levels of CXCR4 but very low CXCR7 surface expression.

HL-60 cells were stained with anti-hCXCR4-APC and anti-hCXCR7-FITC conjugated antibodies and the level of surface expression of each receptor was determined by flow cytometry.

Figure 11.

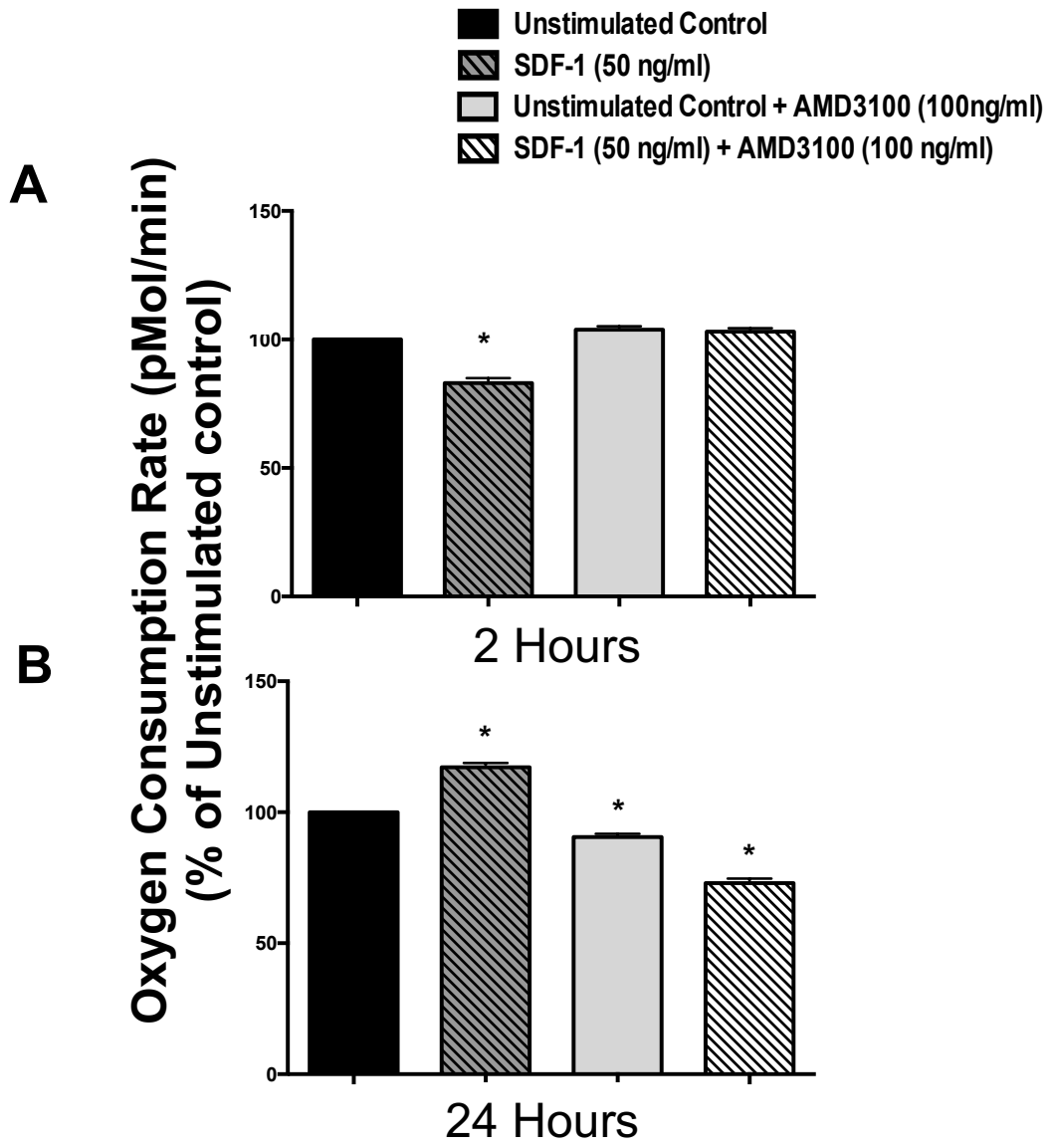


Figure 11. SDF-1/CXCL12 mediated effects on HL-60 cells are CXCR4 specific.

HL-60 cells were pre-treated with 100 ng/ml of the CXCR4 antagonist, AMD3100, for 30 min. After pretreatment, cells were treated with 50 ng/ml of SDF-1/CXCL12 for 2 and 24 h respectively. After each time point, cells were collected and their oxygen consumption rates (OCR) (B) were measured on the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SD of one experiment with triplicates of each group. * $P \leq 0.05$ is significant by one-way ANOVA (Messina-Graham S and Broxmeyer HE, 2016).

To further investigate the mechanism by which SDF-1/CXCL12 mediates changes in mitochondrial respiration, we investigated changes in mitochondrial mass and $\Delta\Psi_m$. We hypothesized that changes in mitochondrial respiration (OCR) and ATP production should be accompanied by concomitant changes in mitochondrial mass and $\Delta\Psi_m$. To assess these changes, we used the mitochondrial specific dyes MitoTracker Green FM (50 nM) and MitoTracker Red CMXRos (50 nM). MitoTracker Green FM is a mitochondrial specific dye that is mitochondrial membrane potential independent and is an indicator of mitochondrial mass, while Mitotracker Red CMXRos is membrane potential dependent and used as an indicator of $\Delta\Psi_m$ (a measure of mitochondrial electron transport chain function). HL-60 cells were cultured for two and 24 hours, respectively, with 50 ng/ml of SDF-1/CXCL12. After each time point, cells were harvested and stained with MitoTracker Green FM or MitoTracker Red CMXRos and mitochondrial mass and $\Delta\Psi_m$ was measured by flow cytometry. Both the mitochondrial mass and $\Delta\Psi_m$ for the two-hour SDF-1/CXCL12 stimulated group were significantly decreased versus unstimulated control (Figure 12A and C). There was a trend toward increased mitochondrial mass at 24 hours (Figure 12B). The $\Delta\Psi_m$ of the 24-hour SDF-1/CXCL12 stimulated group was significantly increased versus control (Figure 12D). Interestingly, we saw a slight but significant increase in ECAR at 2 hours and a small but significant decrease in ECAR at 24 hours (Figure 13A and B). Much like the OCR and ATP production, SDF-1/CXCL12 is also producing biphasic changes in mitochondrial mass and $\Delta\Psi_m$, suggesting that it may be regulating

Figure 12.

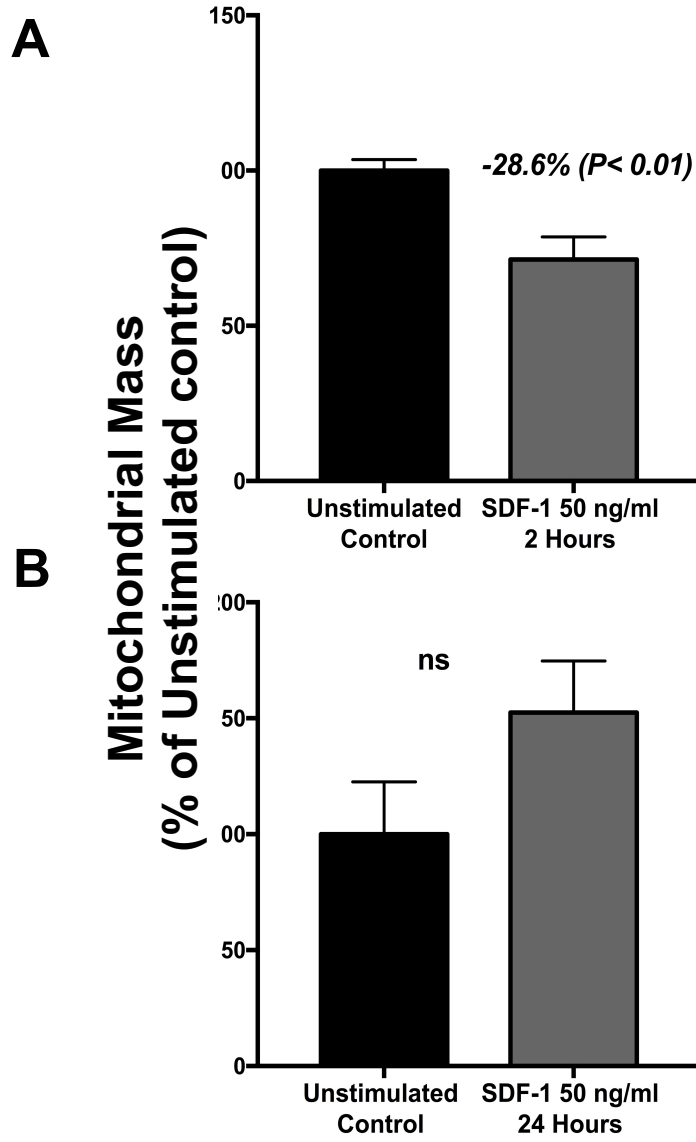


Figure 12. Continued

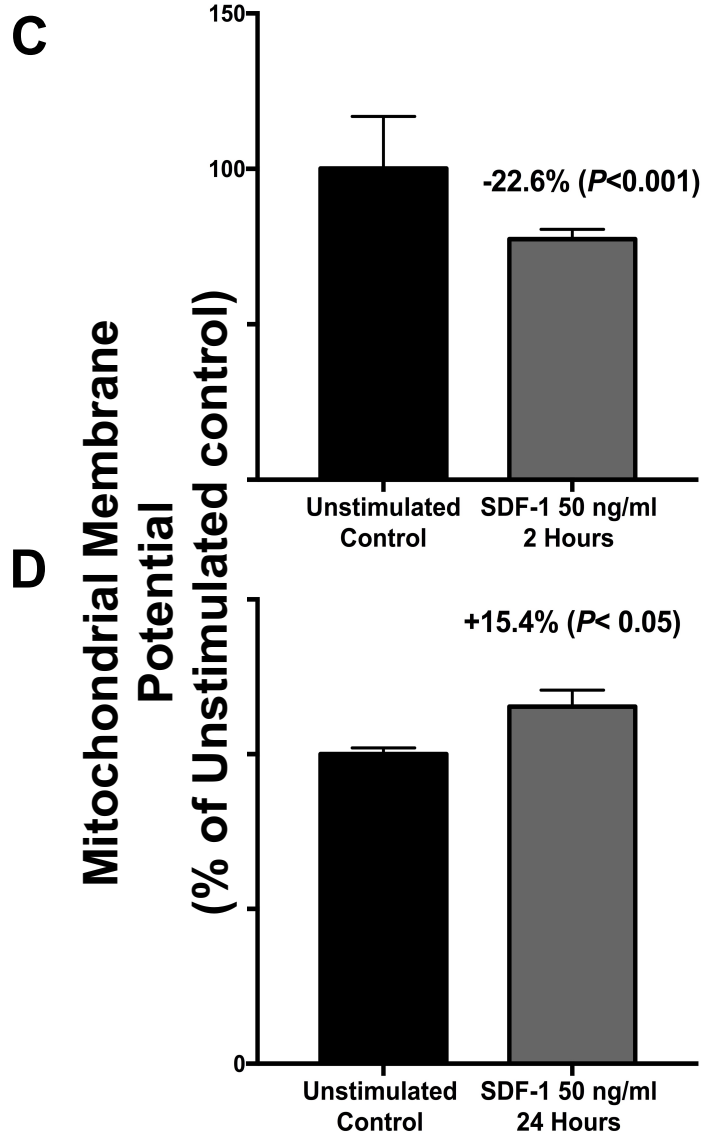


Figure 12. SDF-1/CXCL12 regulates mitochondrial mass and mitochondrial membrane potential of HL-60 cells in a biphasic manner.

HL-60 cells were treated with 50ng/ml of SDF-1/CXCL12 for 2 and 24 h respectively. After each time point, cells were collected and stained with 50nM MitoTracker Green (A) to measure mitochondrial mass or 50nM MitoTracker Red CMXRos (B) to measure mitochondrial membrane potential. Mitochondrial mass and membrane potential were analyzed by flow cytometry. Results are the mean \pm SEM of five independent experiments with significance of $p \leq 0.05$ as compared to unstimulated control (Messina-Graham S and Broxmeyer HE, 2016).

Figure 13.

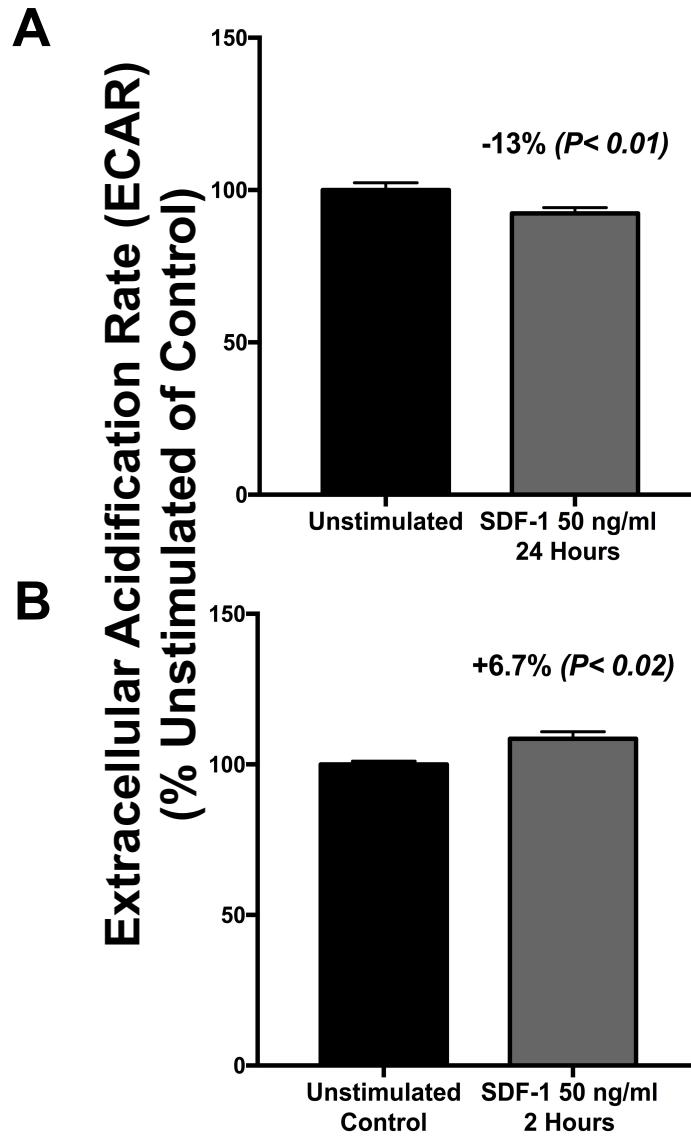


Figure 13. SDF-1/CXCL12 has very little effect on glycolysis in HL-60 cells

HL-60 cells were treated with 50 ng/ml of SDF-1/CXCL12 for 2 (A) and 24 (B) hours respectively. After each time point, cells were collected and the extracellular acidification rate (ECAR), a measure of glycolysis, was measured by the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SEM. Results are the mean \pm SEM of three independent experiments with a significance of $p \leq 0.05$ as compared to unstimulated control (Messina-Graham S and Broxmeyer HE, 2016).

several different aspect of mitochondrial respiration through regulation of electron transport, oxidative phosphorylation, and mitochondrial biogenesis.

Effects on primary lineage negative mouse bone marrow cells

We next determined if SDF-1/CXCL12 effects on HL-60 cells could be mimicked in primary cells, Lin⁻ bone marrow cells (enriched for immature blood cells) from C57BL/6 mice and cultured with or without 50 ng/ml SDF-1/CXCL12 for two and 24 hours, respectively. Cells were then collected after each time point and OCR rates of each group measured. As already noted for the human HL-60 cells (Figure 8), the OCR for the two-hour SDF-1/CXCL12 stimulated group was significantly reduced versus unstimulated two-hour control (Figure 14A). The OCR of the 24-hour SDF-1/CXCL12 stimulated group, however, was significantly increased (Figure 14B). These results are similar to the changes in OCR of the SDF-1/CXCL12 treated HL-60 cells. Next we analyzed mitochondrial-linked ATP production using the Seahorse Bioscience XF96 Extracellular Flux Analyzer (Figure 14C and 14D). Treatment of Lin⁻ cells with SDF-1/CXCL12 produced a significant decrease in the mitochondrial-linked ATP production versus two-hour unstimulated control and treatment of Lin⁻ cells with SDF-1/CXCL12 for 24 hours produced a significant increase in the mitochondrial-linked ATP production as compared to control cells. We also sought to determine the effect of SDF-1/CXCL12 treatment of Lin⁻ cells on glycolysis. We Lin⁻ cells were treated for two and 24 hours, respectively and then ECAR was measured. There were no

Figure 14.

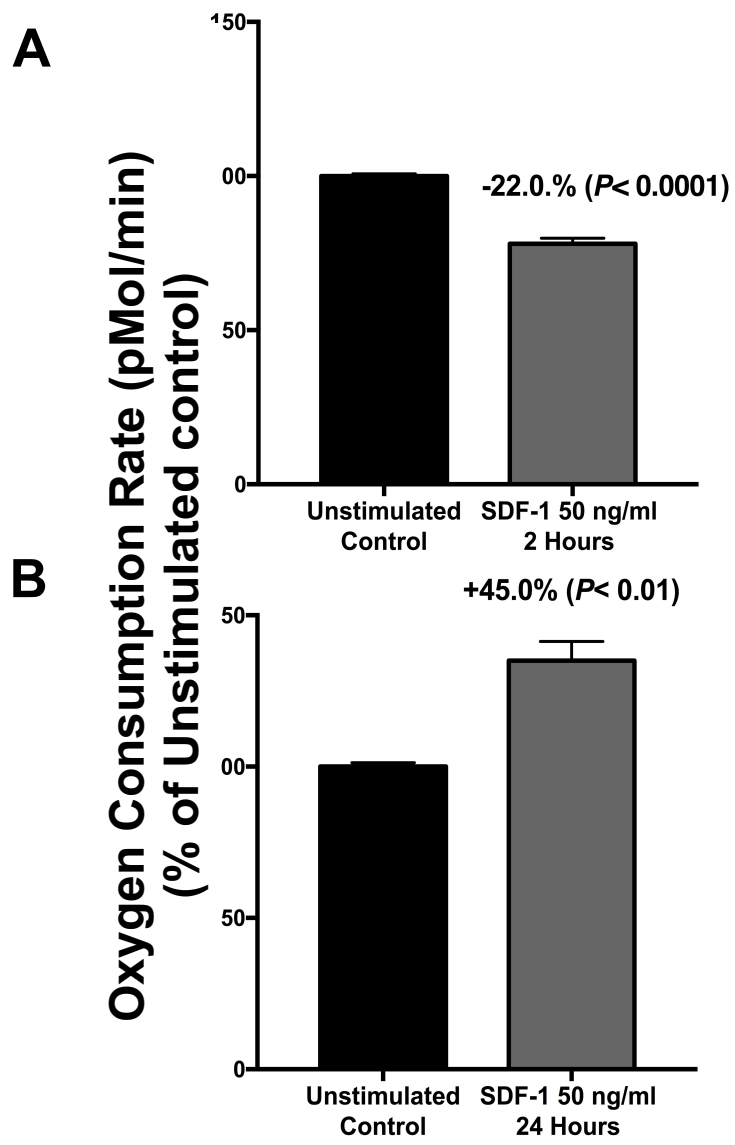


Figure 14. Continued

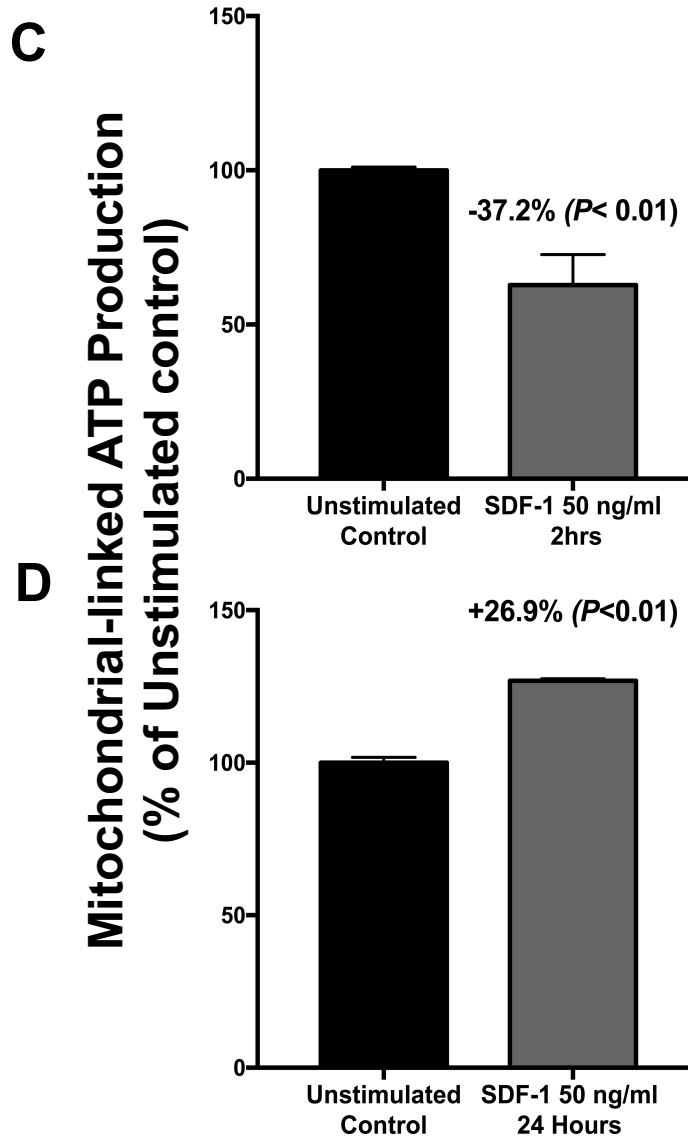


Figure 14. SDF-1/CXCL12 regulates mitochondrial respiration of lineage negative bone marrow cells in a biphasic manner.

Lineage negative bone marrow cells were isolated from C57Bl/6 mice and treated with 50ng/ml of SDF-1/CXCL12 for 2 and 24 h respectively. After each time point the OCR (A and B) and mitochondrial-linked ATP production (C and D) were measured on the Seahorse Bioscience Extracellular Flux Analyzer. Results are the mean \pm SEM of three independent experiments with a significance of $p \leq 0.05$ as compared to unstimulated control control (Messina-Graham S and Broxmeyer HE, 2016).

significant differences between SDF-1/CXCL12 treated and control Lin⁻ cells for both the two and 24-hour time points (Figure 15A and B). This may be due to the more differentiated immature blood cell types in the Lin⁻ population maybe more reliant on mitochondrial respiration and ATP production than on glycolysis. It is well documented that HSCs switch from glycolysis to oxidative phosphorylation as they become more differentiated and lose pluripotency markers (Mantel C et al. 2010). Taken together these results demonstrate that ex vivo treatment of primary mouse immature blood cells in Lin⁻ bone marrow cells with SDF-1/CXCL12 regulates mitochondrial respiration in a biphasic manner similar to that seen in HL-60 cells. This helps to provide evidence that SDF-1/CXCL12 modulates mitochondrial metabolism and supports the notion that SDF-1/CXCL12 regulates mitochondrial metabolism in vivo and possibly functions in fate determination of HSCs in vivo

Figure 15.

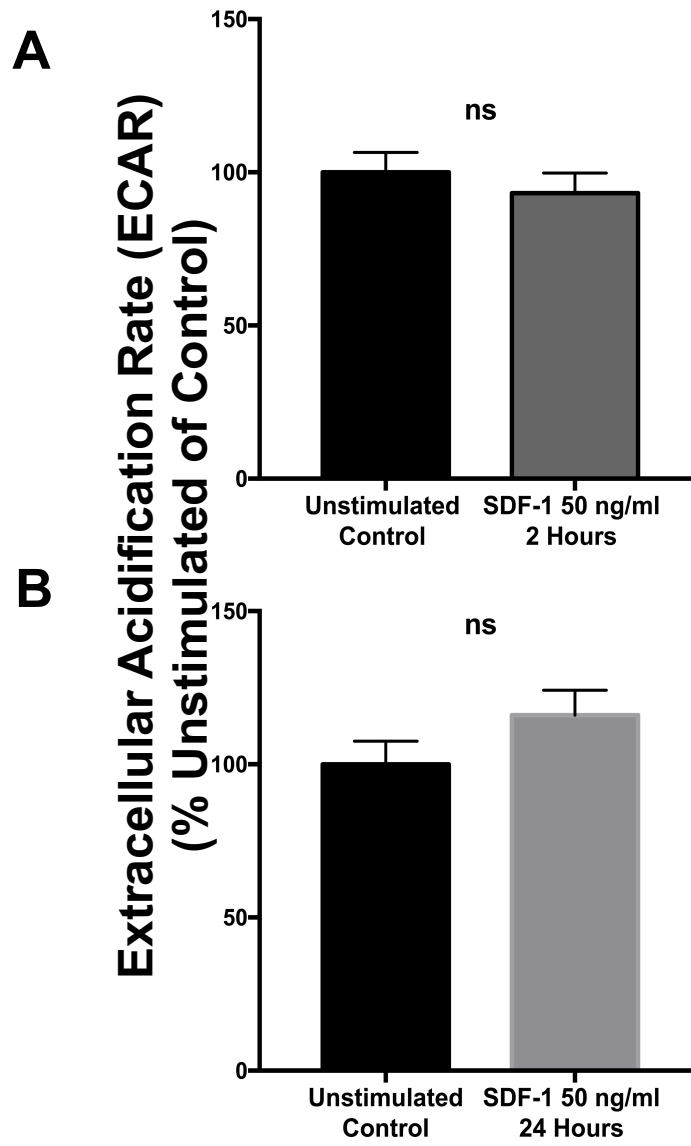


Figure 15. SDF-1/CXCL12 has no significant effect on glycolysis in lineage negative bone marrow cells

Lineage negative bone marrow cells were isolated from C57Bl/6 mice and treated with 50ng/ml of SDF-1/CXCL12 for 2 and 24 hours respectively. After each time point the the OCR (A) and ECAR (B) were measured. Results are the mean \pm SEM of three independent experiments. control (Messina-Graham S and Broxmeyer HE, 2016).

Role of STAT3 in Mitochondrial Function in Hematopoietic Stem and Progenitor Cells

STAT3, is a transcription factor involved in normal cellular function as well as in human malignancies such as cancer and myeloproliferative disorders (Inghirami G et al., 2005). In mouse embryonic and induced pluripotent stem cell lines, it is required for self-renewal (Cartwright P et al., 2005). Much of the work on STAT3 has been done, for the most part, in terms of its phosphorylation, dimerization, nuclear translocation, and transcriptional regulation of target genes. STAT3 is activated by cytokines such as IL-3, IL-5, IL-6, G-CSF, GM-CSF, and SCF, and mediates the effects of cytokine synergy between SCF and G-CSF (Duarte RF and Frank DA, 2000). Knockout of *Stat3* is embryonic lethal and a Cre-Loxp system was developed to generate tissue and conditional *Stat3* knockout mice (Takeda K et al., 1997). Using this method of knocking out *Stat3*, in vivo studies have revealed an important role for STAT3 in T-cell, B-cells and an important role in IL-6 signaling in hematopoiesis (Takeda K et al., 1997; Inghirami G et al., 2005; Oh I-H and Eaves CJ, 2002; Jenkins BJ et al., 2005)

A recent, novel and noncanonical role for STAT3 in mitochondrial function has been identified (Inghirami G et al., 2005). STAT3 influences the function of the electron transport chain (ETC) by interacting with the ETC complex I protein, Grim-19 and also with complex II of the ETC (Reich NC, 2009; Wegrzyn J et al., 2009; Szczepanek K et al., 2011). STAT3 is required for optimal ETC function and oxidative phosphorylation (Wegrzyn J et al., 2009). Mitochondrial STAT3 supports

cellular transformation by oncogenic Ras. This effect seems to be dependent on the localization of STAT3 in mitochondria, as transcriptionally defective STAT3 mutants had no effect on transformation, further strengthening the mitochondrial role of STAT3 in transformation (Gough DJ et al., 2009). There is very little known about the mechanism of STAT3 in mitochondrial function and regulation. There is data, however, that indicates that the ratio of STAT3 to ETC complexes is low, suggesting that the direct binding of STAT3 to mitochondrial respiratory complexes may have a minimal effect on the STAT3 mediated mitochondrial function (Phillips D et al., 2010). In HSCs and HPCs, STAT3 may affect mitochondrial function in a broader, global way rather than direct regulation of ETC components.

STAT3 mitochondrial regulation may also occur by another mechanism. Recent evidence from the field of cardiac research has shown that STAT3 interacts with cyclophilin D, a component of the mitochondrial permeability transition pore, where mitochondrial STAT3 is cardio protective during ischemia and reperfusion (Zorov DB et al., 2009; Miura T and Tanno M, 2012; Boengler K et al., 2010). The outer membrane of the mitochondria is much more permeable than the inner mitochondrial membrane (IMM). This is due to the composition of the outer mitochondrial membrane and the many integral outer mitochondrial membrane (OMM) proteins such as the voltage dependent anion channel (VDAC). VDAC is present in the OMM in abundance and forms large channels and allows various cellular constituents, such as various metabolites, ions, and a limited number of small molecules to enter into the mitochondria (Juhaszova M et al., 2008; Miura T

and Tanno M, 2012; Zorov DB et al., 2009). On the other hand, the IMM is very impermeable and specialized transporters and exchangers are needed to move metabolites, ions and molecules across it (Juhaszova M et al., 2008; Miura T and Tanno M; 2011; Zorov DB et al., 2009). The membrane composition and impermeability of the IMM supports its function as an electric insulator, and the process of mitochondrial electron transport and oxidative phosphorylation drives an electrochemical proton gradient, sometimes called chemiosmosis, across the IMM into the mitochondrial intermembrane space (Zorov DB et al., 2009). This electrochemical gradient drives the synthesis of ATP by ATP synthase.

To begin to understand the potential role STAT3 plays in regulating hematopoiesis we investigated HSC and HPC function and mitochondrial function in a hematopoietic targeted *Stat3*^{-/-} mouse model. First, *Stat3* deletion in the HSC compartment resulted in a significant reduction in the number of phenotypically defined long-term repopulating HSCs (LT-HSCs, CD34⁻ Lin⁻ Sca-1⁺ c-Kit⁺) and an increase in short-term repopulating HSCs (ST-HSCs, CD34⁺ Lin⁻ Sca-1⁺ c-Kit⁺). The more HPCs were increased in the bone marrow, but these progenitors had decreased colony forming capacity, and these cells lost multicytokine responses when plated in colony assays with synergistic combinations of cytokines (Mantel C et al., 2012).

To characterize the effect of *Stat3* deletion in mouse bone marrow we defined the phenotype of bone marrow HSCs and HPCs from WT control and

Stat3^{-/-} mice at 5 weeks of age. We first analyzed the proportions of phenotypically defined long-term repopulating HSCs (LT-HSC) and short-term repopulating HSCs (ST-HSC) based on CD34 expression and the size of cells in the lineage⁻ sca-1⁺, c-kit⁺ (LSK) population (LSK). LT-HSCs are defined as CD34⁻ LSK cells while, ST-HSCs are defined as CD34⁺ LSK cells (Donnelly DS et al., 1999). Total LSK cells are increased in the *Stat3*^{-/-} but within the LSK compartment, the CD34⁻ Lin⁻ Sca-1⁺ c-Kit⁺ LT-HSCs are significantly reduced compared to WT and the CD34⁺ Lin⁻ Sca-1⁺ c-Kit⁺ ST-HSCs are significantly increased compared to WT (Figure 16). This suggests that *Stat3* gene deletion induces differentiation from LT-HSCs to ST-HSCs. When we analyzed the phenotype of the bone marrow progenitor compartments by surface marker expression (Ema H et al., 2006; Dykstra B et al., 2007) we saw a significant reduction in the phenotypically defined megakaryocyte/erythrocyte progenitor population (MEP), while there was significant increase in granulocyte/macrophage progenitors (GMP) and no changes in the phenotyped common lymphoid (CLP) and common myeloid progenitors (CMP) in *Stat3*^{-/-} cells versus WT (Figure 17). Interestingly we consistently found two populations of LSK cells. One population contains c-Kit^{hi} (LSK^{hi}) larger cells and a second population of smaller, c-Kit^{low} (LSK^{low}) cells, results that we have seen in a previous study using an SDF-1/CXCL12 TG mouse model. The first population, LSK^{hi}, were CD34⁻ and the second population, LSK^{low}, were CD34⁺ (Figure 18).

Figure 16.

% of Total LSK Cells in Bone Marrow

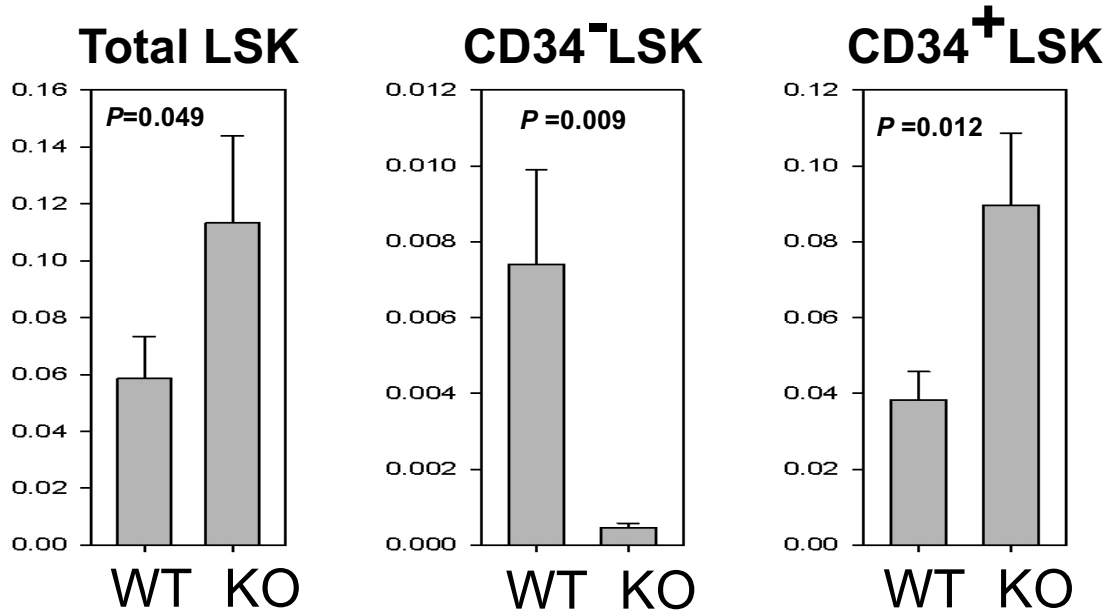


Figure 16. Percentage of total LSK cells in WT and *Stat3*^{-/-} bone marrow

Comparative analysis of subpopulations of cells in the Lin⁻ bone marrow compartment from 4 WT and 4 *Stat3*^{-/-} mice. Data are expressed as means ± SD. (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

Figure 17.

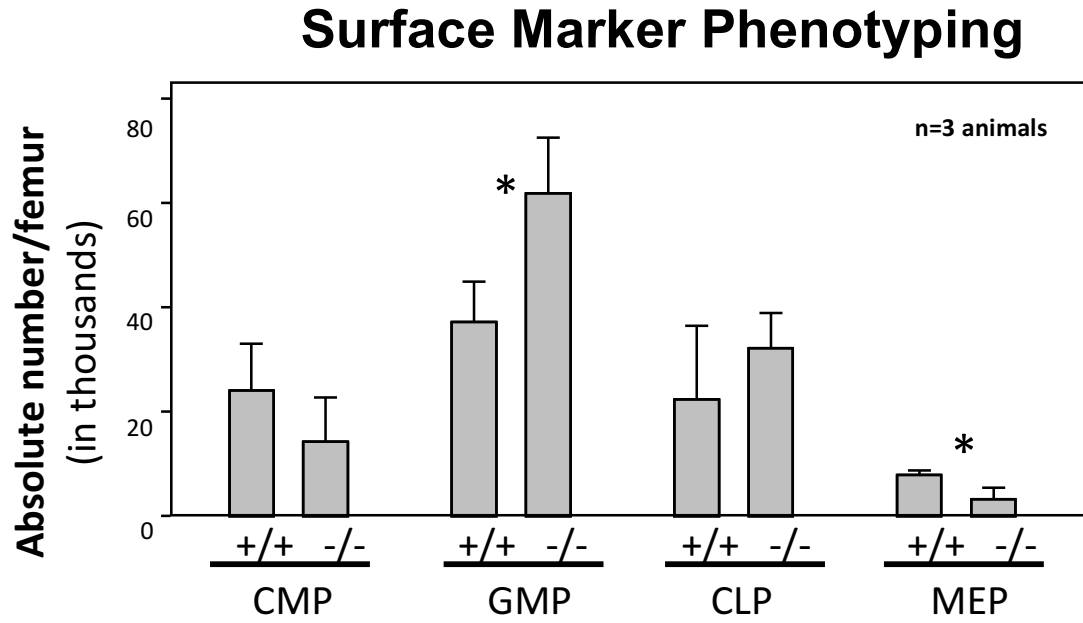


Figure 17. Surface marker phenotype analysis of hematopoietic progenitor cells

Phenotype analysis of hematopoietic progenitor cells from WT and *Stat3*^{-/-} mouse bone marrow. *P<0.001. Flow cytometric surface marker analysis was done as previously described (Ema H et al., 2006; Dykstra B et al., 2007). CMP, common myeloid progenitors; GMP, granulocyte-macrophage progenitors; CLP, common lymphoid progenitors; MEP, megakaryocyte-erythroid progenitors (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

Figure 18.

Lin⁻ Compartment

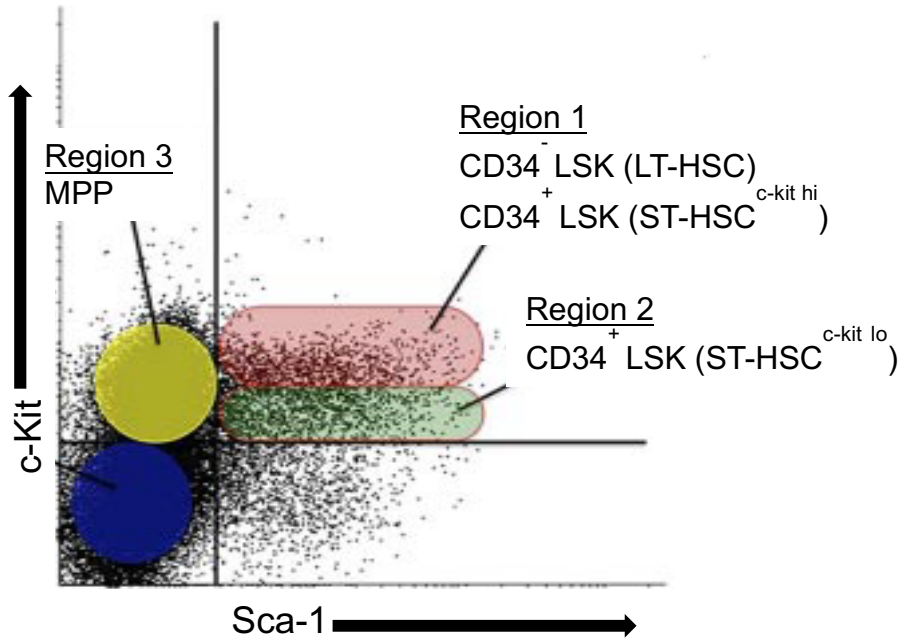


Figure 18. Populations of cells in the bone marrow Lin⁻ compartment

Graphic representation and dot-plot of mouse bone marrow Lin⁻ cells and c-Kit and Sca1 expression showing 3 subpopulations that were gated on and evaluated for mitochondrial mass, ($\Delta\Psi_m$), and ROS levels. R1 and R2 are further subdivided into two discrete subpopulations based on size and CD34 surface expression. MPP, multipotent progenitor (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

To assess the functional capability of *Stat3*^{-/-} bone marrow cells and spleen cells, colony assays were performed using *Stat3*^{-/-} and WT cells. *Stat3*^{-/-} bone marrow and spleen cells showed significantly reduced colony-forming ability (CFU-GM, BFU-E, and CFU-GEMM) (Figure 19A). To further assess the effect of *Stat3* gene deletion on HPCs, the cell cycle status of WT and *Stat3*^{-/-} bone marrow and spleen we performed using a high specific activity tritiated thymidine kill assay. The cycling of *Stat3*^{-/-} cells was significantly lower compared to WT control (Figure 19B). *Stat3*^{-/-} cells had reduced functional myeloid, erythroid and multipotent progenitor cells/femur and these cells had a slower cycling state. Furthermore, the replating capacity of *Stat3*^{-/-} bone marrow was significantly reduced. Replating capacity is used to test the limited self-renewal ability of HPCs (Broxmeyer HE et al., 2007). *Stat3*^{-/-} CFU-macrophage (M), CFU-GM, CFU-GEMM, and BFU-E all had significantly reduced replating capacity, when assessed for numbers of secondary plates with colonies, and numbers of colonies per secondary plate (Mantel C et al., 2012). These data suggest that there are significant deficiencies in HPC numbers and functional capacity. The engraftment capability of *Stat3*^{-/-} is reduced. The engraftment capability was assayed by performing primary competitive-repopulation transplants (2.5X10⁵ donor cells and 2.5X10⁵ competitor cells) and noncompetitive secondary transplants (10⁶ donor cells) into lethally irradiated female mice, respectively. Mice receiving *Stat3*^{-/-} bone marrow had significantly reduced % chimerism as compared to WT donor bone marrow for all time points

Figure 19.

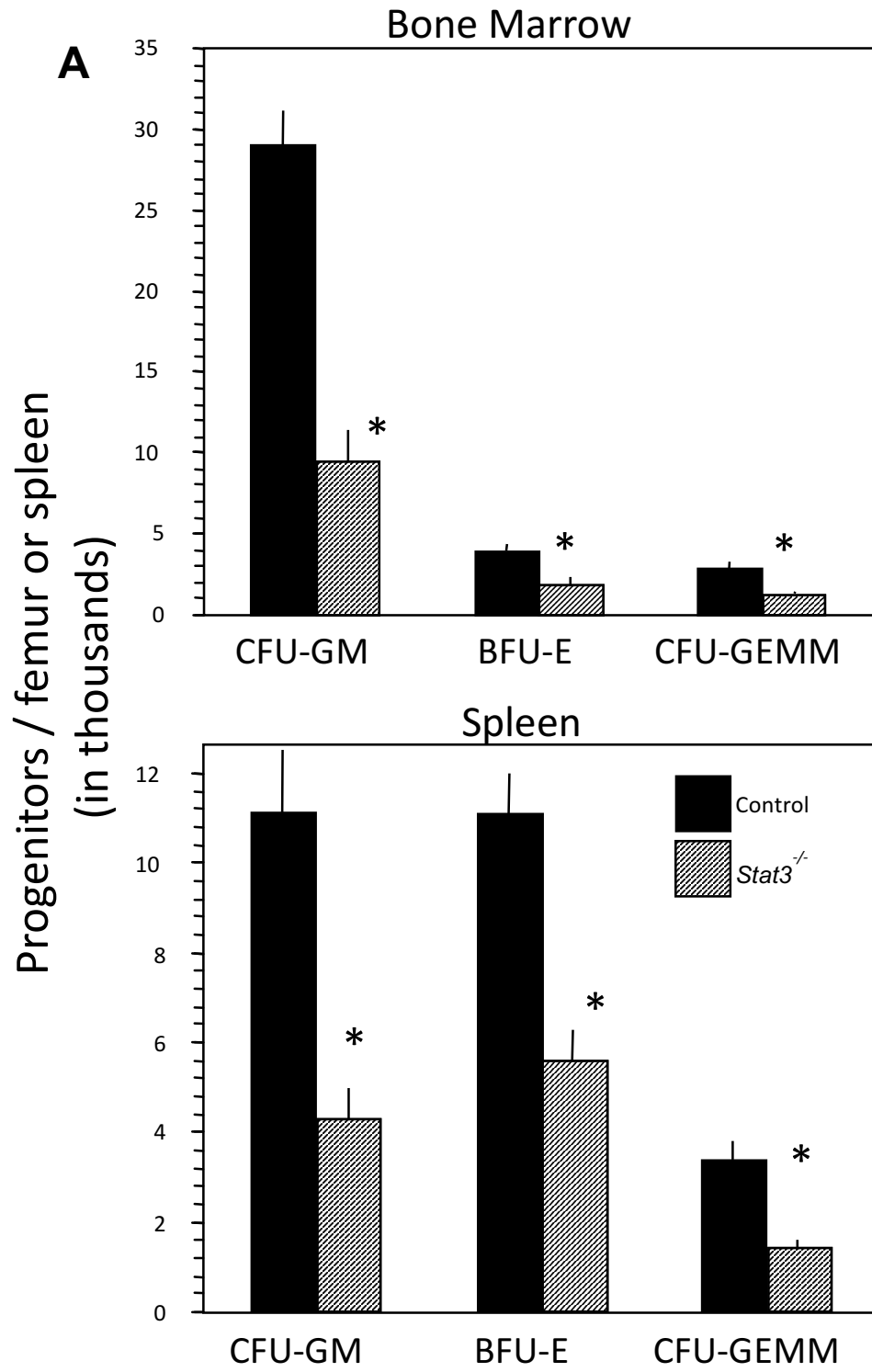


Figure 19. Continued

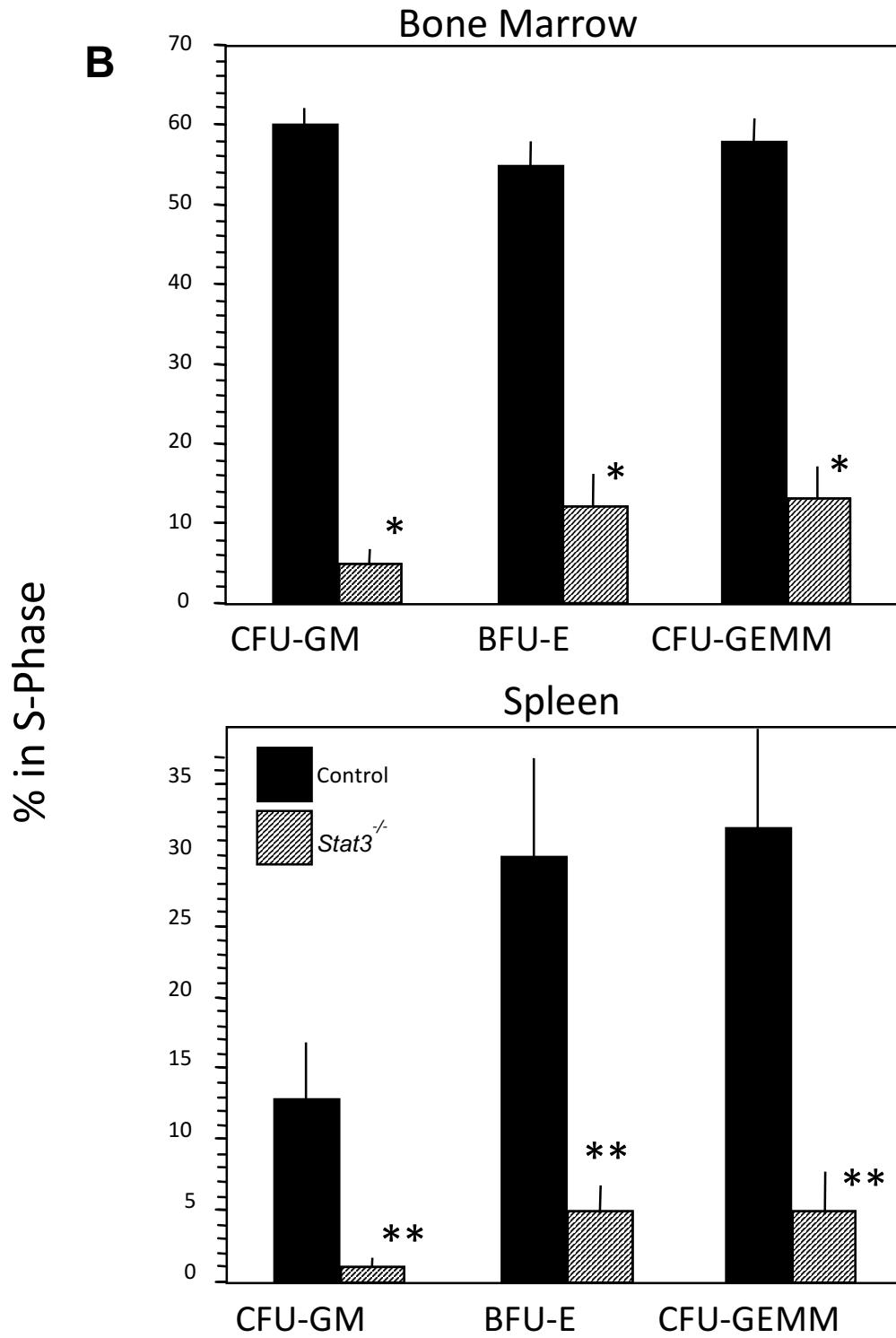


Figure 19. Functional assessment of WT and *Stat3*^{-/-} mouse HPCs

(A) Influence of *Stat3*^{-/-} on absolute numbers of HPCs. Results shown are the average \pm SEM for 11 WT and 10 *Stat3*^{-/-} mice individually assessed from a total of 3 different experiments. Cells were stimulated in vitro with erythropoietin, pokeweed mitogen mouse spleen cell conditioned medium, SCF, and hemin to detect the more immature subsets of progenitors. (B) Influence of *Stat3*^{-/-} on cycling status of HPC for the same cells as in panel A. The percentage of HPCs in the S-phase was determined with the high specific activity tritiated thymidine kill technique. *, $P < 0.001$; ** $P < 0.05$ (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

analyzed post-transplantation. Decreased engraftment in secondary transplant recipients was also seen and suggests that the self-renewal capacity of these cells may have been compromised. Furthermore, we tested proliferative synergy, which can be induced by combinations of certain cytokines in vitro; STAT3 signaling pathways activated by certain cytokines have been shown to be important in the regulation of hematopoiesis (Takeda K et al., 2000). Proliferation induced by cytokine synergy was defective in *Stat3*^{-/-} bone marrow cells as compared to WT. Thus, STAT3 is essential for the synergistic proliferative response in mouse bone marrow progenitor cells. CMP and GMP are thought to be similar to CFU-GEMM and CFU-GM, respectively. CFU-GEMM and CFU-GM were reduced in numbers, cycling status and had significantly reduced response to synergistic cytokine stimulation, but the phenotyping analysis did not reflect the functional defects seen in colony assays which suggests that the numbers of GMP are increased but some of these progenitor cells may have functional defects in response to cytokines.

Several recent reports have shown STAT3 to be critical to mitochondrial function (Wegrzyn J et al., 2009; Bernier M et al., 2011; Shaw PE, 2010; Myers MG Jr, 2009; Szczepanek K et al., 2011). The mechanism by which STAT3 functions in regulating mitochondrial function is still largely unknown. To further investigate the role of STAT3 in the regulation of mitochondrial metabolism the effect that STAT3 deficiency has on mitochondrial mass and $\Delta\Psi_m$ in *Stat3*^{-/-} and WT littermate control LT-HSCs, ST-HSCs and LSK cells, respectively. We previously identified three discrete populations of bone marrow cells were

identified based on size, mitochondrial mass, $\Delta\Psi_m$, and CD34 surface expression (Figure 4 and 5). We next sought to investigate the three distinct populations of bone marrow cells that we uncovered in this current study. The gating scheme and the three populations were shown in Figure 18 and Figure 20. The larger cells of Region 1 are enriched for CD34⁻ LSK cells that are c-Kit^{hi} and we called these cells LT-HSC^{kit-hi}, while the smaller cells of Region 1 are CD34⁺ LSK cells that are also c-Kit^{hi} and were termed ST-HSC^{kit-hi}. Cells of region 2 are a subpopulation of cells comprised of both large and small cells that are almost all CD34⁺ LSK cells that are c-Kit^{low} and were referred to as ST-HSC^{kit-low}. Region 3, Lin⁻c-Kit⁺ (LK) cells, do not express Sca-1 and are not in the LSK compartment. Note the four populations in Figure 18 that are used for the analysis of mitochondrial dynamics.

To examine the mitochondrial mass in these subpopulations, we stained the cells with the mitochondrial specific fluorophore, MitoTracker Green FM, which is taken up in mitochondria in a potential-independent manner and is an indicator of mitochondrial mass (Oubrahim H et al., 2001). The mitochondrial mass was significantly increased in *Stat3*^{-/-} LT-HSC^{kit-hi} versus WT cells. (Figure 21 A) However, LT-HSC^{kit-hi} in general had significantly higher mitochondrial mass than both the ST-HSC^{kit-hi} and ST-HSC^{kit-low} subpopulations. This may be due to the size difference between the larger LT-HSC and smaller ST-HSC subpopulations, which is supported by our previous report. There was no significant increase in

Figure 20.

A

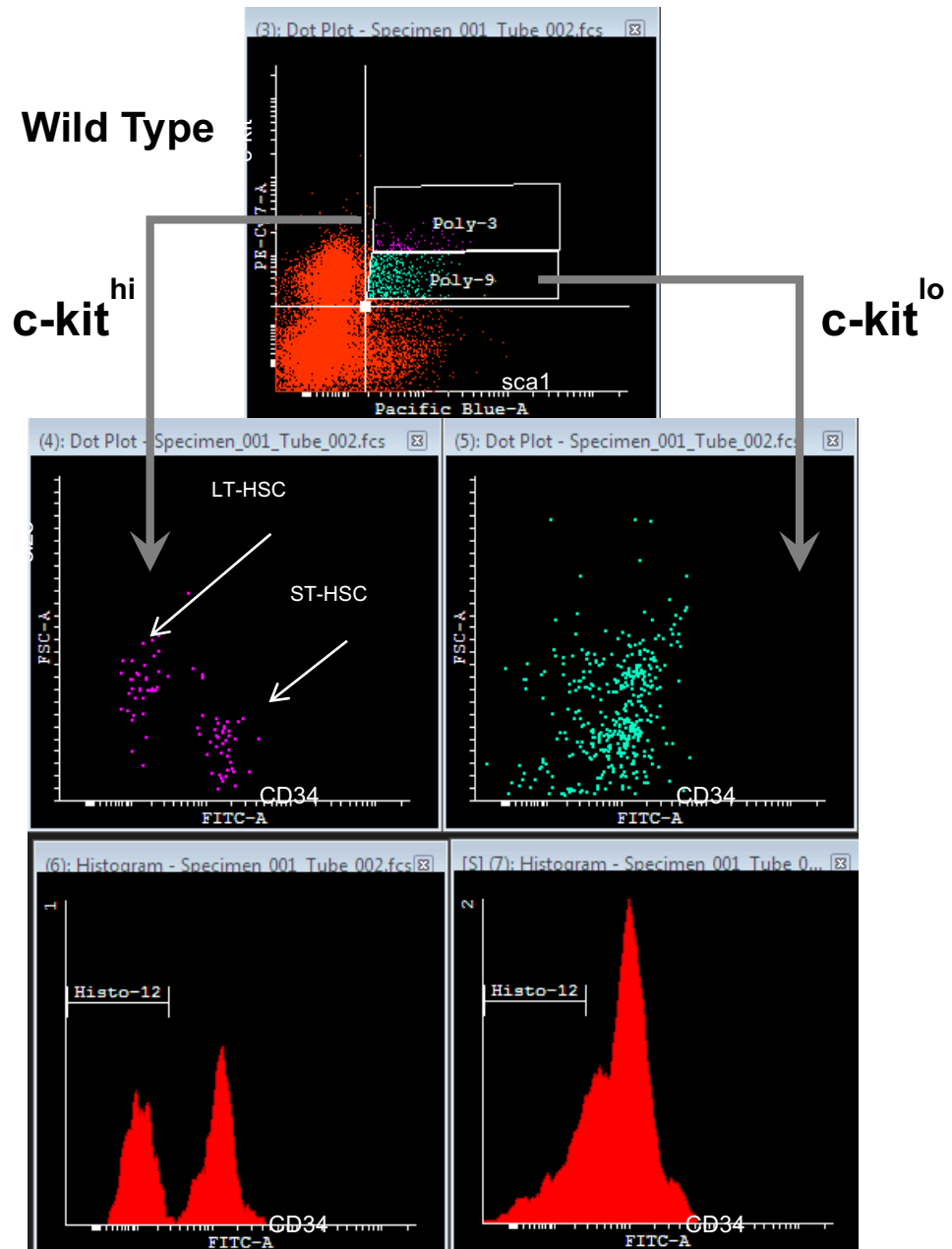


Figure 20. Continued

B

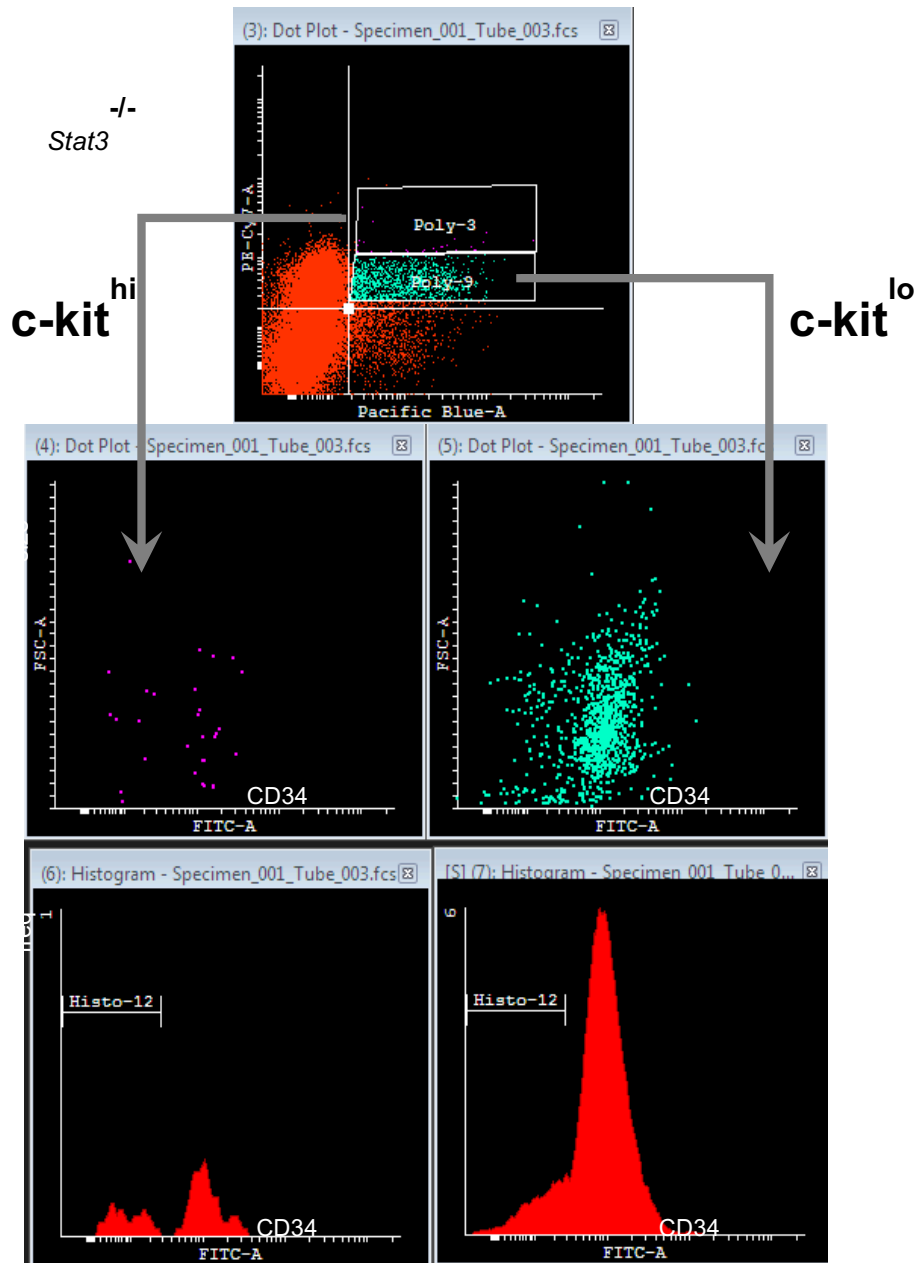


Figure 20. Gating strategy for analysis of HSC and HPCs in the Lin⁻ compartment of WT and *Stat3*^{-/-} mice

Dot-plot of the gating strategy to determine the populations of HSCs and HPCs in the Lin⁻ bone marrow compartment of (A) WT and (B) *Stat3*^{-/-} mice. WT and *Stat3*^{-/-} mouse bone marrow cells are first gated on Lin⁻ cells. Populations are based on CD34 and c-Kit surface expression as well as cell size. Red histograms represent populations with negative CD34 expression (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

mitochondrial mass between WT and *Stat3*^{-/-} ST-HSC^{kit-hi} cells. There was however a significant increase in mitochondrial mass in *Stat3*^{-/-} ST-HSC^{kit-low} versus WT.

Multipotent progenitors (MPP) are enriched in cells of the (Lin⁻ c-kit⁺) LK subpopulation. Both WT and *Stat3*^{-/-} MPP had higher mitochondrial mass as compared to WT and *Stat3*^{-/-} LT-HSC^{kit-hi}, ST-HSC^{kit-hi}, and WT ST-HSC^{kit-low}. *Stat3*^{-/-} produced a small but significant increase in mitochondrial mass in *Stat3*^{-/-} MPPs as compared to WT. The percentage of total LK cells in *Stat3*^{-/-} bone marrow was significantly higher (16.0 ± 2.3%) as compared to WT (3.6 ± 0.5%; P=0.0005). Increases in MPPs in *Stat3*^{-/-} cells suggest that *Stat3* deletion produces a shift from LT-HSCs and ST-HSCs to MPPs. To investigate the mitochondrial activity, we used the mitochondrial specific fluorophore JC-1 that is transported into the mitochondrial in a $\Delta\Psi_m$ -dependent manner (Chazotte B, 2009). JC-1 has two different emission spectra. The monomeric form of JC-1 has a green emission spectrum that is similar to fluorescein isothiocyanate (FITC) while the aggregated form has a red emission spectra similar to phycoerythrin (PE). Once inside the cell monomeric JC-1 can accumulate in the mitochondria in a $\Delta\Psi_m$ -dependent manner and as it accumulates, the emission spectrum shifts from green to red and the ratio of red/green fluorescence intensity is a relative measure of the $\Delta\Psi_m$.

The $\Delta\Psi_m$ of *Stat3*^{-/-} LT-HSC^{kit-hi} is significantly higher than that of the WT cells (Figure 21B). In WT and *Stat3*^{-/-} cells the $\Delta\Psi_m$ of LT-HSC^{kit-hi} is similar to that of both ST-HSC subpopulations, however the lower mitochondrial mass of WT and

Stat3^{-/-} cells in both ST-HSC populations indicate that the $\Delta\Psi_m$ of these two subpopulations is generated by less mitochondria when compared to the LT-HSC^{kit-hi}, suggesting that the mitochondria in the ST-HSC subpopulations have higher activity than those of the LT-HSC subpopulation (Figure 21A and B). This is consistent with the concept that mitochondrial activity increases as cells begin to differentiate. The MPPs in the LK subpopulation had a higher $\Delta\Psi_m$ than the three other subpopulations, but the *Stat3*^{-/-} LK cells had a modest decrease in $\Delta\Psi_m$ as compared to WT, which is opposite of the effect that *Stat3* deletion had on the other LSK populations, suggesting that STAT3 may exert a positive effect on mitochondrial activity in MPPs. Despite a lower $\Delta\Psi_m$ in the *Stat3*^{-/-} LK cells, this result is consistent with a positive role of STAT3 on mitochondrial function (Myers MG Jr, 2009). We next compared the mitochondrial $\Delta\Psi_m$ to the mitochondrial mass which gave us a better indication of the specific mitochondrial activity normalized for mitochondrial mass. In other words, this allows us to assess the contribution the mitochondria in each subpopulation has to mitochondrial activity (Figure 22A). The activity/mass ratio was significantly lower for ST-HSC^{kit-low} and LK cells from *Stat3*^{-/-} bone marrow which further supports the positive regulatory effect of STAT3 on mitochondria (Myers MG Jr, 2009). By analyzing the mitochondrial activity/mass ratio we found that STAT3 deficiency causes changes in the function of mitochondria that may indicate aberrant mitochondrial function and/or dysregulation in HSCs and HPCs.

Figure 21.

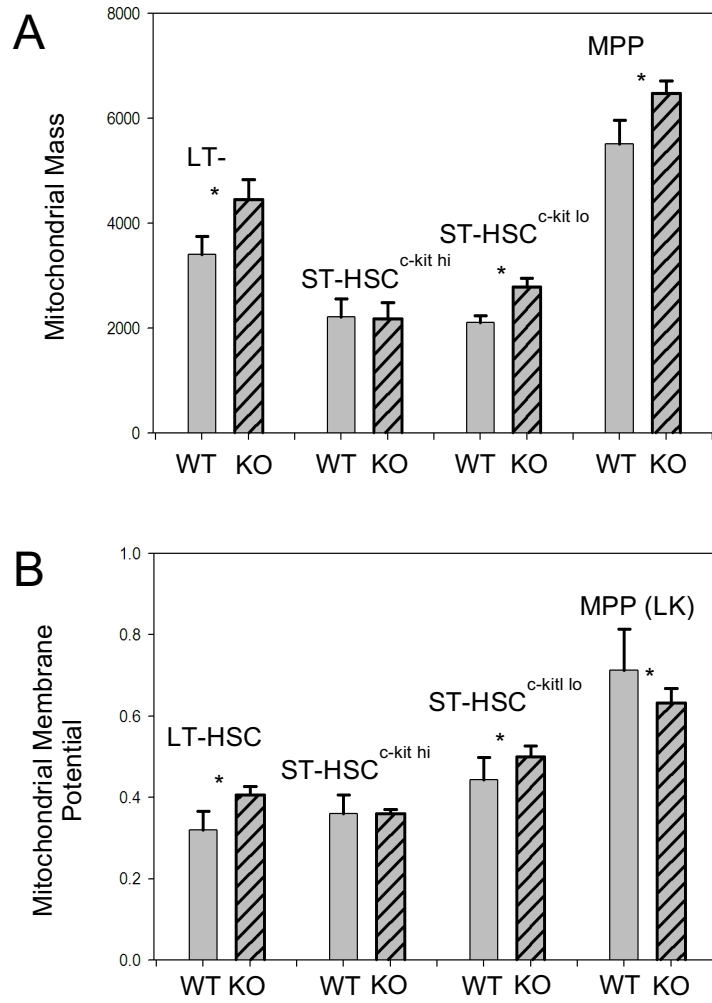


Figure 21. Mitochondrial dysfunction in populations of *Stat3*^{-/-} mouse HSCs and HPCs.

(A) Comparison of average MitoTracker Green staining (MFI) per cell (a measure of mitochondrial mass per cell) on gated subpopulations described in Figure 18.

(B) Staining of the indicated subpopulations with JC-1, a probe that measures mitochondrial membrane potential ($\Delta\Psi_m$), a measure of mitochondrial activity in the 4 defined subpopulations. These results are from 3 WT and *Stat3*^{-/-} pairs and data is presented as the average \pm SD. *P <0.05 by 2-tailed Student t test (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

It is well documented that mitochondria are the primary source of ROS (Halliwell B and Gutteridge JM, 1988; St-Pierre J et al., 2002). We investigated superoxide ($O_2^{\bullet-}$) production, the main form of ROS production in cells, by using another mitochondrial specific fluorophore, MitoTracker Orange CM-H₂TMRos (Chazotte B, 2009; Agnello M et al., 2008). The unoxidized form of MitoTracker Orange is non-fluorescent, but is oxidized by $O_2^{\bullet-}$ to emit red fluorescence and can be measured by flow cytometry much like MitoTracker Green and JC-1. ROS levels were, overall, much higher in LT-HSC^{kit-hi} subpopulation as compared to the ST-HSC populations and the LK population. However, for the ST-HSC^{kit-hi}, and ST-HSC^{kit-low}, and LK population, the *Stat3*^{-/-} bone marrow cells had significantly increased ROS production as compared to WT (Figure 22B). Because there is evidence implicating STAT3 as a regulator of mitochondrial function, we analyzed mitochondrial oxygen consumption rates in WT and *Stat3*^{-/-} splenocytes using a Seahorse Bioscience XF96 Extracellular Flux Analyzer (Gerencser AA et al., 2009). Splenocytes were used as a source of extra-medullary hematopoietic cells because we were unable to obtain enough Lin⁻ bone marrow cells at that time, that were required to reliably measure the mitochondrial oxygen consumption rate in the Seahorse machine. Oxygen can be consumed by increased electron flow through the mitochondrial ETC complexes to finally react with protons to produce water. There is a second means by which oxygen can be consumed in mitochondria. Oxygen can be converted to $O_2^{\bullet-}$ by complexes I and III. Superoxide dismutase 1 and 2 (SOD1 and SOD2) then convert superoxide into hydrogen peroxide, thus consuming oxygen (St-Pierre J et al., 2002). We reasoned that

Figure 22.

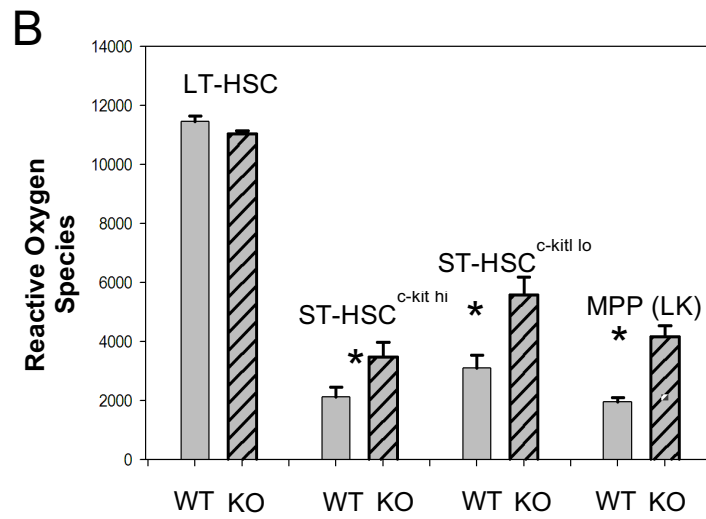
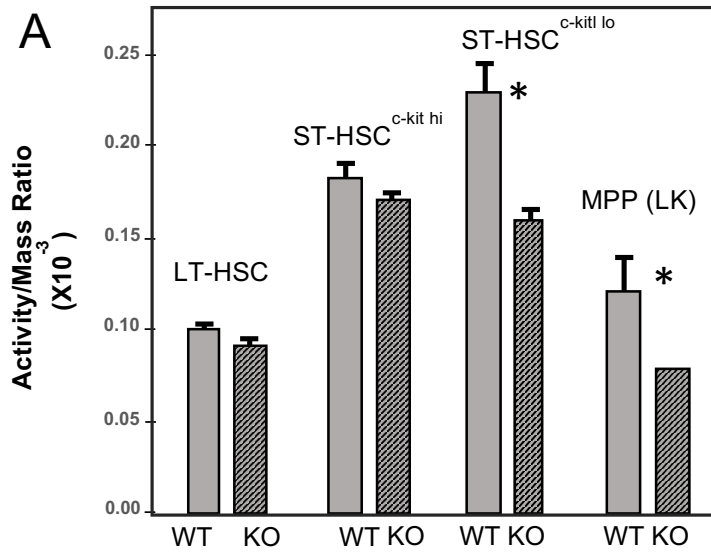


Figure 22. Mitochondrial activity and ROS production in populations of *Stat3*^{-/-} mouse HSCs and HPCs

(A) Comparison of “activity/mass ratio” for subpopulations of *Stat3*^{-/-} and WT bone marrow cells. This ratio is a measure of mitochondrial activity that is normalized for mitochondrial mass (derived from data in Figure 18) and reflects the $\Delta\Psi_m$ and is the average mitochondrial membrane potential irrespective of mitochondrial mass.

(B) Analysis of ROS levels in subpopulations of *Stat3*^{-/-} bone marrow cells. These results are from 3 WT and 3 *Stat3*^{-/-} pairs and data is presented as the average \pm SD. *P <0.05 by 2-tailed Student-t test (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

since there was increased levels in subpopulations of *Stat3*^{-/-} LSK cells, there should be concomitant increases in mitochondrial oxygen consumption.

Stat3^{-/-} splenocytes had significantly higher OCR than WT (Figure 23), which suggests that mitochondrial respiration is increased in *Stat3*^{-/-} cells. The OCR associated with ATP production by ETC complex V (ATP synthase) was also measured by using the complex V inhibitor, oligomycin-A (oligo-A), and the decrease in OCR between the basal OCR and oligo-A inhibited OCR (see Figure 2) was greater for *Stat3*^{-/-} splenocytes as compared to WT. The difference between WT and *Stat3*^{-/-} does not take into account the total increase in OCR of the *Stat3*^{-/-} splenocytes as compared to WT. The increase in OCR of the *Stat3*^{-/-} cells is consistent with increases in OCR being due to increased proton leak/uncoupling of mitochondria, which is supported by increased mitochondrial ROS production. Furthermore, these results suggest that increased mitochondrial respiration (OCR) of splenocytes has been uncoupled from ATP synthesis. These findings support our hypothesis that increased mitochondrial activity in HSCs and HPCs in the absence of STAT3 promotes mitochondrial dysfunction, increased ROS production, and mitochondrial uncoupling (increased proton leak).

We had thus found mitochondrial dysfunction in the bone marrow of *Stat3*^{-/-} mice. We analyzed several hematopoietic criteria in *Stat3*^{-/-} and WT littermate control mice. Although the *Stat3*^{-/-} mice are born normal in regards to appearance and health, by 2-3 weeks after birth a noticeable deficiency in growth is apparent

Figure 23.

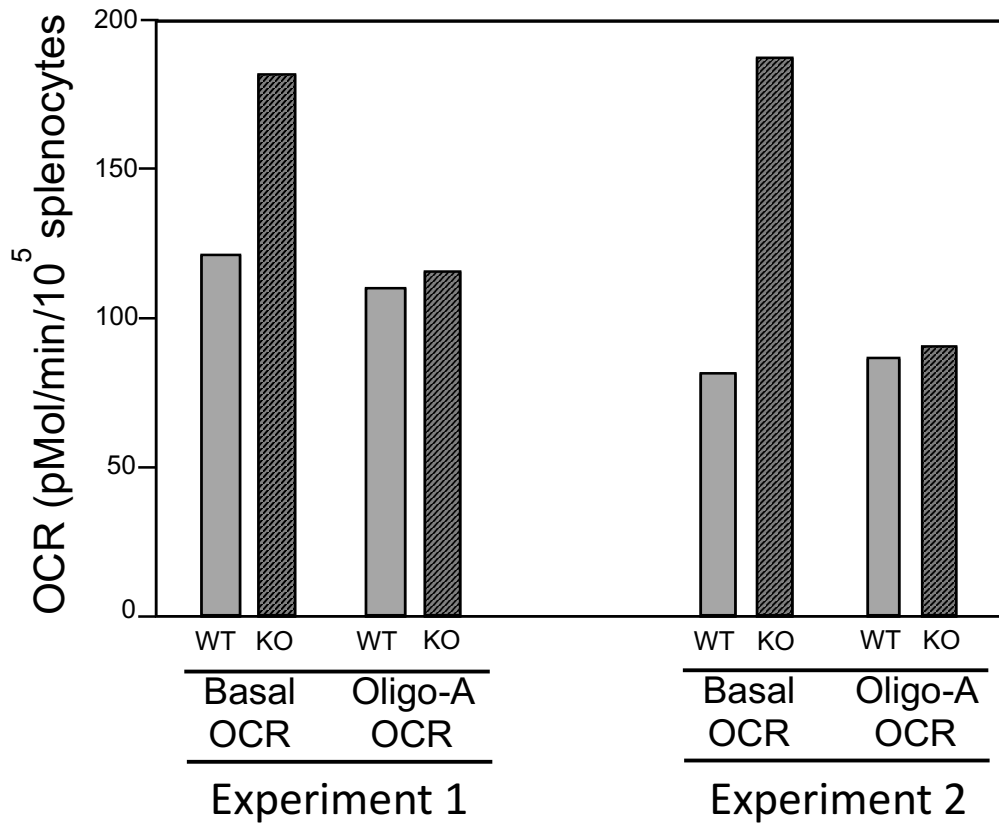


Figure 23. Measurement of OCR in WT and *Stat3*^{-/-} splenocytes

Measurement of OCR using a XF Mito Stress Test Kit and Seahorse XF96 extracellular flux analyzer, using 10^5 splenocytes/well from *Stat3*^{-/-} or WT mouse spleens. Data from 2 separate experiments each with 1 WT and 1 *Stat3*^{-/-} mouse. Basal OCR and OCR after oligomycin-A treatment are shown. Data shown are the average OCR measured from 24 wells of each type of cell where the OCR (pMoles/minute) was measured 16 times in each well simultaneously over a period of 2 hours until the OCRs were stabilized. The last stabilized average OCR measurement is presented (Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

and by 4-6 weeks the *Stat3^{-/-}* are approximately 20% smaller (in body weight) as compared to WT littermate controls and are more lethargic (Mantel C et al., 2012; Welte T et al., 2003).

In female *Stat3^{-/-}* mice, death occurs at approximately 6 weeks of age and male *Stat3^{-/-}* mice may survive 1-2 weeks longer. Splenomegaly was apparent and there was a significant reduction in white pulp structures and large areas of red pulp and increased myeloid infiltration in the *Stat3^{-/-}*. Blood indices indicated a significant myeloid to lymphoid cell ratio, decreases in hemoglobin content accompanied by increased red cell distribution width (an indicator of anemia) and decreased erythrocyte counts in *Stat3^{-/-}* mice as compared to WT controls (Mantel C et al., 2012). By 5 weeks of age, *Stat3^{-/-}* mice have a pronounced lymphoid to myeloid shift and a large expansion of immature myeloid cells (Mantel C et al., 2012). *Stat3^{-/-}* mice seem to be born with normal blood indices but about three weeks after birth begin to right shift from lymphoid to myeloid and this shift is accompanied by severe erythroid dysplasia and anemia.

Increased splenomegaly suggests that the spleen has become a source of extramedullary hematopoiesis or defective erythrocytes could be removed from the peripheral blood, causing splenic hypertrophy. Age related diseases such as myelodysplasia (MDS) and myeloproliferative neoplasms (MPN) are seen in humans. *Stat3^{-/-}* mice exhibit defects in neutrophil morphology and in bone marrow cellularity and morphology as well as erythroid dysplasia (Hoekstra J et al., 2011;

Polednak AP 2011). There are several hallmarks of normal aging in mice. These include anemia, increased LSK cells and a lymphoid to myeloid shift and are typically seen in mice of about 1-2 years of age (Chambers SM et al., 2007; Sudo K et al., 2000). *Stat3*^{-/-} exhibit hematopoietic and blood defects that are more commonly associated with disease and/or seen in aged mice. Table 2 summarizes abnormalities observed in *Stat3*^{-/-} mice as compared to WT.

Based on the abnormalities we found in the bone marrow and peripheral blood of *Stat3*^{-/-} we hypothesized that the over production of ROS in the HSPC compartment may in part contribute to the abnormal blood and bone marrow phenotype as well as the diminished lifespan of *Stat3*^{-/-} mice. Injecting mice with the ROS scavenger, N-acetyl cysteine (NAC), increased the life span of *Stat3*^{-/-} mice by two fold (Mantel C et al., 2012), suggesting that increased ROS production (from mitochondrial dysfunction) contributes to the shortened life span of *Stat3*^{-/-} mice. Furthermore, injection of NAC decreased the lymphoid to myeloid shift in the peripheral blood smears of *Stat3*^{-/-} animals 40 days post birth (Mantel C et al., 2012), as compared to that which was previously seen. These results indicate a partial reversal of the *Stat3*^{-/-} blood phenotype by NAC with no effect of on the mature blood cell indices of WT mice (Mantel C et al., 2012).

Together, these results suggest that a hematopoietic-specific deletion of *Stat3* in mice produces blood indices similar to some observed in human malignancies such as myeloproliferative neoplasms. HSCs from *Stat3*^{-/-} are

Table 2.

	WT	<i>Stat3</i> ^{-/-}
Body size at birth	normal	normal
Body Weight after 4 weeks	normal	~20% smaller
Activity	normal	lethargic
Spleen	normal	splenomegaly
Red cell distribution width	normal	increased
Erythrocyte counts	normal	reduced
Erythroid dysplasia	no	severe
Early death	no	4-8 wks

Table 2. Abnormalities observed in *Stat3*^{-/-} mice compared to WT

Abnormalities observed in *Stat3*^{-/-} include reduced body weight and activity 4 weeks after birth as well as abnormal peripheral blood counts and mortality between 4-8 weeks (Adapted from Mantel C, Messina-Graham S, Moh A, Cooper S, Hangoc G, Fu X-Y, and Broxmeyer HE, 2012).

decreased in numbers in the bone marrow, have diminished bone marrow repopulating ability and HPCs have decreased functionality in vitro. We found that *Stat3*^{-/-} animals have LSK cells that are almost all c-kit^{low}, which is indicative of older mice and are marks of deeply quiescent HSCs (Matsuoka Y et al., 2011). Our results show that *Stat3*^{-/-} HPCs are not actively cycling and do have a cell surface marker phenotype consisted with older mice, however we saw no difference in the efficiency of repopulating capacity between c-kit^{low} and c-kit^{hi} cells and cell cycle status also had no effect on repopulation (primary and secondary). Therefore, *Stat3*^{-/-} mediated defects in the cell cycle do not account for the defects in repopulating ability, suggesting a more complex role for STAT3 in repopulation, cytokine signaling, and cell cycle. *Stat3* deletion in HSCs and HPCs yields aberrant mitochondrial function, including overproduction of ROS. ROS has been shown to contribute to normal hematopoiesis and linked to pathological disorders such as leukemia and MDS (Sattler M et al., 2000; Abdel-Wahab O et al., 2010). We noted a myeloproliferative phenotype in *Stat3*^{-/-} mice, which may be due in part to ROS overproduction and dysfunction of mitochondrial respiration and the electron transport chain (Wegrzyn J et al., 2009). Dysfunctional electron transport chain function can increase oxygen consumption through increased proton leak which uncouples mitochondrial ATP production, and our results are consistent with this.

DISCUSSION

HSCs have been shown to have fewer and less active mitochondria (Mantel C et al., 2010) with a greater reliance on glycolysis and metabolic quiescence (Kohli L and Passague E, 2014; Simsek T et al., 2010) than more committed progenitors and mature blood cells. Metabolic quiescence and a dependence on glycolysis is fundamental to HSC maintenance in the bone marrow and serves to prevent increased cell cycling and oxidative damage. HSCs possess the ability to undergo self-renewing divisions. HSCs can undergo asymmetric or maintenance division in which one daughter cell becomes a committed progenitor and the other one remains a stem cell. HSCs can also undergo an expansion division in which both daughter cells remain HSCs or HSCs can divide to produce two HPC progeny. Both intrinsic and extrinsic stimuli govern HSC cell fate decisions (Siller KH, et al. 2009). Localization of cell fate determinates prior to division is believed a major determinant of HSC self-renewal or differentiation (Siller KH, et al. 2009). More recently, mitochondria, and mitochondrial metabolism have been implicated as a determinant in stem cell fate decisions (Parker GC et al., 2009; Mantel C et al., 2010; Kohli L and Passague E, 2014). HSCs have low numbers of mitochondria, and this can be used to identify and isolate HSCs (Mckenzie JL et al. 2007; Romero-Moya D et al., 2013). Our data shows that upregulation of mitochondrial biogenesis in an SDF-1/CXCL12 TG mouse model is linked to the loss of pluripotency, and mitochondria are required during the early stages of differentiation (Yu WM et al., 2013), which is consistent with reports in embryonic

stem cells (Facucho-Oliveira JM et al, 2007). Increased energy demands of stem cells during differentiation are met by increasing mitochondrial number and activity. Increasing energy demands during differentiation poses a potential risk of oxidative damage.

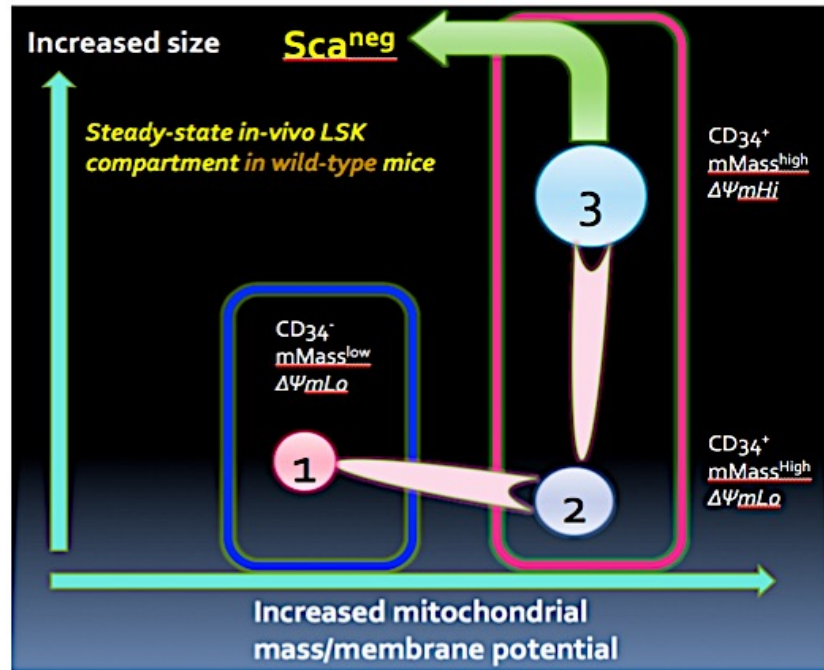
Determining factors that are involved in regulating mitochondria biogenesis and pluripotency in HSCs is critical to understanding HSC function. The relationship of SDF-1/CXCL12 to mitochondrial metabolism in HSCs and HPCs has yet to be firmly established, but, by using an SDF-1/CXCL12 TG mouse model our data shows a strong relationship between the expression of CD34 and mitochondrial biogenesis. We noted two discrete populations of LSK cells based on CD34 surface expression and mitochondrial mass (Figure 4). These populations were either CD34^{lo}/Mt-mass^{lo} and CD34^{hi}/Mt-mass^{hi} in WT mouse bone marrow (Figure 4B) and SDF-1/CXCL12 TG mouse bone marrow contained one discrete population of CD34^{hi}/Mt-mass^{hi} cells (Figure 4C). This suggests that SDF-1/CXCL12 mediated upregulation of CD34 surface expression and mitochondrial biogenesis are associated with loss of pluripotency and an increase in the percent of total LSK cells, as well as an increase in the percent of total number of LSK cells expressing high CD34 levels in the SDF-1/CXCL12 TG mouse bone marrow (Figure 5A). These results are consistent with previously published results showing that as ESCs begin to differentiate, mitochondrial biogenesis is induced and there is a loss of pluripotency and pluripotency markers (Facucho-Oliveira JM et al, 2007). Our results are the first to show a role for SDF-1/CXCL12

in the regulation of mitochondrial dynamics and may have implications for cell-fate determination. We also found a relationship between the size of LSK cells and mitochondrial mass (Figure 6A). Three different populations of LSK cells were discernable (Figure 6A and C). Population 1 is CD34^{lo} and Population 2 and 3 are CD34^{hi}. We further characterized these three populations by measuring their mitochondrial activity. We utilized the mitochondrial membrane potential fluorescent dye, JC-1, to assess $\Delta\Psi_m$ in these three populations (Figure 6B). Figure 24 is a schematic representation of the in vivo steady state LSK compartment in the WT and SDF-1/CXCL12 TG mouse bone marrow. The shift to larger LSK cells with increased mt-mass and reduced $\Delta\Psi_m$ can be seen in the SDF-1/CXCL12 TG LSK compartment. This suggests that SDF-1/CXCL12 not only functions in modulating mitochondrial respiration and biogenesis, but SDF-1/CXCL12 TG expression reveals that the three distinct populations of LSK cells may play a role in the asymmetric segregation of mitochondria during differentiation, which may be a strategy that HSCs employ to protect themselves from oxidative damage from increase mitochondrial activity (Figure 25). During an asymmetrical (maintenance) HSCs division, one daughter cells remains an HSC, while the other begins its journey of differentiation.

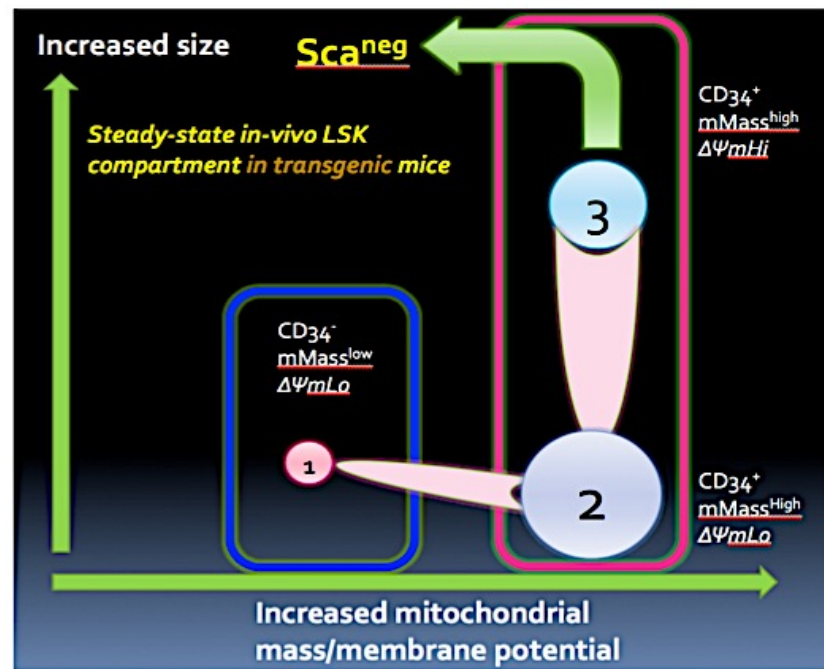
Based on size, CD34 expression, mitochondrial mass, and $\Delta\Psi_m$, we have devised a model of asymmetrical HSC cell division that suggests asymmetrical mitochondrial segregation during cell division (Figure 25). Asymmetrical mitochondrial segregation during asymmetric HSC division ensures that the

resulting HSC daughter cell is protected from oxidative damage, while ensuring upregulated mitochondrial biogenesis required for the differentiation of the progenitor daughter cell. Furthermore, our results suggest that SDF-1/CXCL12 has the ability to affect steady state hematopoiesis and changes in SDF-1/CXCL12 expression in the bone marrow microenvironment may affect HSC quiescence and self-renewal by effecting mitochondrial activity. Movement of HSCs from the CD34^{lo} LSK compartment to the CD34^{hi} compartment may be favored due to SDF-1/CXCL12 upregulation and increased progenitor cycling. It is well known that SDF-1/CXCL12 is critical for the survival of hematopoietic progenitors and is integral to HSC quiescence, self-renewal, and maintenance in the bone marrow microenvironment (Mendez-Ferrer S et al., 2008; Mendez-Ferrer S et al., 2010; Omatsu Y et al., 2010; Ding L and Morrison SJ, 2013; Ugarte F and Forsberg EC, 2013; Nombela-Arieta C et al., 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014). A published report has shown that SDF-1/CXCL12 expression is regulated in a circadian manner by sympathetic nerves innervating the bone marrow (Mendez-Ferrer S et al., 2008), and SDF-1/CXCL12 expressed by the many different stromal cell populations in the bone marrow microenvironment may be responsible, in vivo, for regulating HSC mitochondrial metabolism and fate determination. Our studies are the first to show the potential role of SDF-1/CXCL12 in the regulation of mitochondria in HSCs, and the potential role for both SDF-1/CXCL12 and mitochondria in HSC fate determination.

Figure 24.



WT



SDF-1 TG

Figure 24. Potential model mitochondrial distinct types of LSK cells

The influence of global SDF-1/CXCL12 transgene expression compared to WT on the LSK compartment is modeled Based on data from Figures 4–7 The relationships compartment to the CD34^{hi} LSK compartment may be favored due to SDF-1/CXCL12 upregulation and increased progenitor cycling, cell size, mitochondrial mass, and CD34 expression are indicated experiments (Mantel C, Messina-Graham S, and Broxmeyer H, 2010).

Figure 25.

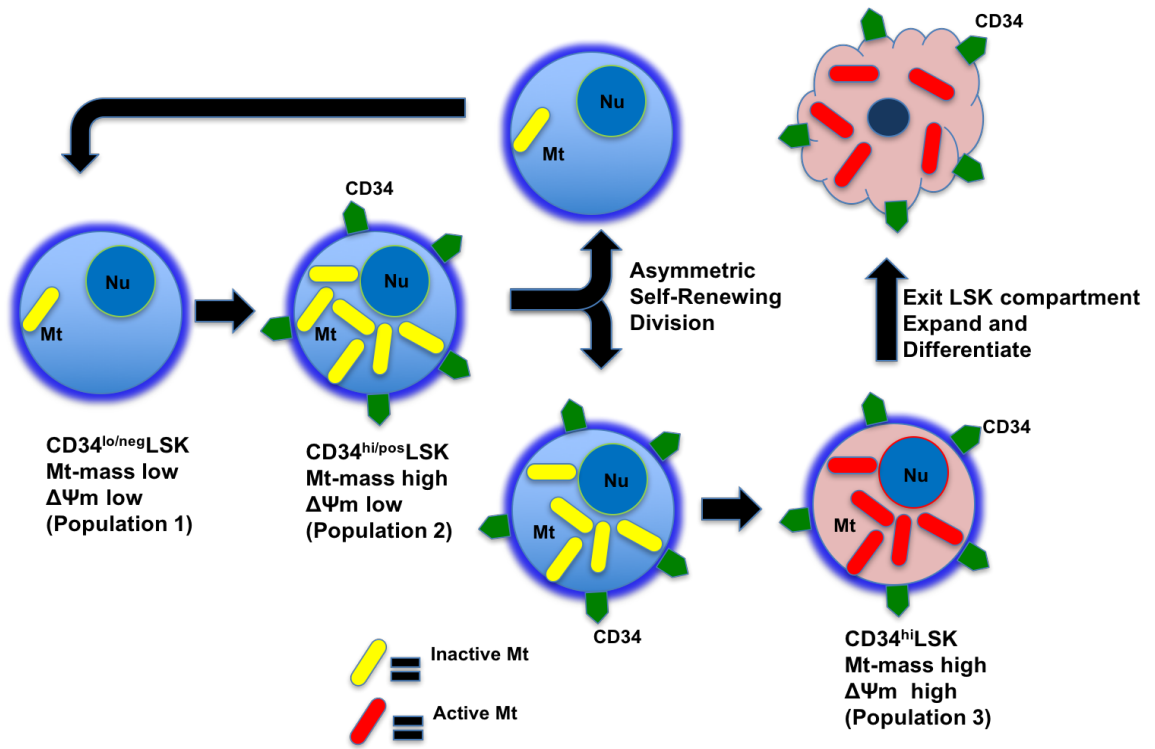


Figure 25. Model of HSC mitochondrial upregulation without exposure to increased oxidative risk.

This model shows mitochondrial upregulation during HSC self-renewing cell divisions and represents asymmetric co-segregation of CD34 and mitochondria.

This model produces self-renewed HSC daughter cells that have had no exposure to active mitochondria and potential oxidative damage experiments (Mantel C, Messina-Graham S, and Broxmeyer H, 2010).

As mentioned above, SDF-1/CXCL12 is essential for maintaining stem cell quiescence and self-renewal in the bone marrow (Mendez-Ferrer S et al., 2008; Mendez-Ferrer S et al., 2010; Omatsu Y et al., 2010; Ding L and Morrison SJ, 2013; Ugarte F and Forsberg EC, 2013; Nombela-Arieta C et al., 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014). Despite work showing the importance of SDF-1/CXCL12 in stem cell maintenance in the bone marrow, the actual mechanism of action is unknown. However, we have shown that SDF-1/CXCL12 expression in mouse bone marrow is involved in controlling mitochondrial biogenesis and mitochondrial activity, which may possibly act as a cell-fate determinant during differentiation, but further studies are necessary to determine the role of SDF-1/CXCL12 in fate determination. We sought to further investigate the ability of SDF-1/CXCL12 to affect mitochondrial biogenesis and mitochondrial respiration. We chose an in vitro approach to study the effect of SDF-1/CXCL12 on mitochondrial dynamics. We used the human leukemia cell line HL-60 as a model of immature human blood cells. These cells express high levels of the SDF-1/CXCL12 receptor, CXCR4 and very little of CXCR7 which ensured that the metabolic effect we saw was SDF-1/CXCL12-CXCR4 mediated (Figure 10 and 11). To study early and later SDF-1/CXCL12-CXCR4 mediated metabolic events, we treated these cells with SDF-1/CXCL12 and incubated them for two and 24 hours respectively. We measured the mitochondrial OCR, at each time and found that after 2 hours of SDF-1/CXCL12 treatment, the OCR was significantly reduced in treated cells as compared to unstimulated control (Figure 9A). At first, we considered that this reduction in OCR may be an artifact of recovery since the cells

are cultured in fresh media, but since the reduction in OCR is only seen in the treated group we are confident that this reduction is in fact due to SDF-1/CXCL12 treatment and not a lag in OCR due to recovery. SDF-1/CXCL12 treatment of HL-60 cells for 24 hours produced a significant increase in OCR as compared to unstimulated control cells. These results suggest that SDF-1/CXCL12 does induce changes in mitochondrial respiration in vitro in a model of human immature blood cells. The ability of SDF-1/CXCL12 to produce time dependent and differential changes in mitochondrial respiration could potentially mean that under different circumstances, temporal changes in SDF-1/CXCL12 expression in the bone marrow microenvironment could cause metabolic changes in HSCs and HPCs. These metabolic changes were further confirmed when we analyzed the mitochondrial-linked ATP production (Figure 9). Two hours of SDF-1/CXCL12 treatment produced a significant reduction in mitochondrial-linked ATP production similar to that seen in the drop in OCR at two hours (Figure 9A), while the 24 hour treated group had a significant increase in mitochondrial associated ATP production that was similar to OCR at 24 hours (Figure 9B). These result suggest that the oxygen consumption and mitochondrial ATP production are tightly coupled. This means that the oxygen consumption driven by the electron flow and proton motive force of the electron transport chain is tightly linked to ATP production and that changes in OCR and mitochondrial-linked ATP production are due to SDF-1/CXCL12. As OCR decreases or increases, a concomitant decrease or increase in mitochondrial-linked ATP production occurs. These results also suggest that there is very little proton leak contributing to the increase in OCR since

the increase in OCR is accompanied by a concomitant increase in ATP production. Changes in OCR and ATP production suggest that SDF-1/CXCL12 is modulating electron transport chain function, potentially by changes in gene expression of downstream targets involved in mitochondrial metabolism or by activating signal transduction cascades that phosphorylate downstream target proteins, such as STAT3, or a combination of both. By using AMD3100 to block CXCR4 we confirmed that the metabolic effect that we saw is SDF-1/CXCL12 specific (Figure 11A and B), however, we did see small but statistically significant glycolytic compensation/decompensation in HL-60 cells treated with SDF-1/CXCL12 at both two and 24 hours respectively (Figure 13). HL-60 cells are a leukemia cell line, and changes in glycolysis may reflect their reliance on both ATP generating pathways or potentially the Warburg effect.

When we analyzed mitochondrial mass and $\Delta\Psi_m$ we found that both were significantly reduced at 2 hours of SDF-1/CXCL12 treatment as compared to control (Figure 12A and 12C). The 24 hour SDF-1/CXCL12 treated group had significantly increased $\Delta\Psi_m$ (Figure 12D), however, the mitochondrial mass at 24 hours was increased but was not statistically significant (Figure 12B). The changes in mitochondrial mass and $\Delta\Psi_m$ are very similar to the changes seen in OCR and ATP production, further suggesting that SDF-1/CXCL12 treatment regulates mitochondrial biogenesis and metabolism in a biphasic manner. To explore the role of SDF-1/CXCL12 in regulating mitochondrial metabolism in primary cells, we next chose to isolate lineage negative bone marrow cells from C57BL/6 mice and

treat them with SDF-1/CXCL12 for two and 24 hours. We found that SDF-1/CXCL12 treatment of lineage negative cells for two hours significantly reduced the OCR and mitochondrial-linked ATP of lineage negative cells similar to that of the HL-60 cells (Figure 14A and C). However, the percent reduction in mitochondrial-linked ATP production at two hours was greater for lineage negative bone marrow cells than that of HL-60 cells (37.2% versus 29.2%). A more robust reduction in ATP production in Lin⁻ cells may be a reflection of this population being highly enriched in progenitor cells that may be more reliant on mitochondrial metabolism due to their differentiation status and thus SDF-1/CXCL12 treatment may have a greater effect on these cells than on HL-60 cells. On the other hand, Lin⁻ cells treated with SDF-1/CXCL12 for 24 hours had significantly increased OCR and mitochondrial-linked ATP production but both were not increased as significantly in Lin⁻ cells after 24 hours of SDF-1/CXCL12 treatment as HL-60 cells (Figure 14B and 14D). For both HL-60 cells and Lin⁻ we did not determine if there was any differentiation. Interestingly, when glycolysis was analyzed in Lin⁻ bone marrow cells, there was no significant difference at two or 24 hours of SDF-1/CXCL12 treatment as compared to unstimulated control cells (Figure 15). Furthermore, when the basal glycolytic rates of the SDF-1/CXCL12 treated HL-60 groups are compared to the SDF-1/CXCL12 treated Lin⁻ groups, the glycolytic rates of the HL-60 cells are about 5-10 times higher than that of the Lin⁻ cells for both 2 and 24 hour time points (data not shown). This suggests that the lineage negative cells enriched for progenitor cells have begun to differentiate and metabolically have switched from glycolysis to oxidative phosphorylation. There

may be a small proportion of HSCs in the Lin⁻ cells at 24 hours that are still dependent on glycolysis but this may not be seen because the population is much too small and the effect is masked by the respiring progenitor cells. The biphasic regulation of mitochondrial respiration by SDF-1/CXCL12 in Lin⁻ cells may serve to reduce oxidative stress and delay the differentiation of HSCs at early time points *ex vivo*, while at later time points may serve to promote mitochondrial biogenesis and increased respiration and ATP generation during differentiation. Furthermore, these findings suggest that in the bone marrow microenvironment, time-dependent changes in SDF-1/CXCL12, as well as changes in the expression of SDF-1/CXCL12 by various niche cells, may differentially affect hematopoietic cells in the bone marrow and potentially influence HSC maintenance and differentiation. This would support recent published findings that show the regulation of SDF-1/CXCL12 expression by sympathetic neurons innervating the bone marrow (Mendez-Ferrer S et al., 2008). Furthermore, the bi-phasic regulation of mitochondrial respiration may suggest that SDF-1/CXCL12 may play a role in a circadian manner in the hematopoietic system. It is clear that mitochondrial metabolism of HSCs and HPCs is important (Qian p et al., 2015; Mantel C et al., 2012; Yalcin S et al., 2010; Mantel CR et al., 2015; Broxmeyer HE et al., 2015; Mantel C et al., 2010; Yu WM et al., 2013) and these results may have important implications for the regulation of mitochondrial metabolism of HSCs and HPCs in the determinate of fate choices during differentiation and in HSC maintenance in the bone marrow microenvironment. By continuing to study the role of SDF-1/CXCL12 in mitochondrial regulation of HSCs in the bone marrow

microenvironment, these studies may elucidate the role that it plays in regulating HSC and HPC function by various SDF-1/CXCL12 expressing niche cells.

Mitochondria are the main ATP producing organelle within cells. Recent reports indicate that mitochondria play a vital part in the regulation of different types of stem cells (Tessitore et al., 2015). There is also evidence that HSCs have lower numbers of mitochondria, lower mitochondrial activity and lower ATP production than more differentiated and committed blood cell types (Romero-Moya D et al., 2013; Simsek T. et al., 2010; Norddahl G.L. et al., 2011; Takubo K. et al. 2013; Mantel CR et al., 2012). Furthermore, mitochondria have been implicated in HSC fate decisions and HSC quiescence may serve as a means to prevent damage by mitochondrial respiration and to prevent HSC exhaustion through increased cell cycling and proliferation (Orford, K.W. and Scadden D.T., 2008; Bakker, S.T. and Passegue, E., 2013). Mitochondrial dysregulation in HSCs can also contribute to increased age related blood disorders such as bone marrow failure, myeloproliferative neoplasms and leukemia (Rossi DJ et al., 2008; Mantel C et al., 2012).

Here we presented evidence that confirms that the nuclear transcription factor, STAT3, plays a critical role in HSC and HPC maintenance and function that seems to be in part due to a noncanonical function of STAT3 in mitochondrial regulation, as has been previously described (Wegrzyn J et al., 2009; Reich NC, 2009). STAT3 has been found in mitochondria where it interacts with components

of the electron transport chain and the mitochondrial permeability transition pore (mPTP) component, Cyclophilin D (Wegrzyn J et al., 2009; Reich NC, 2009). In a hematopoietic-targeted *Stat3*^{-/-} mouse model, the blood indices of knockout mice are similar to those found in human patients with myeloproliferative neoplasms. HSCs from *Stat3*^{-/-} mice begin to develop defects in HSC function several weeks after birth. There is a marked decrease in HSC numbers in these mice and have a decreased ability to repopulate a lethally irradiated recipient mouse and these cells also have a reduced capacity for homing, engraftment and repopulation in secondary recipients. *Stat3*^{-/-} HSCs and HPCs have a reduced proliferative response to synergistic cytokines in vitro. STAT3 is involved in several cytokine receptor signaling pathways and defects in the synergistic proliferative response may help to explain why there is a reduced repopulation capacity in primary and secondary transplants. LSK cells with lower c-kit surface expression have been shown to be more quiescent than cells expressing high levels of c-kit, which is indicative of older mice (Matsuoka Y et al., 2011). Our findings are consistent with this. *Stat3*^{-/-} results in bone marrow that is almost all c-kit^{low} with HPCs that are not in cycle (Figure 19B). Furthermore, these *Stat3*^{-/-} mice have surface marker phenotypes that resemble aged mice at only 4-6 weeks of age (Figure 16). Our study also found that there was no difference in the ability of c-kit^{low} and c-kit^{hi} to repopulate primary and secondary recipients, suggesting that cell cycle status cannot account for the defects seen in repopulation. This supports the hypothesis that STAT3 plays a more complex role in HSC maintenance in mouse HSCs and HPCs beyond that seen in defective repopulation and defective synergistic

proliferative response to cytokines and cell cycle status. Previous reports have shown that aged mice have several functional defects in their HSCs (Florian MC et al., 2012; Chen, J et al., 1999). Aged mice have a severe reduction in their ability to self-renew (Florian MC et al., 2012; Chen, J et al., 1999). Also, aged mice have increased numbers of HSCs, however, the increase in number fails to compensate for their decreased functional capacity (Florian MC et al., 2012; Chen, J et al., 1999) and they also have age-related impaired responses to secreted niche factors such as cytokines (Wagner W et al., 2008). Indeed, our findings are consistent with age-related defects in HSCs.

We have also shown that HSCs and HPCs from *Stat3*^{-/-} mice have dysfunctional mitochondria (Figure 21 and 22). These mitochondria have increased mitochondrial membrane potential and ROS production and increased oxygen consumption that is uncoupled from ATP production. It is well established that ROS is necessary for normal hematopoietic function (Sattler M et al., 2000) and overproduction of ROS can lead to pathologies such as myelodysplastic syndrome, myeloproliferative neoplasms or leukemia (Abdel-Wahab O and Levine RL, 2010). Deletion of *Stat3* did in fact cause a myeloproliferative phenotype similar to that seen in humans, with pronounced defects in HSC maintenance. Defects in HSC function in *Stat3*^{-/-} mouse bone marrow and myeloproliferation can be due at least in part to mitochondrial dysfunction and overproduction of ROS, which is supported by our oxygen consumption experiments. Results from our oxygen consumption experiments also support the idea that the source of ROS

over production is due to defects in the ETC, which produces increased proton leak and uncoupling of oxygen consumption from ATP production (Figure 23). Lack of STAT3 in HSCs could be effecting the ETC and also the mPTP as previously reported (Boengler K et al., 2010). Further ROS release from the mitochondria can be induced by ROS release by the mPTP by a phenomenon called ROS-induced ROS release which in which ROS induces more ROS release by the opening of the mPTP (Zorov DB et al., 2000; Boengler K et al., 2010; Mantel C et al; 2011). STAT3 has been shown to inhibit opening of the transition pore in mitochondria and prevent ROS release and oxidative damage (Boengler K et al., 2010). Mitochondria and various antioxidant enzymes are required for cells to keep tightly regulated and balanced ROS levels (Mantel C et al., 2011; Dröge W, 2002). It is evident that the *Stat3*^{-/-} mediated overproduction of ROS plays a role in the myelodysplastic-like phenotype in mice due to the fact that NAC had a pronounced pro-survival effect that lessened pathology in *Stat3*^{-/-} mice (Mantel C et al., 2012). To our knowledge, the noncanonical role of STAT3 in the mitochondrial ETC and mPTP function in relation to the pathophysiology of diseases such as myelodysplasia and leukemia has yet to be investigated, but our results have begun to show that the lack of proper STAT3 activity in mitochondria may interfere with the proper balance and regulation of ROS production, which may lead to disease. A shift in STAT3 equilibrium between nuclear localization and the mitochondria may contribute to mitochondrial dysfunction similar to what we have previously shown for p53 (Han M-K et al., 2008). HSCs are known to reside in a hypoxic environment in the bone marrow and in this context, mitochondrial

localization of STAT3 may be a part of a complex metabolic program that favors low mitochondrial respiration to reduce ROS production and oxidative damage while favoring dependence on glycolysis and quiescence (Kohli L and Passague E, 2014).

Many cell types, cytokines, and chemokines in the bone marrow microenvironment may contribute to the mitochondrial function of STAT3. One such factor is the chemokine, SDF1/CXCL12, of which STAT3 is a known target (Shen HB et al., 2013; Liu X et al., 2014). SDF-1/CXCL12 is known to be essential in HSC maintenance and quiescence, and is expressed by many different stromal cell types in the bone marrow and has also recently been shown to regulate mitochondrial respiration in hematopoietic progenitor cells (Mendez-Ferrer S et al., 2008; Mendez-Ferrer S et al., 2010; Omatsu Y et al., 2010; Ding L and Morrison SJ, 2013; Ugarte F and Forsberg EC, 2013; Nombela-Arieta C et al., 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014), thus mitochondrial STAT3 could conceivably be regulated by SDF-1/CXCL12 in bone marrow, but further studies are needed to determine this. Also, mitochondrial localization of STAT3 may be niche dependent, but since our data shows that defects in HSC function cannot be accounted for by lack of nuclear function alone, it is possible that mitochondrial localization of STAT3 may be regulated in a niche autonomous manner in which the regulation of mitochondrial STAT3 may be dependent on cell intrinsic factors rather than the niche. Furthermore, STAT3 has been shown to be a downstream target of SDF-1/CXCL12 (Huang C et al., 2011). Further studies

need to be conducted to determine the relationship between SDF-1/CXCL12-CXCR4 signaling and activation of STAT3 and mitochondrial regulation through this pathway.

FUTURE DIRECTIONS

SDF-1/CXCL12 plays an important role in regulation HSC function in the bone marrow microenvironment (Mendez-Ferrer S et al., 2008; Mendez-Ferrer S et al., 2010; Omatsu Y et al., 2010; Ding L and Morrison SJ, 2013; Ugarte F and Forsberg EC, 2013; Nombela-Arieta C et al., 2013; Greenbaum A et al., 2013; Anthony BA and Link DC, 2014) however, there is no information on how SDF-1/CXCL12 may be functioning to regulate mitochondria in HSCs in the bone marrow microenvironment. Our lab has shown that SDF-1/CXCL12 can regulate mitochondrial biogenesis and mitochondrial function (Mantel C et al., 2010; Messina-Graham S and Broxmeyer HE, 2016) and further studies to elucidate the role of SDF-1/CXCL12 in mitochondrial function are necessary. Some suggested studies are presented below.

Analysis of OCR and ECAR in SDF-1/CXCL12 transgenic and CXCR4 knockout mouse bone marrow cells

To further investigate the role of SDF-1/CXCL12 in mitochondrial regulation in HSCs and HPCs, experiments should be conducted to measure the OCR and ECAR of SDF-1 TG, CXCR4 knockout and WT mice. Using an inducible CXCR4 knockout mouse will be valuable since the phenotypes of SDF-1/CXCL12 and CXCR4 knockout mouse are very similar (Ma Q et al., 1998). Lin⁻ cells will be isolated from mouse bone marrow by lineage depletion and immediately analyzed

on the Seahorse Bioscience XF 96 Extracellular Flux Analyzer. This will allow determination of the potential effect that SDF-1/CXCL12 has on primary Lin⁻ bone marrow cells in vivo. Isolation of more purified populations of bone marrow cells, potentially by flow cytometry, such as Lin⁻ Sca-1⁺ and LSK cells may be used for these seahorse experiments as well. By using more purified populations of bone marrow cells, we may get a better idea of what SDF-1/CXCL12 is doing to effect mitochondrial function in HSCs and HPCs.

Analysis of genes associated with mitochondrial function in mouse bone marrow cells

We have shown that SDF-1/CXCL12 regulates mitochondrial biogenesis and function, and may potentially play a role in fate determination in HSCs and HPCs (Mantel C et al., 2010), and SDF-1/CXCL12 modulates mitochondrial function in a biphasic manner (Messina-Graham S and Broxmeyer HE, 2016). To further understand how SDF-1/CXCL12 regulates mitochondrial function, it is necessary to analyze the expression of genes related to mitochondrial biogenesis and function (oxidative phosphorylation), such as PGC1 α , ATP6, SOD1 and SOD2. Gene expression should be measured by quantitative real-time PCR (qRT-PCR) in phenotypically defined (LSK, CD34⁻ LSK) and sorted, populations of bone marrow cells from SDF-1/CXCL12 TG, CXCR4 knockout, and WT mice as well as ex vivo SDF-1/CXCL12 treated WT mouse bone marrow cells. To determine which genes related to mitochondrial function are up or down regulated, an alternative

approach would be to analyze the effects of SDF-1/CXCL12 on phenotypically defined, sorted populations of HPCs and HSCs using RNASeq.

The peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 α (PGC-1 α) is a master regulator of mitochondrial biogenesis (Wanet A et al., 2015; Villena JA, 2015) and based on our results showing changes in mitochondrial mass mediated by SDF-1/CXCL12, measuring the expression of PGC-1 α should provide insight into the effect that SDF-1/CXCL12 has on regulating mitochondrial biogenesis. Our data also showed that SDF-1/CXCL12 affects mitochondrial function. To analyze changes in genes involved in mitochondrial function, such as oxidative phosphorylation, one can begin to elucidate roles of these genes, that can then be up or down regulated by various techniques, such as siRNA, in order to determine the functional significance of these genes. PTPMT is a phosphatase that is essential for mitochondrial function and is also required for differentiation of HSCs and is nuclear encoded (Yu WM et al., 2013). One could analyze gene expression of PTPMT1 to determine if it is regulated by SDF-1/CXCL12. Furthermore, one could measure the expression of several mitochondrial genes that encode proteins which are part of the electron transport chain. Candidate genes to evaluate include: COXI (mitochondrially encoded cytochrome c oxidase I) and COXII (mitochondrially encoded cytochrome c oxidase II), as well as COXIV (cytochrome c oxidase subunit IV), a subunit of respiratory complex IV encoded which is nuclear encoded (Sriskanthadevan S et al., 2015). Measuring the gene expression levels of these genes should provide

further information on the role that SDF-1/CXCL12 plays in regulating mitochondrial function in HSCs and HPCs.

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CURRICULUM VITAE

Steven V. Messina-Graham

EDUCATION

Indiana University
PhD., Microbiology and Immunology, Cancer Biology Minor
October, 2016

San Diego State University
BS., Microbiology 2004

RESEARCH EXPERIENCE

Indiana University School of Medicine, Indianapolis, IN Department of Microbiology and Immunology

Graduate Student (Laboratory of Hal E. Broxmeyer, Ph.D.) 2010-2016

As a member of the Broxmeyer lab, my thesis project involved studying mitochondrial function in hematopoietic stem and progenitor cells. By using an SDF-1/CXCL12 transgenic mouse model I investigated the role of the chemokine, SDF-1/CXCL12, in mitochondrial function in hematopoietic stem and progenitor cells. I found increased mitochondrial mass is linked to CD34 surface expression in hematopoietic stem and progenitor cells, suggesting that mitochondrial biogenesis is linked to loss of pluripotency. I also tested the ability of SDF-1/CXCL12 to modulate mitochondrial function in vitro by treating the human leukemia cell line, HL-60, and primary mouse lineage⁻ bone marrow cells with SDF-1/CXCL12 which significantly reduced mitochondrial function at two hours while mitochondrial function was significantly increased at 24 hours. By using a *Stat3* knock out mouse model, I investigated STAT3 in hematopoietic stem and progenitor cells and found that *Stat3* knockout hematopoietic progenitor cells had reduced colony forming ability, slower cell cycling status, and loss of proliferation in response to multicytokine, and mitochondrial dysfunction. I was also involved in several other research projects. My research endeavors in the Broxmeyer lab led to a first author publication and several coauthor publications.

University of California San Diego, San Diego, CA

Staff Research Associate I (Laboratory of Robert Mattrey M.D.) 2007-2009

As part of a radiology research lab, my duties included, preparation of animals for imaging via CT, MRI, SPECT, PET, US, Optical Imaging. I assisted with the operation of radiology scanners (CT, MRI, US, Optical Imager), as well as Nuclear Medicine experiments (SPECT & PET). I was also responsible for the monitoring of experiments, injection of animals with contrast material, tumor cell xenografts, and anesthesia administration in animal subjects. I performed animal procedures such as surgery, euthanasia and necropsy to harvest tissues for analysis and other post mortem procedures. Other duties included cell culture, tumor passaging, research and development, data entry and general lab maintenance.

San Diego Miramar College, San Diego, CA

Instructional Lab Technician

Department of Biology 2006-2007

As an Instructional Lab Technician, I prepared and set up labs for the Biology and Biotechnology program. I also supported faculty and assisted in teaching both theory and application of basic lab skills such as aseptic technique, as well as advanced molecular biology skills and techniques such as, bacterial isolation streaks, bacterial transformations, PCR, western blots, mammalian tissue culture, restriction digests and ligations, and flow cytometry. I also maintained equipment and managed department budgets and vendor accounts.

La Jolla Institute for Allergy and Immunology, San Diego, CA

Research Technician (Laboratory of Alex Sette, Ph.D.) 2006

As part of a vaccine development team I screened viral peptides for the Vaccine Discovery Division and the Immune Epitope Database project. I was responsible for maintenance and passaging of cell lines and isolation of primary mouse splenocytes, as well as human leukocytes used to screen viral peptides by ELISPOT assay. I also performed animal handling and surgeries, human whole blood processing.

Howard Hughes Medical Institute

University of California San Diego, San Diego, CA

Research Technician (Laboratory of Scott Emr, Ph.D.) 2005-2006

Maintained lab and prepared media and reagents for research staff. By using standard cloning techniques, I created new fluorescent protein tagging vectors for yeast. I also investigated a novel yeast "arrestin-like protein" using the above cloning techniques as well as yeast transformations, tetrad analysis, yeast genomic DNA isolation, and genotyping.

Applied Biotech Inc., San Diego, CA
Research Associate

2005

Involved in quality control of drug test products following company SOP's to generate data for an FDA inquiry into company procedures and practices.

RELEVANT UNDERGRADUATE EXPERIENCE

Ferring Research Institute, Inc., San Diego, CA
Vivarium Manager and Lab Assistant

2003-2005

Performed basic husbandry and handling of mice and rats used in research studies, cleaned and sterilized equipment, vivarium and surgical facilities. Managed protocols, database upkeep, and supervised research staff.

San Diego State University, San Diego, CA

Independent Research Study (Laboratory of Stanley Maloy, PhD) 2004

Student research project designed to study mutations in Salmonella typhimurium RpoS protein and the effects those mutations had on invasion using a macrophage and Acanthamoeba polyphaga model, as well as the effects on resuscitation from a viable but non-cultivable state. The project was carried out using basic microbial techniques such as isolation streaks, growth curves and spot counting, as well as tissue culture.

San Diego State University, San Diego, CA

Independent Research Study (Laboratory of Chris Glembotski, Ph.D.) 2002

Student research project designed to find the transcriptional activation domain of ATF6-beta. C- and N- terminal truncations of ATF6-Beta were done using PCR. Tissue cultured mouse cardiomyocytes were transfected with expression vectors containing the truncated ATF6-Beta and transcriptional activation was measured using a GAL4-luciferase assay.

San Diego State University Microchemical Core Facilities, San Diego, CA

Student Intern 2002

Custom synthesized DNA primers on an ABI Expedite and ABI 392 DNA synthesizer. I checked the quality and size of primers by both polyacrylamide gel electrophoresis and HPLC. I set up DNA sequencing on an ABI Prism 3100 capillary electrophoresis DNA sequencer using samples prepared by a chain-termination, primer extension PCR sequencing reaction.

Arizeke Pharmaceuticals, Inc., San Diego, CA

Lab Support 2000-2001

Washed and sterilized laboratory glassware and supplies, ordered and stocked supplies, prepared media, and managed lab and supported research staff. I also performed basic cloning, restriction digests and diagnostic gel electrophoresis for protein expression project.

The Scripps Research Institute, San Diego, CA

Scientific Volunteer/ Intern (Laboratory of Peter K. Vogt, PhD) 1999-2000
Washed and sterilized laboratory glassware and supplies, ordered and stocked supplies, prepared media, and supported research staff. Also performed basic cloning, restriction digests and diagnostic gel electrophoresis for post-doctoral researchers.

TEACHING EXPERIENCE

Teaching Assistant, J210 Microbiology and Immunology, Spring 2012
Indiana University School of Medicine, Indianapolis, IN

Instructor, Camp Medical Detectives-Biomedical Research Section 2010-2012

AWARDS AND HONORS

AAAS/Science Program for Excellence in Science, 2011-2013

Outstanding Presenter Award at the Midwest Blood Club Symposium, March 2012

Judge- Charles A. Tindley Accelerated School Senior Capstone Project, 2016

Pre-Doctoral Fellowship- NIH T32 DK07519, 2011-2016

ABSTRACTS

Newton, I., Messina-Graham, S., Shih, A., Abrahamsson-Schairer, A., Jamieson, C., & Mattrey, R. Optical Imaging of Neural Progenitor Cell Homing to Patient-Derived Chronic Myelogenous Leukemia. American Journal of Roentgenology. Vol. 194. No. 5. American Roentgen Ray Society Meeting May 2010.

Mantel, C., Messina-Graham, S., Moh, A., Fu, X-Y., Broxmeyer, HE. STAT3 Deletion in Hematopoietic Tissue Causes Mitochondrial Dysfunction with Increased ROS Leading to LT-Repopulating Stem Cell Depletion, Erythroid Dysplasia, Peripheral Neutrophilia, and a Rapid Aging-Like Phenotype. Poster Presentation at the 52nd Annual American Society of Hematology meeting December 2011, Orlando Florida.

Mantel, C., Messina-Graham, S., Moh, A., Fu, X-Y., Broxmeyer, HE. STAT3 Deletion in Hematopoietic Tissue Causes Mitochondrial Dysfunction with Increased ROS Leading to LT-Repopulating Stem Cell Depletion, Erythroid Dysplasia, Peripheral Neutrophilia, and a Rapid Aging-Like Phenotype. Poster Presentation at the Midwest Blood Club annual meeting March 2012, Indianapolis, Indiana.

Messina-Graham, S. and Broxmeyer, HE. SDF-1/CXCL12 Modulates Mitochondrial Respiration of HSPCs in a Bi-phasic Manner. Poster Presentation at the 56nd Annual American Society of Hematology meeting December 2015, Orlando Florida.

PUBLICATIONS

Mantel, C., S. Messina-Graham and H. E. Broxmeyer (2010). "Upregulation of nascent mitochondrial biogenesis in mouse hematopoietic stem cells parallels upregulation of CD34 and loss of pluripotency: a potential strategy for reducing oxidative risk in stem cells." Cell Cycle 9(10): 2008-2017.

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Farag, S. S., S. Srivastava, S. Messina-Graham, J. Schwartz, M. J. Robertson, R. Abonour, K. Cornetta, L. Wood, A. Secrest, R. M. Strother, D. R. Jones and H. E. Broxmeyer (2013). "In vivo DPP-4 inhibition to enhance engraftment of single-unit cord blood transplants in adults with hematological malignancies." Stem Cells Dev 22(7): 1007-1015.

Velez de Mendizabal, N., R. M. Strother, S. S. Farag, H. E. Broxmeyer, S. Messina-Graham, S. D. Chitnis and R. R. Bies (2014). "Modelling the sitagliptin effect on dipeptidyl peptidase-4 activity in adults with haematological malignancies after umbilical cord blood haematopoietic cell transplantation." Clin Pharmacokinet 53(3): 247-259

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