G PROTEIN-COUPLED RECEPTOR KINASE 3 (GRK3) REGULATES G PROTEIN-COUPLED RECEPTORS ON MURINE BONE MARROW NICHE MESENCHYMAL STEM CELLS AND HEMATOPOIETIC STEM-PROGENITOR CELLS

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ABSTRACT

Jaime Marie Brozowski: G protein-coupled receptor kinase 3 (GRK3) regulates G protein-coupled receptors on murine bone marrow niche mesenchymal stem cells and hematopoietic stem-progenitor cells
(Under the direction of Teresa K. Tarrant)

The bone marrow microenvironment, termed *niche*, supports hematopoietic cell development and thus, is vital for establishment of the immune system. Within the niche reside bone marrow mesenchymal stem cells (BmMSCs) that surround the hematopoietic stem-progenitor cells (HSPCs) to support their development, maintenance, and function; however, the intracellular regulatory mechanisms of BmMSCs and HSPCs are still being defined. The goal of this dissertation work is to provide further insight into the regulatory mechanisms that modulate functionality of BmMSCs and HSPCs. Our data suggest *G protein-coupled receptor kinase 3* (GRK3) functions as a negative regulator of G protein-coupled receptors (GPCRs) on BmMSCs and HSPCs.

BmMSCs isolated from GRK3-deficient (*Grk3-/-*) mice have enhanced proliferation and osteogenic differentiation *ex vivo* compared to wildtype (WT) BmMSCs. *Grk3-/-* BmMSC cultures also have higher levels of CXCL12, an essential chemokine for HSPC development, and interestingly, *Grk3-/-* mice have increased hematopoietic cell numbers isolated from the bone marrow. Both *Grk3-/-* BmMSC proliferation and osteogenic differentiation were reduced to WT level upon reduction of sphingosine-1-phosphate (S1P), and *Grk3-/-* BmMSCs have sustained ERK1/2 signaling upon stimulation of sphingosine-1-phosphate receptor (S1PR) with S1P in comparison to WT BmMSCs. In addition, we report

GRK3 recruits β-arrestin, a protein necessary for receptor internalization, to the C-terminus of S1PR1, and we demonstrate BmMSCs lacking GRK3 regulation have impaired S1PR1 internalization. Our findings suggest GRK3 regulates GPCR S1PR on BmMSCs.

Grk3-/- mice have increased bone marrow lineage negative (Lin^-) $Sca1^+$ $c\text{-}Kit^+$ (LSK) HSPC and oligopotent progenitor numbers, as well as increased total leukocytes in the peripheral blood. Since increased stem cell numbers and function potentiate cellular engraftment and hematopoiesis, we tested whether GRK3 deficiency enhances hematopoietic cell function *in vivo* after short-term transplantation, termed *colony forming unit-spleen* (CFU-S) assay. Transplanted *Grk3-/-* LSK HSPCs or *Grk3-/-* whole bone marrow increases colony counts on the explanted spleen in comparison to WT controls, suggesting hematopoiesis of *Grk3-/-* HSPC is enhanced. Further, both *Grk3-/-* hematopoietic myeloid granulocytic and monocytic (CFU-GM) and lymphoid (CFU-Pre-B) colony counts increased *ex vivo* upon CXCR4 ligand stimulation (CXCL12), and *Grk3-/-* myeloid colony counts reduced to WT levels with CXCR4 antagonist treatment (AMD3100). Taken together, *in vivo* and *ex vivo* CFU data suggest GRK3 regulates bone marrow HSPC numbers, and this is, at least in part, mediated through CXCL12/CXCR4 stimulation.

Herein, we describe a newly elucidated pathway of regulation on two niche cells, BmMSCs and HSPCs. Specifically, our data suggest GRK3 functions as a negative regulator of GPCRs on both BmMSCs and HSPCs and can modulate stem cell function.

To my unborn child, everything I aim to accomplish in life is now for you.

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LIST OF ABBREVIATIONS AND SYMBOLS

-/-	Deficient
±	Plus/minus
≤	Less than or equal to
μ	Micro
μCT	Micro-computed tomography, micro-CT
α	Alpha
AAALAC	Association for assessment and accreditation of laboratory animal care
ALP	Alkaline phosphatase
AMD3100	CXCR4 antagonist
ANOVA	Analysis of variance
APC	Allophycocyanin
β	Beta
BCA	Bicinchoninic acid
BmMSC(s)	Bone marrow(-derived) mesenchymal stem cell(s)
BV/TV	Bone volume/total volume (bone volume fraction)
C57BL/6	C57 Black 6 or "B6" (mice)
CAR	CXCL12-abundant reticular
CCK-8	Cell counting kit-8
CD	Cluster of differentiation
CD11b	Mac-1, Integrin α M (ITGAM)
CD16/CD32	FcγRIII/FcγRII
CD29	Integrin β1

CD31	PECAM-1
CD34	Hematopoietic progenitor cell antigen CD34
CD44	Hermes, H-CAM
CD45	Leukocyte common antigen
CD45R	B220
CD48	Slamf2
CD73	5’-nucleotidase
CD105	Endoglin
CD106	VCAM-1
CD150	Slamf1
cDNA	Complementary deoxyribonucleic acid
CEM	Complete expansion media
CFU	Colony forming unit
CIM	Complete isolation media
c-Kit	Stem cell factor receptor (CD117)
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO_2	Carbon dioxide
CXCL12	C-X-C motif chemokine ligand 12/ stromal cell-derived factor-1
CXCR4	C-X-C motif chemokine receptor 4 (CD184)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate-buffered saline

ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FTY720	S1PR agonist
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase, G3PDH
G-CSF	Granulocyte-colony stimulating factor
GMP	Granulocytic-monocytic progenitor
GPCR(s)	G protein-coupled receptor(s)
GRK	G protein-coupled receptor kinase
Gy	Gray, unit of radiation
HBSS	Hank’s balanced salt solution
HCT	Hematopoietic cell transplantation
HRP	Horseradish peroxidase
HS	Horse serum
HSC(s)	Hematopoietic stem cell(s)
HSPC(s)	Hematopoietic stem-progenitor cell(s)
IACUC	Institutional animal care and use committee
IDUA	Iduronidase, alpha-L-
IMDM	Iscove’s modified dulbecco’s medium
IU	International unit

KD, kDa	Kilodalton
LIC	Ligation independent cloning
Lin	Lineage (Lin^-, lack of specific lineage commitment)
LSK	Lin^- $Sca1^+$ $c\text{-}Kit^+$
LT-	Long-term
Ly-6G	Neutrophil marker
m	Milli
M	Molar
MAPK	Mitogen-activated protein kinase
MEP	Myeloid erythroid progenitor
mL	Milliliter
MPP	Myeloid progenitor population
mRNA	Messenger ribonucleic acid
η	Nano
NaF	Sodium fluoride
$NaVO_4$	Sodium orthovanadate
NT	Non-target
ρ	Pico
PDGFR	Platelet-derived growth factor receptor
PE	R-phycoerythrin
PMSF	Phenylmethylsulfonyl fluoride
P/S	Penicillin/Streptomycin
PTH	Parathyroid hormone

PTHR	Parathyroid hormone receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction (real-time)
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute medium
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
Sca1	Stem cell antigen 1, Ly-6A/E
SEM	Standard error of the mean
SEW2871	S1PR1 agonist
SFM	Serum free media
shRNA	Short hairpin ribonucleic acid
SKI	Sphingosine kinase inhibitor
SphK	Sphingosine kinase
ST-	Short-term
TBS(/T)	Tris-buffered saline (/Tween 20)
Ter119	Erythroid cell marker, Ly-76
tTA	Tetracycline-controlled transactivator
V	Volt(s)
VPC23019	S1PR1 and S1PR3 antagonist
WT	Wildtype
YFP	Yellow fluorescent protein

CHAPTER 1: INTRODUCTION— THE BONE MARROW NICHE[1]

Overview

The bone marrow microenvironment, termed *niche*, is an active area of ongoing research, since the niche is critical in maintaining and directing hematopoietic stem cells (HSCs)—the most primitive cells that undergo self-renewal activity and differentiate into all the hematopoietic lineages that comprise the immune system. Mesenchymal stem cells, osteoblasts, and CXCL12-abundant reticular (CAR) cells are key cellular components of the bone marrow niche that surround HSCs and direct cell development and functionality, which is mediated, at least in part, through G protein-coupled receptors (GPCRs). Here, we discuss such complexity of the bone marrow, including historical and modern-day revelations, different regions of the bone marrow and key niche cells, mechanistic GPCR interactions within the niche, and targeting such GPCRs for therapeutic applications.

Healing Properties of Hematopoietic Cell Transplantation

In the 1950s, Dr. E. Donnall Thomas discovered infusion of human bone marrow cells could establish new blood cells and repopulate the bone marrow. In 1959, Dr. Thomas performed the first successful hematopoietic cell transplantation (HCT) among monozygotic twins to treat one twin that was diagnosed with leukemia [1]. In 1968, Dr. Robert Good and

[1]Adapted from my first-author review article: Brozowski JM, Billard MJ, Tarrant TK. Targeting the Molecular and Cellular Interactions of the Bone Marrow Niche in Immunologic Disease. Curr Allergy Asthma Rep. 2014 February; 14(2):402, of which *JM Brozowski researched the review article topics, compiled the resources, wrote the journal article, and made all the figures/tables. For this dissertation, JM Brozowski also made Figure 1.1.*

colleagues performed the first successful HCT among individuals whom were not identical twins to treat one sibling diagnosed with an immune deficiency [2]. Further, in 1969, Dr. Thomas and colleagues performed the first HCT using a HLA-matched sibling donor [1]. Due to these historical revelations, HCT has become the standard of care to treat several forms of malignant and non-malignant diseases in modern-day [3].

Historical Relevance of the Bone Marrow Niche

While physicians were discovering the healing properties of the bone marrow during HCT in the 1950s-1960s, research scientists were beginning to understand the cellular rationale and importance of the bone marrow. Research scientists Till and McCulloch first observed proliferative bone marrow cells *in vivo* in the early 1960s [4]. After irradiation and injection of donor bone marrow cells, nodules were visualized on a mouse spleen, and nodules were comprised of cells that visually resembled hematopoietic cells upon microscopy evaluation. Till and McCulloch proposed each nodule stems from one self-renewing cell, which was experimentally tested in 1963 and established the foundation for the current *colony forming unit-spleen* (CFU-S) assay [5]. Today, we understand that due to murine extramedullary hematopoiesis in the spleen, transplanted bone marrow cells with self-renewal capabilities home to the spleen and form clonal nodules, termed *colonies*, which can be easily visible and enumerated by eye [6]. This CFU-S assay is commonly used today as a short-term transplantation assay to evaluate *in vivo* HSC function. Furthermore, in 1968, Friedenstein observed that a single bone marrow cell was capable of forming fibroblastic–like cells, which uncovered the identification of bone marrow stromal cells [7]. The research of Till, McCulloch, and Friedenstein through the 1960s and 1970s demonstrated the bone

marrow is comprised of a heterogenous population composed of hematopoietic [4, 5, 8, 9] and stromal cells [7, 10-15], and later in 1978, Schofield proposed the concept of a *niche,* where these cells associate with one another to elicit cellular behaviors [16, 17]. Thus, in 1978 the bone marrow *niche* was born.

Cellular Components of the Bone Marrow Niche

Hematopoietic stem cells.

The hematopoietic cells Till and McCulloch identified *in vivo* that had created a clonal nodule were, in fact, hematopoietic stem cells (HSCs). HSCs are the most primitive cells within the bone marrow that have the abilities to duplicate themselves over and over, termed *self-renewing*, and differentiate into several (hematopoietic) lineages that constitute the immune system, termed *multipotent.*

Murine hematopoietic stem-progenitor cells (HSPCs) include all multipotent hematopoietic cells, identified by having a lack of markers indicative of specific lineage commitment, termed *lineage marker negative* (Lin$^-$), and two positive markers: Sca1 and c-Kit (CD117), termed *LSK HSPCs*. Within the LSK HSPC population reside the most primitive murine HSCs—*long-term HSCs* (LT-HSCs) identified as LSK Slamf1(CD150)$^+$ and Slamf2 (CD48)$^-$ [18-20] and *short-term HSCs* (ST-HSCs) identified as LSK Slamf1(CD150)$^-$ and CD48$^-$ [18, 21]. ST-HSCs possess less self-renewal capabilities in comparison to LT-HSCs.

Importantly, these multipotent HSCs differentiate into progenitors with more limited differentiation potential, termed *oligopotent* progenitors. The mature myeloid and erythroid lineage blood cells differentiate through an oligopotent progenitor, termed *common myeloid*

progenitor (CMP), which terminally differentiates into neutrophils, monocytes, basophils, eosinophils, erythrocytes and megakaryocytes/thrombocytes. Alternatively, multipotent HSCs can also differentiate into the lymphoid lineage blood cells through an oligopotent progenitor, termed *common lymphoid progenitor* (CLP), which terminally differentiates into T lymphocytes, B lymphocytes, and natural killer cells [22].

Interestingly, while the self-renewing, multipotent HSCs are most commonly located within the bone marrow, they may also mobilize into the peripheral blood upon stress responses, such as during inflammation or bleeding [23, 24], or upon regimen-treatments used to mobilize stem cells as a source for transplantation. Thus, the self-renewal activity (proliferation), multipotency (differentiation), and mobilization (migration/homing) are key functions of HSCs, which will be discussed in Chapter 3.

Mesenchymal stem cells.

Bone marrow-derived MSCs (BmMSCs) are stromal cells that originate within the bone marrow and possess self-renewing and multipotent characteristics, similar to HSCs, though differentiate into mesodermal lineages, such as osteocytes (bone), chondrocytes (cartilage), and adipocytes (fat) [25]. BmMSCs are a heterogenous population that must adhere to three criteria to be deemed a true stromal mesenchymal stem cell, (1) adhere to plastic during culture (with stromal-like morphology), (2) possess multipotency, and (3) express certain surface marker proteins [26]. While the International Society for Cellular Therapy (ISCT) has strict definitions for human BmMSCs marker proteins, those for murine are less well-defined, though may include Sca1, CD44, CD29, CD73, CD105 or CD106 [27-30].

BmMSCs are a key component of the bone marrow niche, as they secrete soluble factors that are essential for HSPC development and function, such as chemokine CXCL12 (ligand for CXCR4) [31-33]. Depletion of either CXCL12 or total numbers of BmMSCs decrease the HSC pool and repopulating activity, as well as affect homing abilities of HSPCs after transplantation [32-34], suggesting BmMSCs impact hematopoiesis. However, further characterization of these BmMSCs is warranted to aid a better understanding of their biological activity, role within the bone marrow, and therapeutic potential. As such, the work within this dissertation further characterizes the regulation and functionality of BmMSCs through GPCR signaling, which will be discussed in Chapter 2.

Osteoblasts.

Osteoblasts are located within the bone marrow alongside of the endosteum and medullary cavity interface and form bone mediated through synthesis, deposition and mineralization of extracellular matrix. Earlier studies demonstrated osteoblasts support *in vitro* HSC expansion and secrete factors, including CXCL12 similar to BmMSCs, to regulate HSC function [35, 36]. Further studies have shown an increase in osteoblast numbers enhances the number of HSPCs *in vivo* [37, 38], and conditional ablation of osteoblasts reduced bone marrow cellularity and thus, the number of HSCs [39], suggesting osteoblasts are key cells within the bone marrow affecting HSPC activity; however, direct interactions between HSCs and osteoblasts are an ongoing area of scientific investigations. More recent findings now suggest osteoblasts may play a more prominent role in supporting lymphoid progenitors within the bone marrow niche rather than a direct role with HSCs [32, 33].

CXCL12-abundant reticular (CAR) cells.

The Nagasawa laboratory identified a distinct population of reticular-like cells with long cellular processes that highly secrete chemokine CXCL12 and were equally distributed throughout the bone marrow. These newly identified reticular cells were termed, *CXCL12-abundant reticular* (CAR) cells, and are located surrounding sinusoidal endothelial cells or near the endosteum [40]. CAR cells have positive cell marker expression of VCAM-1, CD44, PDGFRα/β and are negative for Sca1, CD45, or CD31 cell marker expression [41].

HSCs are found in contact with CAR cells; thus, disruption of the CAR cells impacts the bone marrow niche with decreased size and number of HSCs with specific reduction in lymphoid and erythroid progenitors, as well as reduced secretion of both CXCL12 and stem cell factor and adipogenic and osteogenic differentiation potential of marrow cells. Depletion of CAR cells, however, did not affect osteoblasts or endothelial cells [42]. Thus, CAR cells are believed to be required for the development and maintenance of HSCs and lymphoid/erythroid progenitors [41, 42].

In summary, BmMSCs, osteoblasts, and CAR cells are all key inhabitants of the bone marrow niche that aid hematopoietic cell development and function. As previously described, BmMSCs, osteoblasts, and CAR cells each secrete a key bone marrow niche chemokine, CXCL12. However, conditional-knockout of CXCL12 in each of these cells contributes to a very specific bone marrow phenotype. Depletion of CXCL12 from BmMSCs reduces HSCs, long-term repopulating activity, quiescence, and CLPs; however, depletion of CXCL12 from CAR cells and osteoblasts did not affect HSCs, but reduces lymphoid progenitors. Interestingly, depletion of CXCL12 from mineralizing osteoblasts did not affect HSCs or

lymphoid progenitors [32, 33]. Therefore, such data suggest BmMSCs, osteoblasts, and CAR cells and the soluble factor CXCL12 participate in an intricate complexity within the bone marrow niche during immune cell development.

Regions of the Bone Marrow Niche

The bone marrow niche can be described into two functional regions: the *endosteal* niche and the *perivascular* niche. The endosteal niche is at the endosteum and medullary cavity interface with osteoblasts as a key cellular inhabitant, whereas the perivascular niche resides along the sinusoidal vessels surrounded by endothelial cells and stromal BmMSCs. It has been a long-standing belief that dormant, quiescent HSCs are located near the endosteal niche; however, recent studies now suggest HSCs are distributed throughout the bone marrow with less than 20% within 10 μm of the endosteum in the endosteal niche and instead preferential associate near sinusoid vessels [19, 43-45]. In addition, conditional deletion studies showing endosteal niche osteoblast CXCL12 secretions have little to no effect on primitive HSCs [32, 33]. Interestingly, after irradiation, HSCs preferentially locate to the endosteum, which is believed to be because irradiation causes destruction to the sinusoids leaving only arteriolar vessels near the endosteum [45]. Teasing out the niche location and developmental stages of HSCs within the bone marrow is an active, ongoing area of investigation.

G Protein-Coupled Receptor (GPCR) Signaling within the Bone Marrow Niche

Background on GPCRs.

G protein-coupled receptors (GPCRs) comprise 3-5% of the human genome and are necessary for several physiological functions. As such, GPCRs serve as therapeutic targets in 20-50% of marketed pharmaceuticals [46]. GPCRs have seven transmembrane domains with an intracellular carboxyl terminus tail, which is essential for receptor regulation. Intracellular signaling is induced upon extracellular ligand stimulation, eliciting a conformational change and dissociation of guanine nucleotide binding proteins, termed *G proteins* (α and β/γ) that elicit downstream cellular signaling for effector function [47].

GPCR regulation: G protein-coupled receptor kinases (GRKs) and β-arrestin.

After ligand-stimulation, G protein-coupled receptor kinases (GRKs) can terminate intracellular signaling by phosphorylating the carboxyl terminus of GPCRs, a process termed *desensitization*, which can recruit β-arrestin—protein necessary for GPCR internalization and another pathway of GPCR regulation in addition to receptor desensitization [47].

There are seven GRKs that are classified into three subfamilies: GRK1 (GRK1 and 7), GRK2 (GRK2 and 3), and GRK4 (GRK4-6); however, GRK1/7 and GRK4 are tissue-selective with expression in ocular tissues and testes, respectively. Therefore, only GRK2, GRK3, GRK5 and GRK6 are ubiquitously expressed [48-52]. While there is sequence homology amongst these GRKs, there are clear functional differences with selective phosphorylation sites and receptor-specific GRK regulation. Studies utilizing conventional murine models of GRK3, GRK5, and GRK6 deficiency have aided our initial understanding of the differential role of these GRKs; however, GRK2-deficient mice are embryonically

lethal with cardiac hypoplasia, and thus require either heterozygotic or conditional knockout models for any research purposes [53]. By utilizing GRK3-deficient (*Grk3-/-*) mice [54, 55], the content of this dissertation reveals a newly identified regulation process by GRK3 on sphingosine-1-phosphate (S1P)/S1P receptor (S1PR) signaling to direct the functionality of BmMSCs (Chapter 2) and discusses how GRK3 regulation on HSPCs impacts hematopoiesis mediated, at least in part, through CXCL12/CXCR4 (Chapter 3).

Ligand-induced GPCR signaling within the bone marrow niche.

GPCR signaling in the bone marrow is essential for fundamental processes for immune cell development and function [46]. In particular, cellular survival, proliferation, differentiation, and trafficking may be hindered if GPCR signaling is impaired. Three GPCRs: parathyroid hormone receptor (PTHR), chemokine receptor CXCR4, and S1PR, have been described as essential GPCRs in the bone marrow niche and will be discussed.

***PTH/PTHR signaling*.** Parathyroid hormone (PTH) is produced by the parathyroid glands and binds PTHR located on bone marrow osteoblasts [56], cells derived from mesenchymal stem cells that are responsible for building bone. The Calvi laboratory described that mice injected with PTH had increased numbers of HSCs and enhanced survival post-transplantation, which is believed to be induced by increased levels of ligand Jagged-1 secreted by osteoblasts that binds Notch receptor on HSCs [37, 38]. However, when PTH is constitutively activated in terminally differentiated osteoblastic cells, termed *osteocytes*, HSC function and numbers were not altered, suggesting HSC expansion within the bone marrow niche mediated through PTH stimulation is cell-specific [57]. Interestingly,

despite such promising pre-clinical studies and a phase I study [58], a phase II clinical trial that tested whether PTH treatment after transplantation enhanced HSC growth and ability to produce new blood cells, termed *immune reconstitution*, was terminated prematurely due to early mortality and lack of efficacy [59]. As previously mentioned, more recent findings now suggest osteoblasts may play a more prominent role in supporting lymphoid progenitors within the bone marrow niche rather than a direct role with HSCs [32, 33], which may partially explain the phase II negative outcome.

CXCL12/CXCR4 signaling. GPCR chemokine receptor CXCR4 binds its cognate ligand CXCL12 (stromal cell-derived factor-1, SDF-1) [60], and such CXCL12/CXCR4 signaling is a key signaling mechanism within the bone marrow niche for steady state immune cell development and function. CXCR4 is expressed on HSCs, CMPs, granulocytic-monocytic progenitors (GMPs), neutrophils, monocytes, and T/B lymphocytes, with low expression on myeloid erythroid progenitors (MEPs) [61, 62]. Thus, several leukocyte subsets within bone marrow utilize CXCL12/CXCR4 signaling to modulate development and function [31].

Interestingly, others have demonstrated GRK3 has selective regulation of CXCR4 with phosphorylation at serine residues 346/347 (S346/347) [63, 64]. Our lab has further demonstrated how GRK3 regulation of CXCL12/CXCR4 signaling is particularly vital for mature hematopoietic cell function [55], and the work within this dissertation further suggests GRK3 deficiency impacts the function of immature hematopoietic cells, mediated at least in part through CXCL12/CXCR4 (Chapter 3). GRK2 [65-67] and GRK6 [68, 69] have also been described to regulate CXCR4 in cell lines and mature hematopoietic cells,

respectively; however, recent findings suggest the most prevalent GRK in the LSK HSPC population is GRK3, and data suggest GRK6 phosphorylation does not play a critical role in CXCL12/CXCR4 signaling that is essential during immature hematopoiesis [70].

S1P/S1PR signaling. The precursor sphingosine is phosphorylated by sphingosine kinases (Sphk1 and Sphk2) to produce active ligand S1P that can bind to GPCR S1PRs1-5. Most cells contain sphingosine kinase activity in the cytosol and thus, secrete S1P to act in an autocrine or paracrine manner [71]. S1P levels are controlled by S1P lyase, which is an enzyme that is ubiquitously expressed and irreversibly degrades S1P [72]. S1P has vast physiological roles, including serving as a chemoattractant for HSCs. This is achieved with lower S1P expression within the bone marrow and high concentrations within the blood, thus, creating a chemoattractant gradient for the trafficking of HSCs [73]. S1P also has been shown to affect mesenchymal stem cell osteogenic differentiation [74-76] and proliferation [77, 78], of which is more clearly defined mechanistically within this dissertation (Chapter 2). While physiological roles of S1PRs in the bone marrow niche are becoming more defined, the regulation of signaling mechanisms are less well understood. Though, Arnon et al. elucidated the importance of GRK2 desensitization on S1PR1 to facilitate lymphocyte egress from circulation into the tissues [79], and the work described within this dissertation suggests the importance of GRK3 regulation on S1PR signaling to modulate BmMSC function. Taken together, both studies suggest the importance of S1P/S1PR signaling regulation on immune cells and bone marrow niche cells.

Targeting GPCRs as Therapeutic Strategies in the Bone Marrow

Hematopoietic cells are retained within the bone marrow by retention signals through GPCR signaling. Manipulation of retention signals, such as CXCL12/CXCR4 and S1P/S1PR among hematopoietic cells, has been utilized as therapeutic strategies for the treatment of various diseases and disorders and will be discussed.

Granulocyte-colony stimulating factor (G-CSF).

G-CSF has been commonly utilized to enhance egress of HSCs from the bone marrow into the peripheral blood [80], for the collection of HSCs to be used in HCT. Data have demonstrated G-CSF acts as a HSC mobilizer through disrupting CXCL12/CXCR4 signaling, as G-CSF treatment induces protease activity in the niche that cleaves both CXCL12 and CXCR4 [81]; however, further studies have demonstrated protease-dependent and protease-independent mechanisms decrease CXCL12 in the bone marrow [82]. G-CSF treatment has also increased S1P levels in the blood [83, 84], which may play a large role in facilitating HSC mobilization due to the S1P/S1PR chemotactic gradient.

AMD3100.

CXCL12/CXCR4 signaling has been discussed as a critical GPCR signaling mechanism in the bone marrow niche to aid HSPC development and function. As such, CXCL12/CXCR4 serves as a retention signal that is targeted therapeutically by AMD3100, a CXCR4 antagonist, to also induce HSC egress from the bone marrow into the peripheral blood [85]. Furthermore, combination treatment of G-CSF with AMD3100 enhanced HSC egress into the peripheral blood in comparison to G-CSF treatment alone [86].

FTY720 and SEW2871.

FTY720 binds to the S1PR family as an agonist eliciting receptor internalization and degradation, thus, suppressing S1P/S1PR signaling due to the reduction of S1PR on the cell surface. FTY720 treatment has been shown to suppress the mobilization of HSCs, as well as other cells such as T/B lymphocytes [87-90]. Therefore, in contrast to AMD3100, which antagonizes CXCL12/CXCR4 signaling and initiates release of hematopoietic cells into the periphery, FTY720 suppresses S1P/S1PR signaling through degradation of the receptor and inhibits mobilization. As such, FTY720 was FDA approved in 2010 for the treatment of relapsing multiple sclerosis by blocking immune cell infiltration into the central nervous system [91]. SEW2871 is a S1PR1-specific agonist that recycles the receptor and does not undergo degradation. As such, combination therapy of SEW2871 with AMD3100 substantially enhanced the mobilization of HSCs and progenitors [92].

Conclusion and Research Goals

The bone marrow microenvironment, termed *niche*, supports hematopoietic cell development and thus, is vital for establishment of the immune system. Within the niche reside complex molecular and cellular interactions, evident by the previously discussed regions of the bone marrow (endosteal vs. perivascular), key inhabitants (HSCs, BmMSCs, osteoblasts, CAR cells), and mechanistic GPCR interactions (PTH/PTHR, CXCL12/CXCR4, S1P/S1PR) (**Figure 1.1**). Novel therapies have been developed by beginning to understand the bone marrow niche; however, there is still much to reveal in regards to the intracellular signaling and regulation of bone marrow niche cells.

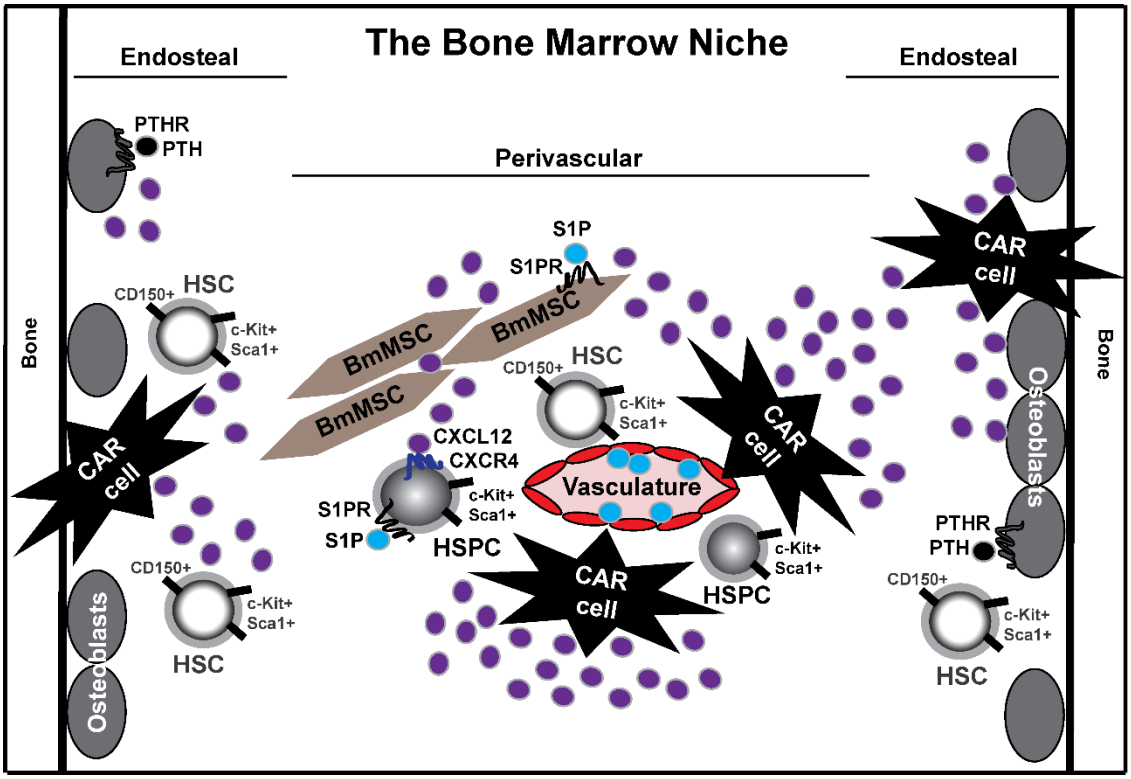


Figure 1.1. The bone marrow niche. The bone marrow is categorized into two regions: endosteal niche and perivascular niche. Hematopoietic stem cells (HSCs, Lin^{-} $Sca1^{+}$ c-Kit^{+} $CD150^{+}$ $CD48^{-}$)/hematopoietic stem-progenitor cells (HSPCs, Lin^{-} $Sca1^{+}$ c-Kit^{+}), bone marrow mesenchymal stem cells (BmMSCs), osteoblasts, and CXCL12-abundant reticular (CAR) cells are key inhabitants of the bone marrow niche. GPCR signaling is prevalent within the bone marrow niche: CXCL12/CXCR4 and sphingosine-1-phosphate (S1P)/S1P receptor (S1PR) signaling is evident on HSC/HSPCs, and the work within this dissertation further elucidates the importance of S1P/S1PR signaling on BmMSCs (Chapter 2). Parathyroid hormone (PTH)/PTH receptor (PTHR) signaling is evident on osteoblasts, cells responsible for bone formation.

Our previous work revealed mice deficient in GRK3 (*Grk3*-/-), an intracellular protein kinase that regulates GPCR signaling, have a hypercellular bone marrow with increased LSK HSPCs, oligopotent progenitors, and evident *leukocytosis*—increased white blood cells (termed *leukocytes*) in the peripheral blood [55]. This observation suggests hematopoiesis is enhanced within *Grk3*-/- mice, though without a clear mechanistic understanding. As such, we proposed (1) GRK3 may be directly impacting HSPC GPCR

signaling to elicit an enhanced phenotype and/or (2) GRK3 may be affecting other surrounding bone marrow niche cells via GPCR signaling that may be contributing indirectly to the enhanced hematopoietic phenotype.

This dissertation initiates the investigations to address these possibilities by exploring regulation of cellular signaling and function of (1) BmMSCs—cells that surround hematopoietic cells to support their development, maintenance, and function, and (2) HSPCs— cells that are essential for the establishment of hematopoietic lineages of the immune system. Specifically, the research goals of this dissertation were to investigate how GPCR regulation by GRK3 may impact these two bone marrow niche stem cell populations with crucial roles in hematopoiesis. As such, I hypothesized regulation of GPCR signaling by GRK3 controls cellular functions of both BmMSCs and HSPCs.

In chapter 2, my work demonstrates the importance of GRK3 regulation of S1P/S1PR signaling in BmMSCs, leading to enhanced BmMSC proliferation and osteogenic differentiation when GRK3 regulation is absent. In chapter 3, my work implicates the importance of GRK3 regulation of CXCL12/CXCR4 on HSPCs, leading to increased colony forming unit (CFU) numbers when GRK3 regulation is absent, evidenced by *in vivo* short-term transplantation and *ex vivo* CFU culture studies.

Given the important role of BmMSCs within the bone marrow niche, it may be possible that such an altered functionality of *Grk3*-/- BmMSCs revealed herein (Chapter 2) has the potential to impact surrounding environments in the bone marrow and affect hematopoiesis, though future investigations are warranted and proposed in Chapter 4. In addition, since GRK3 deficiency enhances CFU numbers (Chapter 3), and given the positive

correlation between the CFU content of a stem cell graft and the success rate of immune reconstitution after HCT, it may be possible that GRK3 deficiency could positively impact HCT, though future investigations are warranted and proposed in Chapter 4.

CHAPTER 2: G PROTEIN-COUPLED RECEPTOR KINASE 3 MODULATES MESENCHYMAL STEM CELL PROLIFERATION AND DIFFERENTIATION THROUGH SPHINGOSINE-1-PHOSPHATE RECEPTOR REGULATION[2]

Overview

The bone marrow niche supports hematopoietic cell development through intimate contact with multipotent stromal mesenchymal stem cells; however, the intracellular signaling, function, and regulation of such supportive niche cells are still being defined. Thus, our study was designed to better define the regulation of cellular signaling and functional mechanisms of bone marrow-derived mesenchymal stem cells (BmMSCs). Our data suggest G protein-coupled receptor kinase 3 (GRK3) functions as a negative regulator of G protein-coupled receptor signaling of sphingosine-1-phosphate receptor (S1PR) on BmMSCs. BmMSCs isolated from GRK3-deficient (*Grk3-/-*) mice have enhanced proliferation and osteogenic differentiation *ex vivo* compared to wildtype (WT) BmMSCs in identical culture conditions and passages. *Grk3-/-* BmMSC cultures also have higher levels of CXCL12, an essential chemokine for hematopoietic stem and progenitor cell development, and *Grk3-/-* mice have increased hematopoietic cell numbers isolated from the bone marrow.

[2]Brozowski JM, Timoshchenko RG, Serafin DS, Allyn B, Koontz J, Ren Y, Eudy AM, Harris TF, Abraham D, Mattox D, Rubin CT, Hilton MJ, Rubin J, Allbritton NL, Billard MJ, Tarrant TK. G protein-coupled receptor kinase 3 modulates mesenchymal stem cell proliferation and differentiation through sphingosine-1-phosphate receptor regulation. Stem Cells. Submitted September 28, 2017. *In revisions for resubmission.*

JM Brozowski performed experiments for Figure 2.1, Figure 2.2, Figure 2.4, Figure 2.5, Figure 2.6B, as well as appendices 1, 2 (performed replicated run to confirm previous data by RGT), 3, 4, 6, 7, 8 and 13. JM Brozowski wrote the journal article, as well as made the figures, including illustration for Figure 2.7. Note: Appendices 3, 7, 8, and 13 were not originally included within the submitted manuscript on September 28, 2017.

However, *Grk3-/-* mice do not have an enhanced trabecular bone volume fraction, suggesting GRK3 deficiency in niche BmMSCs may have more pronounced effects on hematopoiesis as opposed to mature bone development *in vivo*. Both *Grk3-/-* BmMSC proliferation and osteogenic differentiation were reduced to WT level upon reduction of sphingosine-1-phosphate (S1P), and *Grk3-/-* BmMSCs have sustained ERK1/2 signaling upon stimulation of S1PR with S1P in comparison to WT BmMSCs. In addition, we report GRK3 recruits β-arrestin, a protein necessary for receptor internalization, to the C-terminus of S1PR1, and we demonstrate BmMSCs lacking GRK3 regulation have impaired S1PR1 internalization. Our work suggests GRK3 regulates S1PR on BmMSCs, and lack of such regulation affects BmMSC functionality.

Significance Statement

Mesenchymal stem cells are self-renewing cells that have the potential to differentiate into multiple different tissue types. This has prompted pre-clinical and clinical investigations studying mesenchymal stem cells for the treatment of various diseases; however, rigorous studies are ongoing to learn more about tissue-specific signaling, function, and regulation. Our work demonstrates the importance of G protein-coupled receptor kinase 3 (GRK3) in bone marrow-derived mesenchymal stem cell (BmMSC) signaling and during development into pre-osteoblasts through its regulation of sphingosine-1-phosphate receptor. Lack of GRK3 receptor regulation enhances BmMSC proliferation and function toward becoming osteoblast precursors, which can then impact surrounding environments in the bone marrow and affect the production of blood cells.

Introduction

Mesenchymal stem cells are multipotent stromal cells that possess the ability to differentiate into mesodermal tissues, such as chondrocytes, adipocytes, and osteocytes [25]. Mesenchymal stem cells that reside within the bone marrow microenvironment, often referred to as bone marrow *niche* mesenchymal stem cells (BmMSCs), secrete high levels of chemokine CXCL12 that binds to G protein-coupled receptor (GPCR) CXCR4 on hematopoietic stem and progenitor cells (HSPCs) to affect their growth and development [31, 93]. BmMSCs impact hematopoiesis as depletion of either CXCL12 or total numbers of mesenchymal stem cells from the bone marrow decrease the hematopoietic stem cell pool and repopulating activity, as well as affect homing abilities of HSPC after transplantation [32-34].

We previously observed mice deficient in *G protein-coupled receptor kinase 3* (GRK3), an intracellular kinase that negatively regulates GPCRs, have (1) a hypercellular bone marrow comprised of significantly increased Lin^- $Sca1^+$ $c\text{-}Kit^+$ (LSK) HSPCs and selective downstream committed progenitors, and (2) an increase of total white blood cells in the peripheral blood (leukocytosis) compared to WT mice [55]. Our data showed GRK3 deficiency affects CXCR4 regulation on hematopoietic cells [55]; however, due to the importance of stromal cells within the niche that impact HSPC development and function, we were also interested in understanding the regulatory role of GRK3 on BmMSCs, in which GRK3 is expressed [54].

We observed *Grk3-/-* BmMSCs have enhanced osteogenic differentiation, higher levels of CXCL12, and increased proliferation. Accumulating evidence supports that phospholipid sphingosine-1-phosphate (S1P) affects mesenchymal stem cell function [46] in

osteogenic differentiation [74-76] and proliferation [77, 78]. Since the S1P receptors (S1PRs) are GPCRs, our study aimed to elucidate whether GRK3 regulated S1PR on BmMSCs to affect their functionality.

Materials and Methods

Animals.

Wildtype (WT) C57BL/6 and GRK3-deficient (*Grk3-/-*) age-matched (8-12 week-old) mice were used under standard IACUC-approved protocols in the AAALAC-accredited vivarium of UNC, and care of animals was in accordance with institutional guidelines. The *Grk3-/-* mouse strain was provided by Dr. Robert J. Lefkowitz (Duke University) and backcrossed >12 generations on the C57BL/6 background.

Bone marrow-derived mesenchymal stem cell (BmMSC) isolations.

BmMSC were isolated from flushed femurs and tibias of two male mice and cultured in complete isolation media (CIM) containing RPMI (Corning, 10040CV) with 10% fetal bovine serum (FBS, Atlanta Biologicals, S12450), 10% horse serum (HS, HyClone, SH30074.03), 1% 100 IU/mL penicillin G/100 µg/mL streptomycin (P/S, Corning, 30-002-Cl), and 12 µM final concentration of L-Glutamine (Corning, 25-005-Cl), as similar methodologies have been previously described [29, 94, 95]. BmMSC expansion was in complete expansion media (CEM) containing IMDM (Gibco, 12440-053), 10% FBS, 10% HS, 1% P/S, and 12 µM final concentration of L-Glutamine [29, 95], which was followed by hematopoietic CD45 (Stem Cell Technologies, 19771) and CD11b (Miltenyi, 130-049-601 or 130-093-634) depletion, as recommended by [94], via magnetic negative selection at early

passages 1-3. BmMSCs were passaged at 70-80% confluency and used for experiments at passages 4-15.

Chondrogenic, adipogenic, and osteogenic differentiation.

Chondrogenic differentiation. BmMSCs were suspended in CEM at $1.6x10^7$ viable cells/mL. Micromasses were made by adding 5 µL droplets of the cell suspension onto a 6-well plate and given 3-4 hours to attach. Chondrogenic media (Gibco StemPro® Chondrogenesis Differentiation Kit, A10071-01) supplemented with penicillin streptomycin was added to each well and incubated for 21 days. Cells were fixed with 10% formalin and stained using Alcian Blue. *Adipogenic differentiation.* BmMSCs were plated at $1x10^5$ cells/well of a 6-well plate in CEM supplemented with 50 µM indomethacin, 5 µg/mL insulin, and 0.1 µM dexamethasone. Cells were fixed with 10% formalin and stained using Oil Red O. *Osteogenic differentiation.* BmMSCs were plated at $1x10^5$ cells/well of a 6-well plate in CEM supplemented with 50 µg/mL ascorbic acid and 20 mM ß-glycerophosphate. Cells were fixed with 10% formalin and stained using Alizarin Red. For SKI-treated osteogenic differentiation, BmMSCs were plated at $2x10^4$ cells/well in a 24-well plate in CEM, and osteogenic differentiation was induced after an overnight rest. Cells were treated with sphingosine kinase inhibitor 2 (SKI, Cayman Chemical, 10009222) at a final concentration of 5 µM or vehicle (DMSO). Fresh media changes occurred every third day using CEM plus SKI or vehicle. Cells were fixed with 10% formalin and stained using Alizarin Red stain for analysis. Images were captured using the Olympus 1X-81 microscope and MetaMorph software.

Bone marker mRNA expression (qRT-PCR).

Total RNA from BmMSCs undergoing osteogenic differentiation was prepared using the RNeasy Mini/ Plus kit (Qiagen) according to manufacturer's instructions. Reverse transcriptase cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad, 170-8891). qRT-PCR was performed in duplicate (SYBR® Green, Bio-Rad, 172-5271) and normalized to housekeeping gene IDUA. Mean fold change of alkaline phosphatase was determined by $2^{-\Delta\Delta Ct}$ with WT day 0 as control. Primers utilized for qRT-PCR were Alkaline Phosphatase forward: AAG GCT TCT TCT TGC TGG TG, Alkaline Phosphatase reverse: GCC TTA CCC TCA TGA TGT CC; IDUA forward: GCA TCC AAG TGG GTG AAG TT and IDUA reverse: CAT TGA GCA GGT CCG GAT AC.

ELISA.

BmMSCs were plated at $1x10^5$ cells/well of a 6-well plate in CEM and rested overnight for attachment before supernatant collections began at baseline (day 1) and each subsequent collection. Supernatants of undifferentiated BmMSC monolayers were collected and analyzed for CXCL12 protein using the CXCL12 DuoSet ELISA kit (R&D Systems, DY460), as per instructions.

Micro-computed tomography (µCT).

For 8-12 week-old mice, µCT imaging was used to analyze the trabecular bone morphology at the distal femur at 12 micron resolution. The metaphyseal region of the distal femur was scanned beginning 720 microns proximal to the growth plate and extending 1500 microns towards the diaphysis of the femur. An automatic script was used to analyze the

region of interest to separate the trabecular and cortical regions of the bone and quantify bone morphology. Trabecular analysis includes quantification of BV/TV (bone volume/total volume). For 17-20 month-old aged mice, μCT imaging morphology (VivaCT80, Scanco Medical, Basserdorf, Switzerland) was used to analyze the trabecular bone. Briefly, metaphysis region was selected for 100 slices under the femur growth plate. Trabecular analysis includes quantification of BV/TV. Analyses were conducted at 12 μm slice increment with an integration time of 300 ms, a current of 145 mA, and an energy setting of 55 kV. The threshold was chosen using 2D evaluation of several slices in the transverse anatomic plane so that mineralized bone was identified but surrounding soft tissue was excluded.

Cellular proliferation.

Proliferation of $5x10^4$ BmMSCs/well of a 6-well plate was analyzed after exogenous CCK-8 (Dojindo Molecular Technologies) was added to each well at the indicated timepoints. Absorbance was measured after 3-hour incubation using the Promega Glomax® Multi + Detection System. Data were analyzed by deducting background (media and CCK-8) absorbance from raw absorbance reads. For S1P studies, BmMSCs were plated at $1x10^4$ cells/well in a 24-well plate and treated with SKI at a final concentration of 5 μM.

Immunoblotting.

BmMSCs were plated in CEM at $2.25x10^5$ cell density in a 6-well plate and incubated overnight. DPBS rinsed cells were rinsed three times with IMDM containing 20% charcoal-stripped FBS (to remove serum S1P) and 1% P/S, and incubated at 37ºC for 15 minutes.

Fresh media was added, and BmMSCs were stimulated with 1 µM S1P for indicated timepoints. Unstimulated BmMSCs served as 0 minute timepoint control. Following stimulation with S1P, BmMSC cells were rinsed with DPBS and lysed in cold HBSS + 1% TritonX100 lysis buffer containing protease inhibitors (1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL pepstatin, and 1 µg/mL leupeptin) and phosphatase inhibitors (5 mM NaF and 2 mM $NaVO_4$). All WT and *Grk3-/-* BmMSC lysates were normalized via Bicinchoninic acid (BCA) assay, and 6 µg of total protein in laemmli sample buffer (non-reducing) was freshly loaded onto AnyKD Mini-PROTEAN®TGX precast protein gel (Bio-Rad, 4569036). Gels were run at 100V for 1.5 - 2 hours in 1XTris/Glycine SDS buffer. Proteins were transferred overnight at 4ºC onto nitrocellulose membrane in Tris base (25 mM)/Glycine (192 mM) transfer buffer containing 20% methanol. The membrane was blocked in 3% fatty-acid free BSA in TBS plus 0.1% Tween-20 (TBS/T) for 2 hours at 25ºC and incubated with primary antibody 1:2,000 phospho-p44/42 MAPK or 1:2,000-3,000 p44/42 MAPK (Cell Signaling Technologies, 4370/4695) overnight at 4ºC, or 1:10,000 GAPDH (Trevigen, 2275-PC-100) for 2 hours at 4ºC. The membrane was washed three times for 10 minutes in TBS/T, incubated with secondary antibody 1:5,000 anti-rabbit IgG HRP (Cell Signaling Technologies, 7074) for 1 hour at 25ºC, and washed twice for 10 minutes in TBS/T and once in TBS. Detection was performed via ECL Prime or ECL Select (GE Healthcare, RPN2232/ RPN2235) and imaged on GeneSys image acquisition software. Densitometry was obtained by measuring ratio of phospho-ERK (pERK) over total ERK using Image J software.

β-arrestin recruitment assay.

GRK recruitment of β-arrestin to the S1PR1 carboxy-terminus was measured using agonist-stimulation in a modified-TANGO assay, as previously reported [96]. HTLA cells were transfected with a S1PR1-TCS-tTA receptor construct after removing the V2 vasopressin sequence to prevent nonspecific β-arrestin recruitment to the wildtype S1PR1. GRK over-expression was achieved via plasmids: GRK2 pcMyc_LIC and GRK3 pcMyc_LIC, and utilized a separate expression vector encoding yellow-fluorescent protein (YFP) that was simultaneously transfected to serve as a transfection control. HTLA cells were transiently transfected with 6.5 μg of total plasmid DNA (3 μg of S1P-Tango, 0.5 μg of YFP, and either 3 μg of empty-vector control, GRK2, or GRK3) via calcium-phosphate precipitation. Transfection efficiency was determined by YFP epifluorescence to be consistently >70% at 24 hours post-transfection. Cells were serum starved and then stimulated with S1P ligand at varied concentrations up to 1 μM. BriteGlo reagent (Promega, Madison, WI, USA) was added for luminescence detection via Promega Glomax® Multi + Detection System (0.5 sec/ well). Raw data were normalized by subtracting background for each independent run and setting the lowest concentration of the control condition at 0% and highest concentration at 100%.

S1PR1 internalization assay.

BmMSCs cultured in CEM were rinsed with DPBS and incubated in serum-free CEM (SFM) for 2.5 hours. BmMSCs were detached using Accutase® (Sigma, A6964), rinsed once with cold SFM containing 5% charcoal-stripped FBS (to remove serum S1P), and resuspended in cold DPBS. $1x10^5$ BmMSCs were stimulated with 1 μM S1P ligand in

100 µL FACS buffer (DPBS1X + 0.2% fatty acid free BSA + 0.1% sodium azide) at specific timepoints. Unstimulated BmMSCs served as 0 minute timepoint control. S1PR1 internalization was halted with 2 mL of ice cold FACS buffer and sample tubes were placed on ice. BmMSCs were stained for Sca1 (eBioscience clone: D7, APC-conjugated) and S1PR1 (R&D Systems clone: 713412, PE-conjugated) for 30 minutes on ice in 100 µL FACS buffer, rinsed, and analyzed by flow cytometry.

Statistical analyses.

All data were graphed utilizing GraphPad Prism v.7 and statistically evaluated using GraphPad Prism v.7 or Microsoft Office Excel program. Taking into consideration time and strain (WT and *Grk3-/-*), the bone marker qRT-PCR, CCK-8 proliferation, and S1PR1 internalization was statistically analyzed using a RM two-way ANOVA with Sidak's multiple comparison test, a method preferred over Bonferroni due to increased power [97, 98]. Similarly, taking into consideration time and strain (WT and *Grk3-/-*) but with multiple treatment groups, the SKI-treated CCK-8 proliferation was analyzed using a RM two-way ANOVA with Tukey's multiple comparison test for pairwise comparisons [97, 99]. Student's t-test compared two independent groups (WT and *Grk3-/-*) for the CXCL12-detection ELISA, micro-computed tomography data, and western blot densitometry. Taking into consideration three independent groups (empty vector, GRK2, and GRK3), the β-arrestin recruitment assay was analyzed by one-way ANOVA with Dunnett's multiple comparison test, which compared each group to the control (empty vector) [97, 100].

Results

Grk3-/- BmMSCs have enhanced osteogenic differentiation.

In our previous studies, we observed *Grk3-/-* mice had increased HSPC numbers compared to WT mice [55]. Since it is well established that bone marrow stromal cells enhance hematopoiesis [32-34, 101-103], we aimed to test whether there were differences between *Grk3-/-* and WT murine stromal BmMSCs that could contribute to this phenotype. BmMSCs isolated from WT and *Grk3-/-* mice adhered to culture plastic, underwent differentiation into tissue-specific lineages to ensure multipotency (**Figure 2.1A**), and lacked

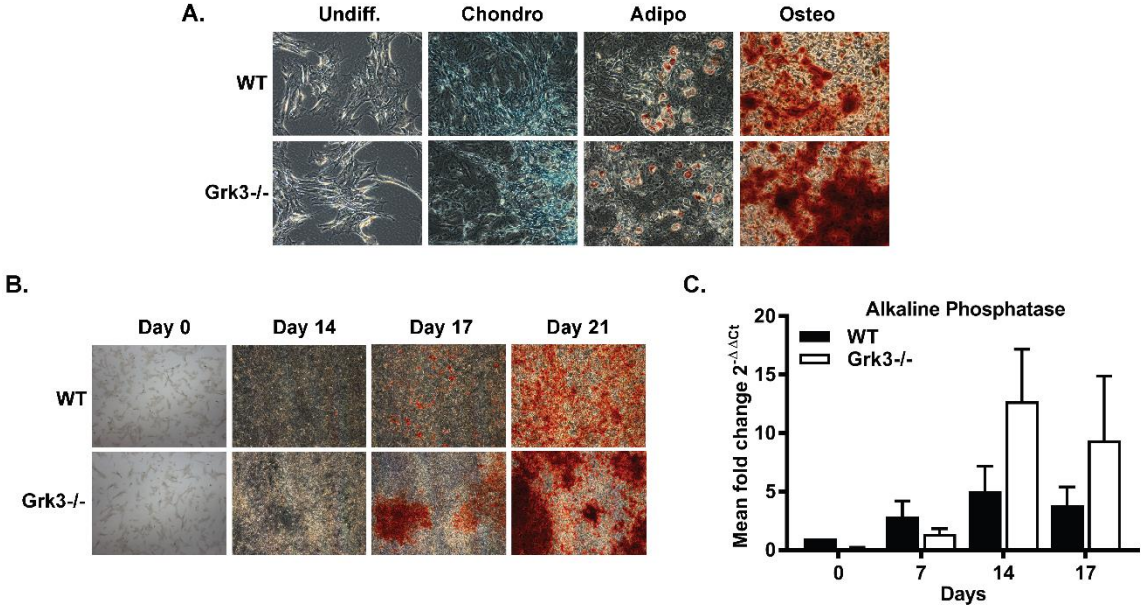


Figure 2.1. Bone marrow-derived mesenchymal stem cells (BmMSCs) deficient in G protein-coupled receptor kinase 3 (*Grk3-/-)* have enhanced osteogenic differentiation in comparison to wildtype (WT) BmMSCs. (A) Multipotent differentiation of BmMSCs isolated *ex vivo* from *Grk3-/-* and WT mice into chondrocytes (Alcian Blue stain), adipocytes (Oil Red O stain), and osteoblasts (Alizarin Red stain) at day 21; images acquired at 10X magnification. Differentiation analyses n=3 chondrogenic/adipogenic and n=10 osteogenic. **(B)** Time-course *Grk3-/-* and WT BmMSC osteogenic differentiation (Alizarin Red stain) at 2X magnification. Representative images, n=4. **(C)** Mineralization of bone marker alkaline phosphatase expression during osteogenic differentiation relative to housekeeping gene IDUA. Data represent mean ± SEM, n=7.

expression of CD45 and CD11b (hematopoietic cell markers) and consistently expressed mouse mesenchymal stem cell markers Sca1, CD106, CD73, CD44, CD29 (**Appendix 1**). During differentiation analysis, we observed no substantial differences between WT and *Grk3-/-* BmMSC chondrogenic or adipogenic differentiation cultures. However, we observed enhanced and earlier osteogenic differentiation in *Grk3-/-* BmMSC cultures in comparison to WT cultures, as demonstrated by positive alizarin red stain (**Figure 2.1B**) and higher mRNA expression levels of alkaline phosphatase (ALP), a marker of osteoblast differentiation that peaks near day 14 (**Figure 2.1C**) [104, 105]. We noticed the enhanced osteogenic differentiation phenotype was repeatedly consistent and was reproduced in four separate isolations. To further ensure there were no isolation differences between WT and *Grk3-/-* BmMSCs that may induce such a phenotype, we utilized shRNA to knockdown GRK3 (GRK3-KD) from WT BmMSCs and induced multipotent differentiation, which again showed the identical phenotype of enhanced osteogenic differentiation in GRK3-KD BmMSCs in comparison to non-target (NT) control BmMSCs (**Appendix 2**).

Grk3-/- BmMSC cultures have higher levels of CXCL12.

Niche BmMSCs, CXCL12-abundant reticular (CAR) cells in the bone marrow, and pre-osteoblasts secrete CXCL12 [32, 33], an essential chemokine in the niche for HSPC development and/or function. Since we observed our *Grk3-/-* mice had increased HSPCs [55], we next tested whether there may be differences in CXCL12 levels between WT and *Grk3-/-* BmMSCs *ex vivo.* Our data revealed *Grk3-/-* BmMSC cultures had higher levels of CXCL12 detected in comparison to WT BmMSC cultures at baseline, as well as each following time point evaluated (**Figure 2.2A**).

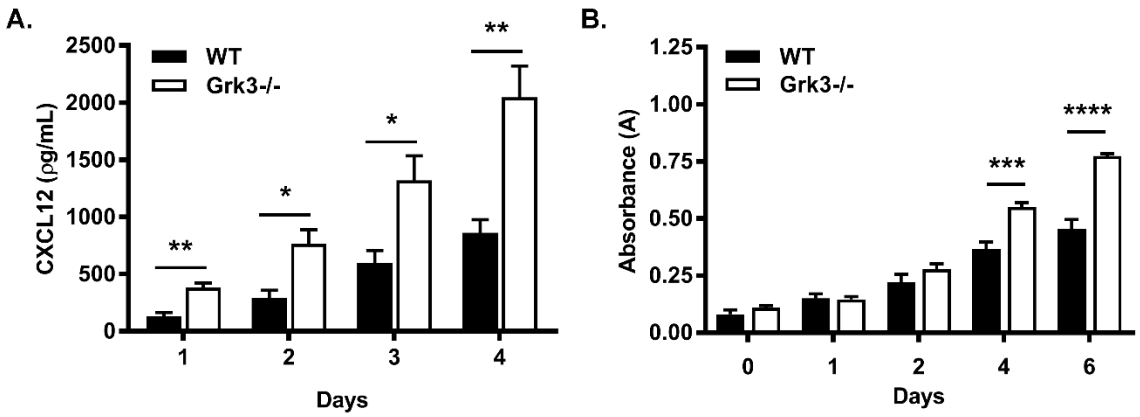


Figure 2.2. *Grk3-/-* BmMSCs have higher levels of CXCL12 detected and proliferate more in comparison to WT BmMSCs. (A) Quantification of CXCL12 protein concentration from supernatant of BmMSC cultures. Lower limit of detection at 46.9 ρg/mL. Data represent mean ± SEM, n=4. **(B)** BmMSC proliferation determined by increased formazan dye production from viable cells over time using CCK-8 proliferation assay. Data represent mean ± SEM, n=3, except day 0 n=2.
* $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ **** $P \leq 0.0001$

GRK3 deficiency increases proliferation *ex vivo.*

Due to the enhanced detection of CXCL12 in *Grk3-/-* BmMSC culture, we wanted to further investigate whether this was the result of enhanced secretory function or a proliferation response, since we also observed increased cell counts when passaging *Grk3-/-* BmMSCs. Cell counting kit-8 (CCK-8) quantitates proliferation by absorbance detection of increased formazan dye production from viable cells. Using this assay of cellular quantification, *Grk3-/-* BmMSCs have enhanced proliferation over time in comparison to WT BmMSCs (**Figure 2.2B**).

GRK3 deficiency does not affect mature bone formation *in vivo*.

As a result of our observed enhancement of *Grk3-/-* BmMSC osteogenic differentiation and proliferation *ex vivo*, we proposed to investigate whether GRK3

deficiency affected mature bone development *in vivo*. Therefore, we performed micro-computed tomography (µCT) for femoral bone analyses on WT and our conventional knockout mouse model of GRK3 deficiency. Despite the propensity of *Grk3-/-* BmMSCs to more readily differentiate into the osteogenic lineage driven by *ex vivo* reagent stimulation, our µCT results did not show differences in the trabecular bone volume fraction of *Grk3-/-* young or aged mice *in vivo* compared to controls (**Figure 2.3A-D**).

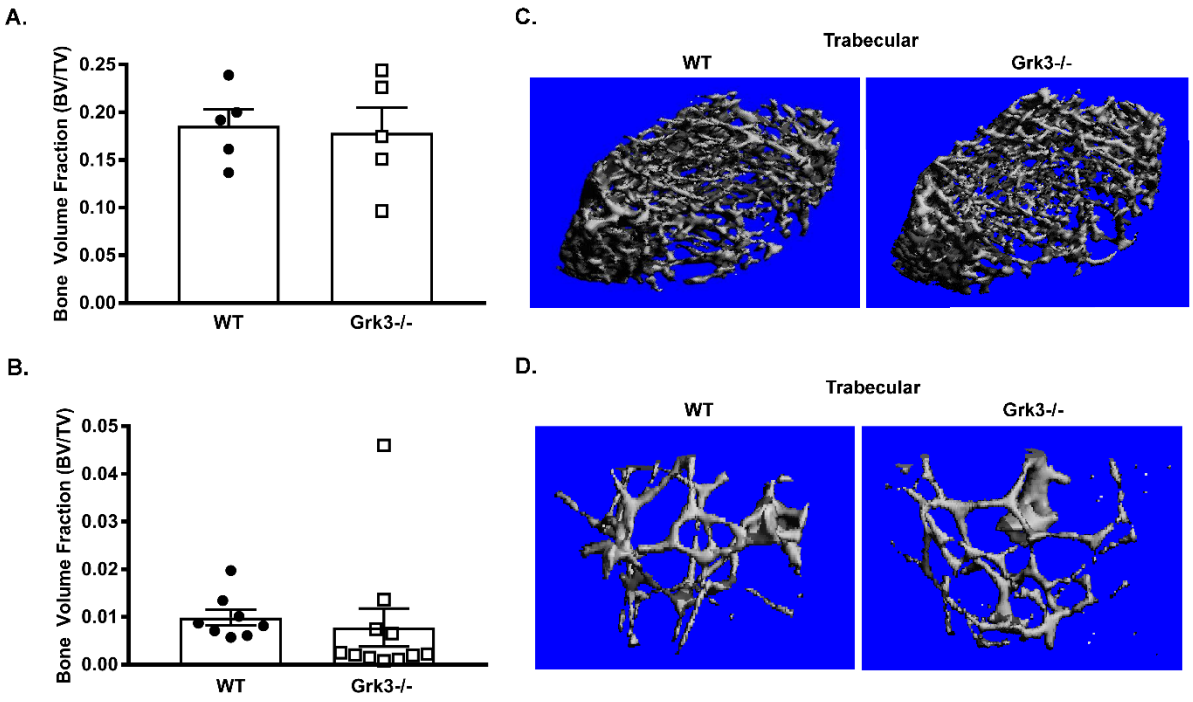


Figure 2.3. Femoral trabecular bone density is not differentially affected by GRK3 deficiency in young or aged mice. Quantification of trabecular bone volume fraction (BV/TV) via micro-computed tomography analysis of **(A)** young and **(B)** aged *Grk3-/-* and WT mouse femurs. Representative 3D image of trabecular bone of **(C)** young and **(D)** aged *Grk3-/-* and WT mouse femurs. Data represent mean ± SEM, n=5 for young mice and n=8 (WT) n=11 (*Grk3-/-*) for aged mice.

Inhibition of S1P reduces the enhanced osteogenic differentiation and proliferation phenotype of *Grk3-/-* BmMSCs.

Since GRK3 regulates GPCRs [54], we next aimed to identify the GPCR that could be contributing to the enhanced *Grk3-/-* BmMSC phenotypes of osteogenic differentiation and proliferation. Since we and others have demonstrated that (1) BmMSCs secrete CXCL12 [32, 33], (2) GRK3 regulates CXCR4 in other cell types [55, 106, 107], and (3) GRK3 regulation of CXCR4 impact bone marrow hematopoietic cell functionality [55, 108], we proposed CXCL12 may be acting via an autocrine CXCL12/CXCR4 signaling loop on BmMSCs. However, only low levels of CXCR4 mRNA expression were detected on *ex vivo* murine BmMSCs (**Appendix 3**) and further functional tests with stimulation or inhibition of CXCR4 signaling resulted in no effect on BmMSCs proliferation (**Appendix 4**).

Accumulating evidence suggests phospholipid S1P affects mesenchymal stem cell function [46], specifically in osteogenic differentiation [74-76] and proliferation [77, 78].

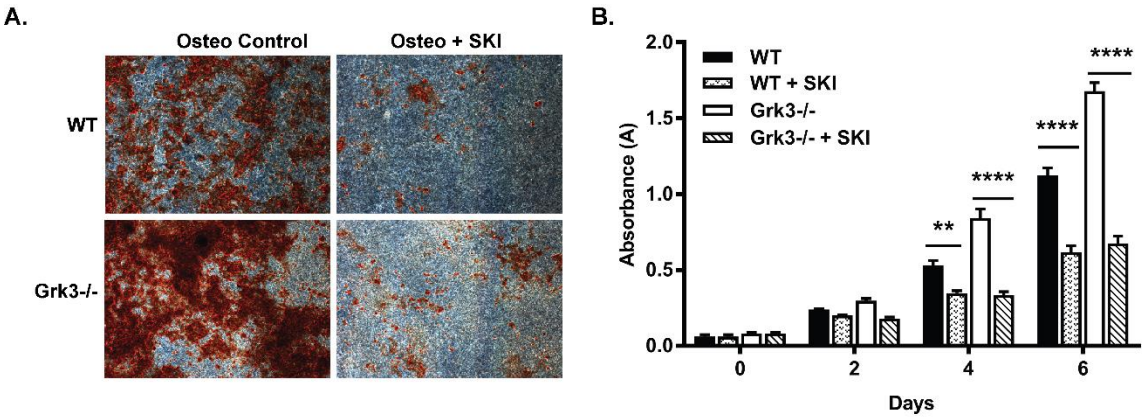


Figure 2.4. Osteogenic differentiation and proliferation of *Grk3-/-* BmMSCs are decreased to WT levels with sphingosine kinase inhibitor treatment. (A) BmMSCs treated with 5 µM sphingosine kinase inhibitor (SKI) while in osteogenic media for differentiation studies and stained with Alizarin Red. Representative images acquired at 2X magnification, n=3. **(B)** BmMSCs cultured with 5 µM SKI in culture media for proliferation detection over time. Data represent mean ± SEM, n=3.
**P≤0.01 ****P≤0.0001

Since S1P binds S1P receptors (S1PR), which are also GPCRs, we next hypothesized GRK3 functioned as a negative regulator of GPCR signaling of S1PR on BmMSCs to affect their functionality. To test this hypothesis, we had to address that (1) S1P is readily available in the BmMSC culture media due to the naturally high presence in fetal bovine and horse serum [109] and that (2) murine BmMSCs secrete S1P [110]. Therefore, to effectively neutralize S1P in culture, we utilized a sphingosine kinase inhibitor (SKI), which reduces the conversion of sphingosine to active ligand S1P. We also verified that WT and *Grk3-/-* BmMSCs had comparable sphingosine kinase activity before manipulation of this pathway (**Appendix 5**). Titration of SKI to an optimized dose of 5 μM reduced both *Grk3-/-* osteogenic differentiation (**Figure 2.4A**) and proliferation (**Figure 2.4B**) to comparable levels with WT BmMSCs, suggesting S1PR, and not CXCR4, has a role in the enhanced phenotypes.

***Grk3-/-* BmMSCs have enhanced ERK1/2 signaling after S1P stimulation.**

The ERK signaling pathway has been linked to both mesenchymal stem cell [111] and pre-osteoblastic cell line [112] osteogenic differentiation, as well as mesenchymal stem cell proliferation [78]; therefore, we hypothesized ERK signaling may be enhanced in *Grk3-/-* BmMSCs with S1P stimulation. We show via densitometry ERK1/2 early signaling at 0, 1, and 5 minutes is comparable between WT and *Grk3-/-* BmMSCs (**Figure 2.5A, B**); however, during late signaling, while WT signaling wanes after 30 minutes, *Grk3-/-* BmMSCs show sustained signaling still at 60 minutes (**Figure 2.5A, C**).

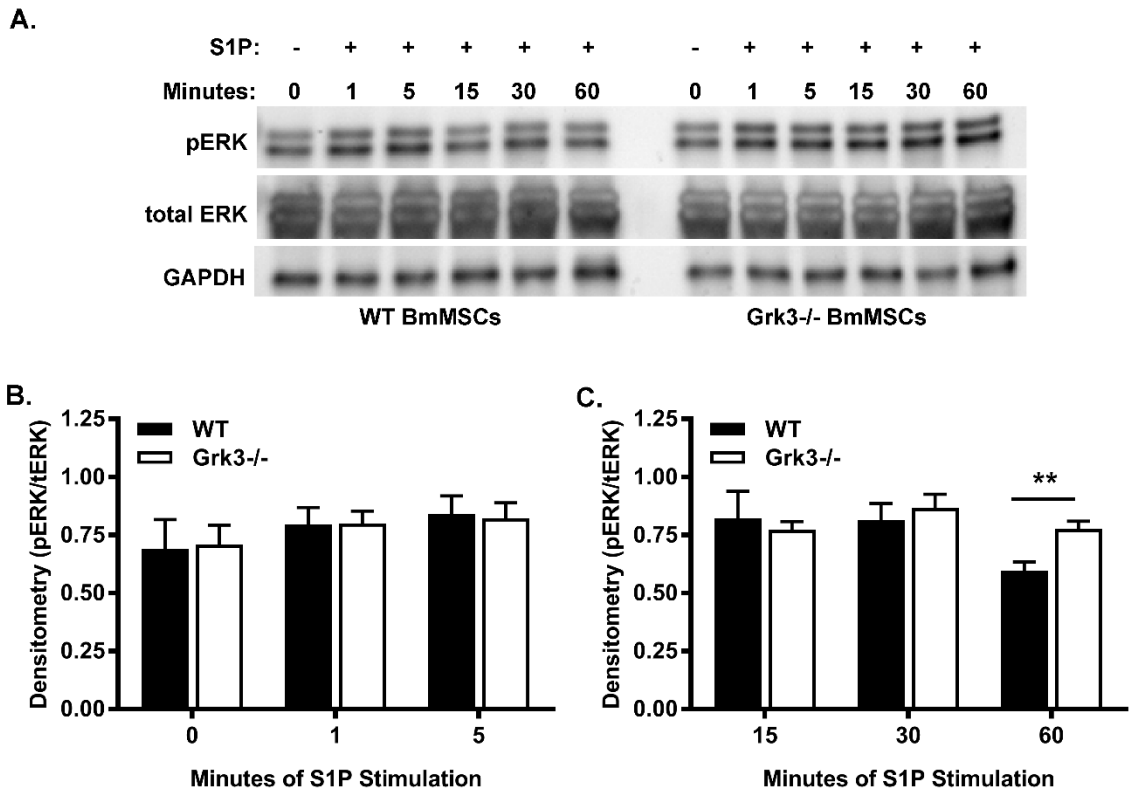


Figure 2.5. *Grk3-/-* BmMSCs have enhanced ERK1/2 signaling after S1P stimulation at later timepoints. (A) WT (left) and *Grk3-/-* (right) BmMSCs were stimulated with 1 μM S1P for designated times for analysis of ERK1/2 signaling; phospho-ERK (pERK) and total ERK (tERK). Representative blot, n=4. The pERK/tERK ratio for each blot was quantified via densitometry for **(B)** early-stage ERK signaling at 0, 1, and 5 minutes and **(C)** late-stage ERK signaling at 15, 30, and 60 minutes. Data represent mean ± SEM, n=4. ** $P \leq 0.01$

GRK3 recruits β-arrestin to the C-terminus of S1PR1 and affects S1PR1 internalization.

While BmMSCs express S1PRs1-3 [113-115], the intracellular signaling, function, and regulation of each specific receptor on BmMSCs is still being defined. Recent studies have shown stimulatory BmMSC function through ligation of S1PR1 and S1PR3 in contrast to inhibition of function after stimulation through S1PR2 [114]. We hypothesized our enhanced GRK3-regulatory phenotype may be mediated via S1PR1, since our *Grk3-/-* BmMSCs phenotype showed enhanced proliferation and prolonged ERK1/2 signal activation

analogous to previously reported S1PR1 activation in adipose-derived mesenchymal stem cells [78] and S1PR1 was more readily detected on WT and *Grk3-/-* BmMSCs by protein lysate analysis and flow cytometry (**Appendix 6**), in comparison to S1PR3 that was minimally detected (**Appendix 7**).

We hypothesized that GRK3 could be involved in S1PR1 β-arrestin recruitment—a protein necessary for GPCR internalization and another pathway of GPCR regulation in addition to receptor desensitization. Using the modified-TANGO assay [96, 116] with GRK2 as a positive control [79], we demonstrated GRK3 recruits β-arrestin to the C-terminus of S1PR1 (**Figure 2.6A**). We next hypothesized that receptor internalization was an additional means of GRK3/S1PR1 regulation, and thus S1PR1 internalization would be impaired in *Grk3-/-* BmMSCs. In S1P-activated receptor internalization assays, S1PR1 surface receptor expression by flow cytometry was prolonged over time in *Grk3-/-* BmMSCs compared to controls (**Figure 2.6B**), suggesting a defect in β-arrestin-mediated internalization.

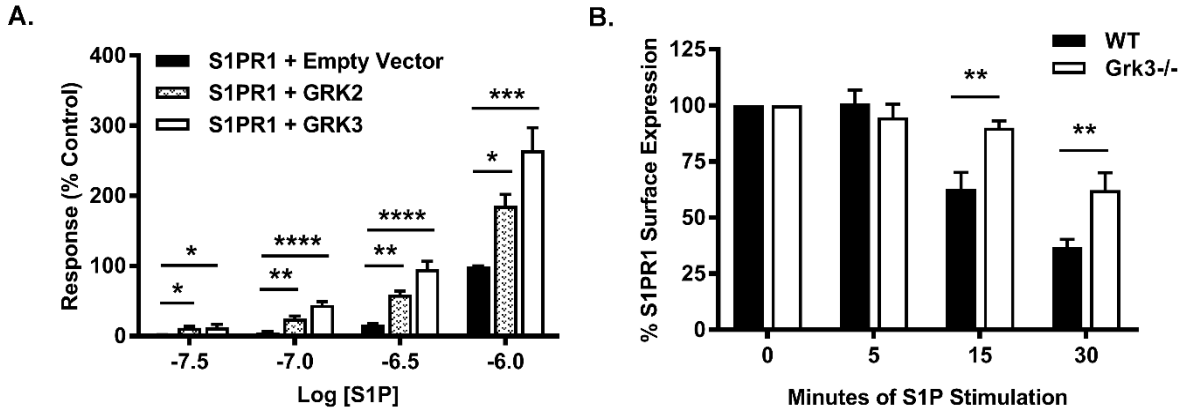


Figure 2.6. GRK3 recruits β-arrestin to the C-terminus of S1PR1 and affects S1PR1 internalization. (A) HTLA cells transfected with S1PR1-TCS-tTA receptor construct and empty vector (negative control), GRK2 (positive control), or GRK3 plasmids and stimulated with 1 μM S1P for detection of β-arrestin recruitment response via luminescence. Data represent mean ± SEM, n=6. **(B)** Sphingosine-1-phosphate receptor 1 (S1PR1) surface expression as measured by median fluorescence intensity compared to unstimulated cells (0 min) by flow cytometry. WT and *Grk3-/-* BmMSCs were stimulated with 1 μM S1P at designated timepoints. Data represent mean ± SEM, n=5. *P≤0.05 **P≤0.01 ***P≤0.001 ****P≤0.0001

Discussion

Mesenchymal stem cells are under active investigation for use in regenerative medicine and cell therapy; therefore, there is an essential need for better cellular characterization and understanding of their signaling, function, and regulation [117-119]. Challenges within the field include *ex vivo* expansion studies utilizing different tissue-derived sources of cells, growth media conditions, serum sources and lots, seeding densities, and sample variations [117, 120], which can be potential confounders in interpreting data. To reduce such confounders, we utilized media previously published to best support BmMSCs from C57BL/6 mice [29], maintained the same serum sources and lots throughout all studies, seeded and cultured cells at comparable densities, and minimized sample-variation findings by design and testing of several different BmMSC isolations from WT and *Grk3-/-* mice.

Here, we demonstrate that BmMSCs deficient in *Grk3* more readily differentiate into osteoblasts upon osteogenic supplementation (**Figure 2.1A-C, Appendix 2**), produce more CXCL12 (**Figure 2.2A**), and proliferate more *ex vivo* (**Figure 2.2B**). We hypothesize that these *in vitro* findings may additionally contribute toward mechanisms underlying the enhanced hematopoiesis phenotype that we observed in the *Grk3-/-* mouse [55]. Previous studies have demonstrated depletion of CXCL12 or total numbers of mesenchymal stem cells decrease the bone marrow hematopoietic stem cell pool and repopulating activity [32-34]. Thus, taken together, we might conclude the enhanced numbers of HSPCs observed in the *Grk3-/-* mouse bone marrow [55] could be partially attributed to the *Grk3-/-* BmMSC enhanced phenotypes described here. However, we recognize that selective *Grk3* gene targeting of mesenchymal stem cells and/or osteoblasts *in vivo* would be necessary to definitively conclude this, which are the goals of future studies.

Here, we demonstrate *Grk3-/-* BmMSC cultures had higher levels of CXCL12 at baseline upon equal seeding densities, and one interpretation is that *Grk3-/-* BmMSCs have an enhanced CXCL12 secretory function, such as has been described of CAR stromal cells in the bone marrow [33]. However, our study also detected enhanced proliferation of *Grk3-/-* BmMSCs at later timepoints (**Figure 2.2B**). This suggests the levels of CXCL12 detection at most time points are likely due to increased number of *Grk3-/-* BmMSCs in culture.

Despite increased proliferation and enhanced osteoblast differentiation during *ex vivo* supplementation, neither young (8-12 week-old) nor aged (17-20 month-old) *Grk3-/-* mice show enhancement of mature trabecular bone formation *in vivo* (**Figure 2.3A-D**). Possible explanations of this finding may be attributed to our *Grk3-/-* conventional knockout mouse model, where there may be other compensatory cellular interactions at play. Another conclusion to be considered is that GRK3 deficiency within niche BmMSCs may have more pronounced effects on hematopoiesis as opposed to mature bone development *in vivo*. Indeed, others have shown that early mesenchymal progenitors that produce high levels of CXCL12 are required for hematopoietic stem cell maintenance [33] as opposed to mature osteoblasts, which would be supportive of this conclusion. Additionally, *Grk3* expression levels have been found to be either undetectable or minimally expressed in osteoblastic cell lines and osteoblasts [121, 122], which could also explain why differences were not observed in trabecular bone *in vivo*.

To test whether GRK3 may regulate S1P-stimulated S1PR, our studies utilized sphingosine kinase inhibitor (SKI) to efficiently reduce the active S1P ligand in extended time-course studies and thereby block ligand activation for all S1PR family members expressed on BmMSCs (**Figure 2.4, Appendix 6 and 7**). These results did show that the

enhanced proliferation and osteogenic differentiation of *Grk3-/-* BmMSCs can be reduced to WT levels with effective removal of the S1PR ligand. Of note, we also tested specific S1PR antagonism targeting S1PR1 and S1PR3 [123]. However, we observed BmMSC cellular toxicity in our extended time-course studies while using similar concentrations of vehicle (DMSO) alone that was present in the solubilized S1PR1 and S1PR3 antagonist (VPC23019, Cayman Chemical) group (**Appendix 8**). Consequently, our culture studies with BmMSCs and S1PR antagonism could not identify which S1PR subtype expressed on BmMSCs elicit the enhanced functionality due to concerns about cell viability.

GRK3 is a ubiquitously expressed kinase that regulates a number of GPCRs, including parathyroid hormone receptor (PTHR) [124]. Although our cellular characterization studies suggest loss of GRK3 function affects S1PR signaling leading to enhanced BmMSC functionality *ex vivo*, we considered GRK3 regulation of parathyroid hormone/parathyroid hormone receptor (PTH/PTHR), since PTH/PTHR has been linked to osteoblast differentiation and enhanced hematopoiesis [37, 38]. However, ligand PTH is not readily available in *ex vivo* cultures, and PTH-R was absent from a RNA-seq database evaluation of readily expressed GPCRs on (human) BmMSCs (ENCODE GEO accession: GSE90273) [125]. Alternatively, the RNA-seq database showed S1PR expression on BmMSC, and it is well established that S1P is readily available in horse and fetal bovine serum (i.e.: BmMSC culture media) [109].

Contrary to our finding, Hosogane et al. proposed CXCL12 regulates bone morphogenetic protein 2-induced osteogenic differentiation of primary mesenchymal stem cells demonstrated in part by treatment with AMD3100 (CXCR4 antagonist) to show reduction of osteogenic differentiation [126]. However, our data show low levels of CXCR4

mRNA expression detected on *ex vivo* murine BmMSCs (**Appendix 3**) and functional tests with CXCL12 stimulation or CXCR4 signaling inhibition with AMD3100 resulted in no effect (**Appendix 4**). While CXCR4 is readily detected on human BmMSCs [127, 128], basal level CXCR4 on murine BmMSCs in culture have been less readily detected [129]. Other possible explanations for outcome differences may be that the Hosogane et. al experimental sub-populations were different based on isolation and/or cell surface marker expression and/or differences in AMD3100 concentration.

To investigate whether the enhanced functionalities of *Grk3-/-* BmMSCs were due to changes in downstream signaling, we tested the ERK signaling pathway since previous studies suggest ERK activation promotes both mesenchymal stem cell [111] and pre-osteoblastic cell line [112] osteogenic differentiation, as well as mesenchymal stem cell proliferation [78]. Our previous work has shown loss of GRKs elicit sustained ERK signaling due to prolonged GPCR surface expression [55]; therefore, we hypothesized loss of GRK3 in BmMSCs may elicit sustained ERK activation through S1P/S1PR. Our results show that stimulation with S1P prolongs *Grk3-/-* BmMSC ERK1/2 signaling (**Figure 2.5**), and thus suggest S1P-ligand induced signaling of *Grk3-/-* BmMSCs may elicit enhanced cell functions mediated through the ERK1/2 signaling pathway.

S1PR1 and S1PR3 have been shown to stimulate BmMSC function [114], and S1PR1-mediated enhanced proliferation has been demonstrated in adipose-derived mesenchymal stem cells [78]. Furthermore, since we found S1PR1 more readily expressed on BmMSCs compared to S1PR3 (**Appendix 7**), we focused our receptor signaling regulation studies on S1PR1 to determine whether GRK3 enhances recruitment of β-arrestin, a protein necessary for GPCR internalization and another pathway of GPCR regulation in addition to

receptor desensitization by GRKs. Previous studies have shown GRK2, which shares high homology with GRK3, phosphorylates the C-terminus of S1PR1 [130, 131], and activated S1PR1 recruits β-arrestin for receptor internalization [131, 132]. Furthermore, cells deficient in GRK2 have high levels of surface S1PR1 [79]. Consequently, we compared GRK2 as a positive control for our modified-TANGO assay. We demonstrate GRK3 enhances β-arrestin recruitment to the C-terminus of S1PR1, as does GRK2 (**Figure 2.6A**). Furthermore, our continued investigation shows BmMSCs deficient in GRK3 had decreased S1PR1 internalization (i.e. more surface expression) (**Figure 2.6B**). Taken together, we propose loss of GRK3 affects S1PR1 regulation and internalization and elicits prolonged surface receptor expression that may sustain late-phase ERK1/2 activation (**Figure 2.5**).

Conclusion

In summary, our study reveals how *Grk3-/-* BmMSCs have enhanced functions of osteogenic differentiation, CXCL12 production, and proliferation *ex vivo*, and our data suggest GRK3 regulates these functionalities through S1P/S1PR (**Figure 2.7A**). We demonstrate GRK3 recruits β-arrestin, a protein necessary for receptor internalization, to the C-terminus of S1PR1, and BmMSCs lacking GRK3 regulation have impaired S1PR1 internalization and prolonged ERK1/2 signaling (**Figure 2.7B**). Our work suggests GRK3 regulates S1PR on BmMSCs, and we propose lack of such regulation affects BmMSC functionality.

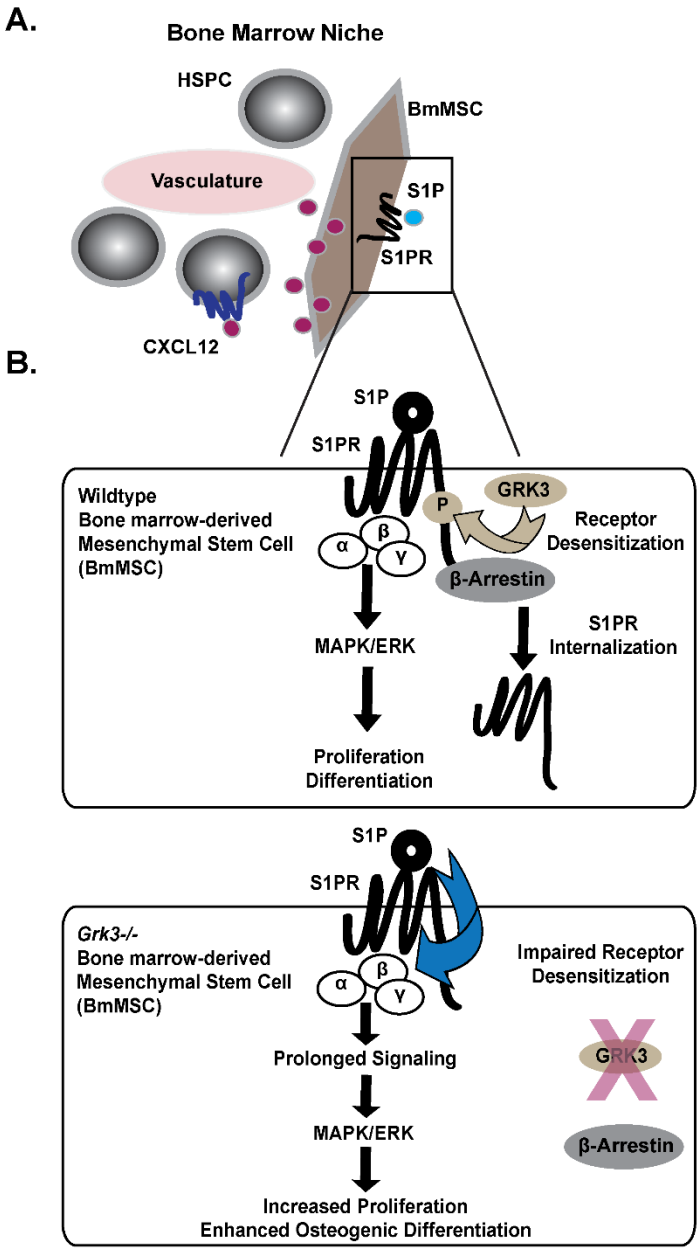


Figure 2.7. Proposed model for GRK3-deficient BmMSCs within the niche. (A) Our studies suggest BmMSCs deficient in GRK3 secrete more CXCL12 and proliferate more rapidly, which may increase support to hematopoietic stem and progenitor cell (HSPC) development. **(B, top)** We propose GRK3 desensitizes S1PR and recruits β-arrestin to affect ERK1/2 signaling and receptor internalization. **(B, bottom)** *Grk3-/-* BmMSCs have enhanced functions of proliferation and osteogenic differentiation, and this may be mediated through decreased S1PR1 internalization (i.e. more surface expression) and prolonged late-phase ERK1/2 activation.

CHAPTER 3: G PROTEIN-COUPLED RECEPTOR KINASE 3 REGULATES HEMATOPOIETIC STEM-PROGENITOR CELL FUNCTION MEDIATED THROUGH CXCL12/CXCR4[3]

Overview

Hematopoietic cell transplantation (HCT) treats malignant and non-malignant diseases; however, patient immune reconstitution after HCT is variable and associated with morbidity and mortality. Thus, HCT improvements are warranted for the enhancement of therapeutic outcomes.

Hematopoietic stem cells (HSCs) are primitive cells that differentiate into all the hematopoietic lineages (erythroid, myeloid, lymphoid) to reconstitute the immune system after HCT. The goal of this study is to better understand HSC and progenitor cell function. Mice deficient in G protein-coupled receptor kinase 3 (GRK3), an intracellular kinase that regulates G protein-coupled receptors, have a hypercellular bone marrow with increased hematopoietic stem-progenitor cells (HSPCs) and oligopotent progenitors, as well as increased leukocytes in the blood. We hypothesize GRK3 regulates bone marrow HSPC numbers and thus, impacts hematopoiesis. We predict if GRK3 is absent, then HSPC numbers will increase, which may positively influence HCT.

[3]The work in chapter 3 has not been submitted or published as a research manuscript. The project work is ongoing. Contributors to this project include: Brozowski JM, Timoshchenko RG, Serafin DS, and Tarrant TK.

Figure 3.1 reused with copyright permission granted by FASEB/Journal of Leukocyte Biology on November 8, 2017. JM Brozowski made illustration Figure 3.2 and performed experiments for Figure 3.3 (JMB, DSS), Figure 3.4, Figure 3.5 (TKT, RGT, JMB), Figure 3.6, Figure 3.7 and appendices 9-12.

To test whether GRK3 deficiency enhances hematopoietic cell function *in vivo*, we utilized a short-term transplantation assay, termed *colony forming unit-spleen (CFU-S)* assay. In the CFU-S assay, transplanted HSPCs with self-renewal capabilities form clonal colonies on the spleen, which can be enumerated to measure extramedullary hematopoiesis. Our data suggest hematopoiesis of GRK3-deficient (*Grk3-/-*) HSPC is enhanced since transplanted *Grk3-/-* Lin^- $Sca1^+$ $c\text{-}Kit^+$ (LSK) HSPCs or *Grk3-/-* whole bone marrow increase colony counts on the explanted spleen in comparison to wildtype (WT) controls.

Our previous studies have demonstrated GRK3 regulates CXCL12/CXCR4 signaling interactions and functional outcomes on mature hematopoietic cells. Since CXCR4 is highly expressed on HSCs, common myeloid progenitors (CMPs), granulocytic monocytic progenitors (GMPs), and B cells (cells also increased in number in our *Grk3-/-* mouse), we tested whether *Grk3-/-* HSPCs would have increased colony counts when grown in these lineage-specific pathways, and if it was dependent upon CXCL12/CXCR4 interactions. We utilized the well established *ex vivo colony forming unit-granulocytic and monocytic* (CFU-GM) and *-pre-B* (CFU-Pre-B) assays to assess numbers and types of progenitor colony formations in standard growth conditions and in the presence of CXCR4 agonist (CXC12) and antagonist (AMD3100). Both *Grk3-/-* hematopoietic myeloid and lymphoid colony counts *ex vivo* increased with CXCR4 ligand stimulation (CXCL12), and *Grk3-/-* CFU-GM colony counts reduced to WT levels with CXCR4 antagonist treatment (AMD3100).

Taken together, our *in vivo* and *ex vivo* CFU data suggest GRK3 regulates bone marrow HSPC numbers, and this is at least in part, mediated through CXCL12/CXCR4 stimulation. Given the positive correlation between the stem cell graft CFU colony counts

and the success rate of immune reconstitution after HCT, we propose the enhanced CFU readouts from *Grk3-/-* HSPCs may positively influence HCT.

Significance Statement

HCT is a treatment used to replenish the immune system by transferring donor hematopoietic cells into an immunologically-destroyed irradiated recipient. Erythroid and myeloid reconstitution occurs within weeks; however, lymphoid lineage reconstitution may be delayed for years after HCT. The delayed immune reconstitution jeopardizes immunologically compromised patients and makes them vulnerable to opportunistic infections. The delay in immune reconstitution may benefit from increased stem cell numbers, enhanced stem cell proliferation, or improved stem cell homing, all of which potentiate cellular engraftment and hematopoiesis. Potentiated hematopoiesis and stem cell engraftment is demonstrated by *Grk3-/-* HSPCs— specifically since transplanted *Grk3-/-* HSPCs have increased CFU numbers. We further show *Grk3-/-* CFU numbers can be affected through CXCR4 agonist and antagonist treatment. Thus, we propose GRK3 deficiency enhances HSPC numbers, which may potentially influence immune reconstitution after HCT.

Introduction

HCT treats hematological cancers, immune deficiencies, autoimmunity, and other diseases by replacing the patients' compromised immune system with a functional immune system [133]. Replenishing the immune system is possible since the transplanted HSCs, which differentiate into the erythroid, myeloid, and lymphoid hematopoietic lineages, are

self-renewing and reconstitute the immune system through proliferation and differentiation after HCT. Erythrocyte, granulocyte, monocyte, and megakaryocyte/platelet lineage reconstitution takes place within the first weeks after HCT; however, complete immune reconstitution of the B and T lymphocytes can be delayed for years after transplantation [134, 135]. Delayed immune reconstitution compromises the immune system and increases patient susceptibility to opportunistic infections. Therefore, the effectiveness of HCT is variable and associated with morbidity and mortality.

Understanding stem cell function may identify mechanisms to improve immune reconstitution after HCT. Cellular engraftment from HCT may be affected by increased stem cell numbers, enhanced stem cell proliferation, or improved stem cell homing [136]. Therefore, we propose immune reconstitution after HCT may benefit from increased stem cell numbers, enhanced stem cell proliferation, or improved stem cell homing. Since *Grk3-/-* mice have increased HSPC numbers in the bone marrow and increased numbers of leukocytes in the blood, we aimed to examine the effects of GRK3 on HSPC function and engraftment in models of HCT.

Engraftment and hematopoiesis of HSPCs occur in the bone marrow microenvironment, termed *niche*, or also in extramedullary hematopoietic sites such as the spleen. The niche supports HSPC development by interactions with the niche stromal mesenchymal stem cells, osteoblasts, and CXCL12-abundant reticular (CAR) cells, which secrete soluble factors, importantly chemokine CXCL12 [32, 33]. CXCL12 is a ligand for GPCR CXCR4 on HSPCs and involved in stem cell retention, migration, and proliferation functionality [31, 137]. We and others have demonstrated that CXCR4 on mature

hematopoietic cells is regulated by GRK3 [55, 106]; however, it is unknown whether GRK3 has a functional effect mediated by CXCR4 on HSPCs.

We observed *Grk3-/-* mice, in comparison to wildtype (WT) control mice, have increased total white blood cells in the peripheral blood and a hypercellular bone marrow with increased LSK HSPCs and oligopotent progenitors [55]. Therefore, we hypothesize bone marrow HSPC numbers are regulated by GRK3 and mediated through CXCL12/CXCR4 interactions, and we predict absence of GRK3 increases CXCL12/CXCR4 function resulting in increased numbers of HSPCs.

Materials and Methods

Animals.

Wildtype (WT) C57BL/6 and GRK3-deficient (*Grk3-/-*) age-matched (8-12 week-old) mice were used under standard IACUC-approved protocols in the AAALAC-accredited vivarium of UNC, and care of animals was in accordance with institutional guidelines. The *Grk3-/-* mouse strain was provided by Dr. Robert J. Lefkowitz (Duke University) and backcrossed >12 generations on the C57BL/6 background.

Flow cytometry bone marrow lineage analyses.

Femur and tibia of WT and *Grk3-/-* mice at 8-12 weeks were crushed using mortar and pestle for bone marrow extraction. After red blood cell (RBC) lysis, white blood cells (WBCs) were counted using hemocytometer and trypan blue exclusion to obtain a total bone marrow cell count. WBCs were stained with anti-CD3, CD4, CD8, Ter119, CD45R (B220), Ly-6G, CD11b (PE-Cy5) for lineage positive exclusion and stained for experimental marker

analysis of c-Kit (APC-e780, clone 2B8), Sca1 (PE-Cy7, clone D7), CD34 (FITC, clone RAM34), CD16/CD32 (PE, clone 93), CD150 (Pacific Blue, clone TC15-12F12.2 Biolegend), CD48 (FITC, clone HM48-1), and Interleukin-7 receptor-α (PE, IL-7Rα, clone A7R34) and acquired on the LSRII or CYAN. All antibodies were purchased from eBioscience unless otherwise noted. Appropriate isotype controls for each marker were analyzed and used to set gates using FlowJo version X. Hematopoietic stem and progenitor populations were identified using markers and gating strategies previously published (LT-HSC [18-20], ST-HSC [18, 21], LSK HSPC/CMP/GMP/MEP [138], CLP [139-141]).

***In vivo* colony forming unit-spleen (CFU-S) assays.**

For LSK HSPC transplants, recipient mice were irradiated with 950 rads (9.5 Gy) with a split dose and rested at least 3 hours. Donor bone marrow was flushed, RBCs lysed, and depleted of lineage positive cells via EasySep^{TM} mouse hematopoietic progenitor cell isolation kit (19856, Stem Cell Technologies). Lin$^-$ cells were stained with c-Kit (PE, clone 2B8 eBioscience) and Sca1 (APC, clone D7, eBioscience) for at least 30 minutes and sorted using the MoFlo or MoFlo XDP cytometer. Cells were injected by tail vein of 0.1 cc (200 LSK HSPCs in DPBS). Mice were treated with Sulfamethoxazole/Trimethoprim in water supply (antibiotic water) until assay endpoint. Spleens were fixed on day 12 in Telly's fixative and nodule colony formations were counted.

For whole bone marrow transplants, recipient mice were irradiated with 1,000 rads (10 Gy) and rested 4-6 hours. Donor bone marrow was flushed, RBCs lysed, and washed with DPBS. Cells were injected by tail vein of 0.1 cc ($5x10^4$ cells in DPBS). Spleens were fixed on day 8 in Telly's fixative and nodule colony formations were counted.

***Ex vivo* colony forming unit-granulocytic and monocytic (CFU-GM) progenitor cell assay.**

Methocult (GM-M3534, Stem Cell Technologies) was supplemented with mouse CXCL12 (Shenandoah) at 50 ηg/mL alone or in combination with AMD3100 (Sigma) at 1,000 ηg/mL in conical tubes, and 300 µL of $1x10^5$ bone marrow cells/mL were added to 3 mL of methocult and vortexed for 10 seconds to ensure thorough mixing of cells into the viscous methocult reagent. Methocult (containing cells) was plated onto $35mm^2$ petri dishes. Plates were incubated at 37°C at 5% CO_2. Colonies were counted on day 12, as per manufacturer's instructions.

***Ex vivo* colony forming unit-pre-B (CFU-Pre-B) progenitor cell assay.**

Methocult (M3630, Stem Cell Technologies) was supplemented with mouse CXCL12 (Shenandoah) at 50 ηg/mL alone or in combination with AMD3100 (Sigma) at 1,000 ηg/mL in conical tubes, and 300 µL of $2x10^6$ bone marrow cells/mL were added to 3 mL of methocult and vortexed for 10 seconds to ensure thorough mixing of cells into the viscous methocult reagent. Methocult (containing cells) was plated onto $35mm^2$ meniscus-free petri dishes (SmartDish™, Stem Cell Technologies). Plates were incubated at 37°C at 5% CO_2. Colonies were counted on day 7, as per manufacturer's instructions.

Statistical analyses.

All data were graphed utilizing GraphPad Prism v.7 and statistically evaluated using GraphPad Prism. Student's t-test compared two independent groups (WT and *Grk3-/-*) for subset bone marrow lineage analyses via flow cytometry, LSK HSPC CFU-S transplant, and

whole bone marrow CFU-S transplant assays. Taking into consideration strain (WT and *Grk3-/-*) and multiple treatment groups, the CFU-GM and CFU-Pre-B assays were statistically analyzed using a two-way ANOVA with Tukey's multiple comparisons test [97, 99].

Results

Grk3-/- HSPC and oligopotent progenitor cell numbers are increased within the bone marrow.

In a previous study, we observed *Grk3-/-* mice have a hypercellular bone marrow with increased numbers of total bone marrow cells ([55], **Figure 3.1B, C)**. A basic subset analysis showed significantly increased LSK HSPCs and oligopotent progenitors ([55], **Figure 3.1D)**. Here, we expanded our lineage subset analyses (**Figure 3.2**) to include short-term HSCs (ST-HSCs), myeloid progenitor population (MPPs), and common lymphoid progenitors (CLPs). Due to the varying views on what parent population CLPs may originate [139-141], we evaluated CLPs that are believed to arise from LSK HSPC population, as well as those believed to arise from the Lin^{-} $Sca1^{lo}$ $c\text{-}Kit^{lo}$ population. Our preliminary findings suggest *Grk3-/-* mice have an increase trend of total numbers of LSK HSPCs **(Figure 3.3A)**, common myeloid progenitors (CMPs) and granulocytic monocytic progenitors (GMPs) **(Figure 3.3B)**, reflective of our previous report ([55], **Figure 3.1D**). However, our expanded subset analyses also showed an increase trend in total numbers of MPPs **(Figure 3.3B)**, $Sca1^{lo}$ $c\text{-}Kit^{lo}$ cells (**(Figure 3.3C)** and CLPs **(Figure 3.3C, D)**. However, long-term HSCs (LT-HSCs) and ST-HSCs were comparable between WT and *Grk3-/-* mice **(Figure 3.3A)**, suggesting GRK3 may play a more pertinent role on HSPCs than the most primitive LT- and

ST-HSCs. Interestingly, the percent of populations remained comparable throughout all lineages **(Appendix 9)**.

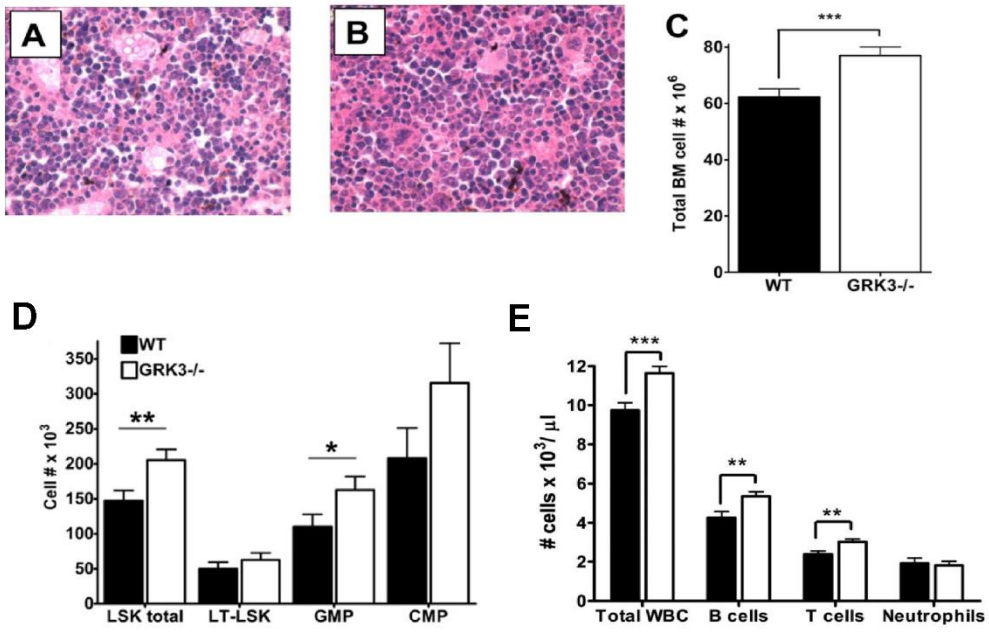


Figure 3.1. G protein-coupled receptor kinase 3 deficient (*Grk3-/-*) mice have enhanced hematopoiesis. Representative femur bone marrow histopathology from **(A)** WT and **(B)** *Grk3-/-* mice (40X). **(C)** Total viable bone marrow cell numbers counted with trypan blue exclusion. Data represent mean ± SEM, n=14 (WT) and n=13 (*Grk3-/-*). **(D)** Enumeration of bone marrow-derived lineage negative (Lin^-) hematopoietic cell subsets. Data represent mean ± SEM, n=13 (WT) and n=12 (*Grk3-/-*). LSK: Lin^- $Sca1^+$ $c\text{-}Kit^+$; LT-LSK (LT-HSC): Lin^- $Sca1^+$ $c\text{-}Kit^+$ $CD48^-$ $CD150^+$; GMP: Lin^- $Sca1^-$ $c\text{-}Kit^+$ $CD16/32^+$ $CD34^+$; CMP: Lin^- $Sca1^-$ $c\text{-}Kit^+$ $CD16/32^-$ $CD34^+$. **(E)** Peripheral blood analysis enumerating leukocyte subsets from WT and *Grk3-/-* mice using leukocyte markers $CD45^+$ and further subset identification with $B220^+$ (B cells), CD3 (T cells), and Ly-6G (neutrophils). Data represent mean ± SEM, n=23 (WT) and n=25 (*Grk3-/-*). * $P \leq 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$ *Figures from Tarrant et al. J Leukoc Biol. 2013 Dec; 94(6): 1243-1251. Copyright © 2013 Society for Leukocyte Biology. Reused with copyright permission. Figure editing (re-arrangement only) permission granted by corresponding author.*

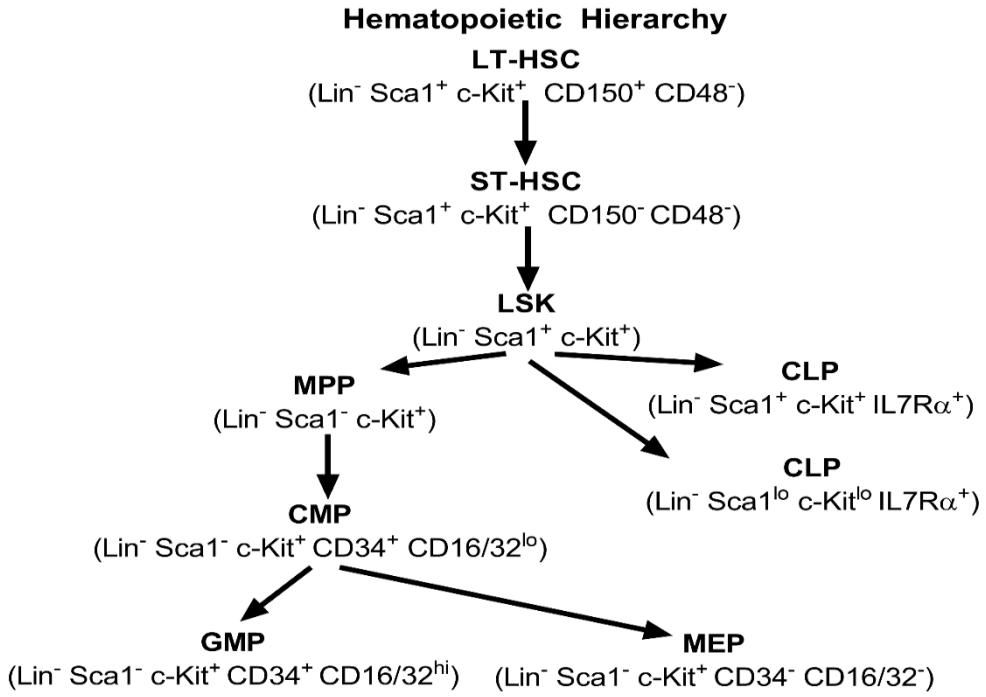


Figure 3.2. Hematopoietic hierarchy of stem and progenitor cell differentiation within the bone marrow. Long-term hematopoietic stem cell (LT-HSC); Short-term hematopoietic stem cell (ST-HSC); Lineage negative (Lin^-) $Sca1^+$ $c\text{-}Kit^+$ hematopoietic stem-progenitor cell (includes LT-HSC and ST-HSC); Myeloid progenitor population (MPP); Common myeloid progenitor (CMP); Granulocytic monocytic progenitor (GMP); Myeloid erythroid progenitor (MEP); Common lymphoid progenitor (CLP).

Transplantation of *Grk3-/-* LSK HSPCs increases hematopoietic stem cell colonies on the spleen.

Since cellular engraftment from HCT may be improved by increased stem cell numbers, proliferation, and/or homing [136], and our flow cytometry data suggest GRK3 deficiency affects the hematopoietic hierarchy (**Figure 3.2**) with increased numbers of LSK HSPCs (**Figure 3.1D and 3.3A**), we wanted to further test whether GRK3 deficiency specifically enhances LSK HSPC function *in vivo*. Thus, we utilized the short-term transplantation assay, termed *colony forming unit-spleen (CFU-S) assay*. Unlike humans, mice commonly undergo both hematopoiesis within the bone marrow and extramedullary

sites, such as the spleen. Due to murine extramedullary hematopoiesis in the spleen, transplanted cells with self-renewal capabilities home to the spleen and form clonal colonies, which can be easily visible and enumerated by eye. In the CFU-S assay, a single cell is responsible for each spleen colony, suggesting the transplanted cells undergo self-renewal and expansion [6]. Therefore, there is a linear relationship between the number of

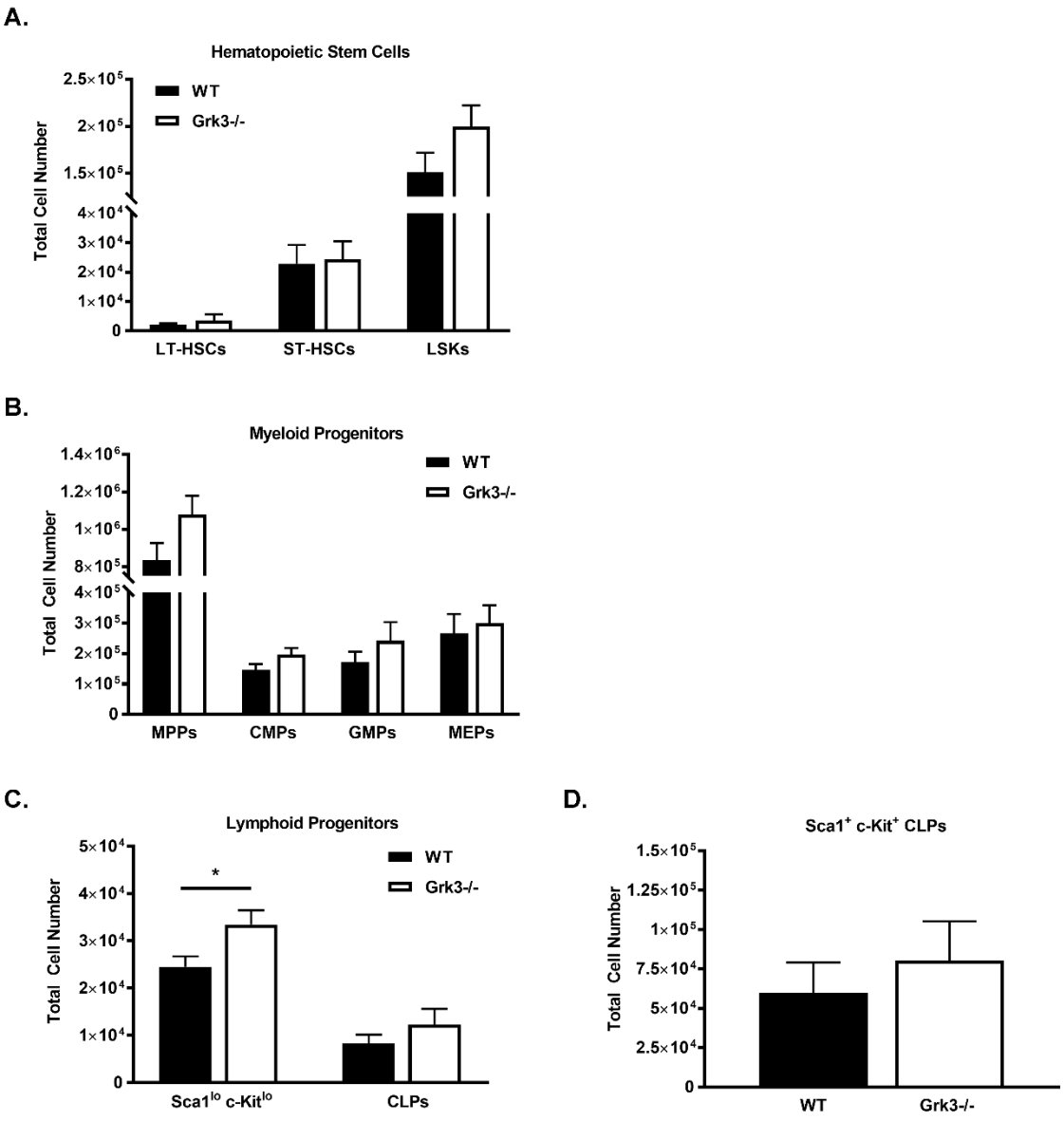


Figure 3.3. *Grk3-/-* hematopoietic stem and progenitor cell numbers are increased within the bone marrow. (A) Total cell numbers of hematopoietic stem cells, **(B)** myeloid progenitors, **(C,D)** lymphoid progenitors from WT and *Grk3-/-* femurs enumerated by flow cytometry. Data represent mean ± SEM, n=8. * $P \leq 0.05$

transplanted cells and the colony spleen count [6]. However, when we transplanted identical numbers of donor WT or *Grk3-/-* LSK HSPCs into WT recipient mice, the *Grk3-/-* LSK HSPC donor group had increased stem cell colony counts on the spleen at day 12, in comparison to the WT LSK HSPC donor group **(Figure 3.4)**.

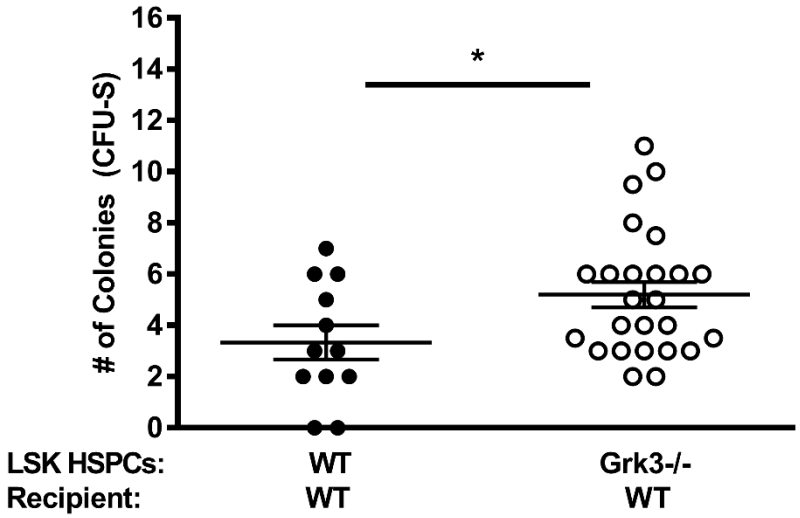


Figure 3.4. Transplantation of *Grk3-/-* LSK HSPCs increases hematopoietic stem cell colonies on the spleen. Colony forming unit-spleen (CFU-S) assay day 12 harvest selectively measures individual HSC homing to the spleen and proliferation. Each dot represents the number of colonies on the spleen of one mouse. Data represent mean ± SEM, n=12 (WT) and n=25 (*Grk3-/-*). * $P \leq 0.05$

Transplantation of *Grk3-/-* bone marrow cells increases hematopoietic progenitor colony counts on the spleen.

Interestingly, spleen colonies evident at earlier timepoints are believed to be derived from progenitors rather than from HSPCs, which present at later timepoints [142]. Since our subset analysis data demonstrated *Grk3-/-* mice had increased HSPCs *and* oligopotent progenitor cells, we wanted to next test whether GRK3 deficiency specifically enhances

progenitor cell function *in vivo*. When we transplanted identical numbers of donor WT or *Grk3-/-* bone marrow cells into WT recpients, the *Grk3-/-* donor group had increased progenitor colony counts on the spleen at day 8, in comparison to WT donors **(Figure 3.5)**.

CXCL12 stimulation increases hematopoietic granulocytic and monocytic-GM progenitor colony formation (CFU-GM) of *Grk3-/-* bone marrow cells.

Our results thus far suggest *Grk3-/-* mice have enhanced hematopoiesis in the bone marrow **(Figure 3.1 and 3.3)** and transplantation of *Grk3-/-* LSK HSPCs or whole bone marrow increase HSPC numbers on the spleen (**Figure 3.4 and 3.5**); however, the mechanism of the observed phenotype is undefined. Since (1) previous findings demonstrate GRK3 regulates CXCL12/CXCR4 on mature hematopoietic cells [55], and (2) CXCR4 is highly expressed on HSCs, CMPs, GMPs, and B cells [61], we hypothesized GRK3 may be regulating CXCL12/CXCR4 on immature hematopoietic cells to induce this phenotype.

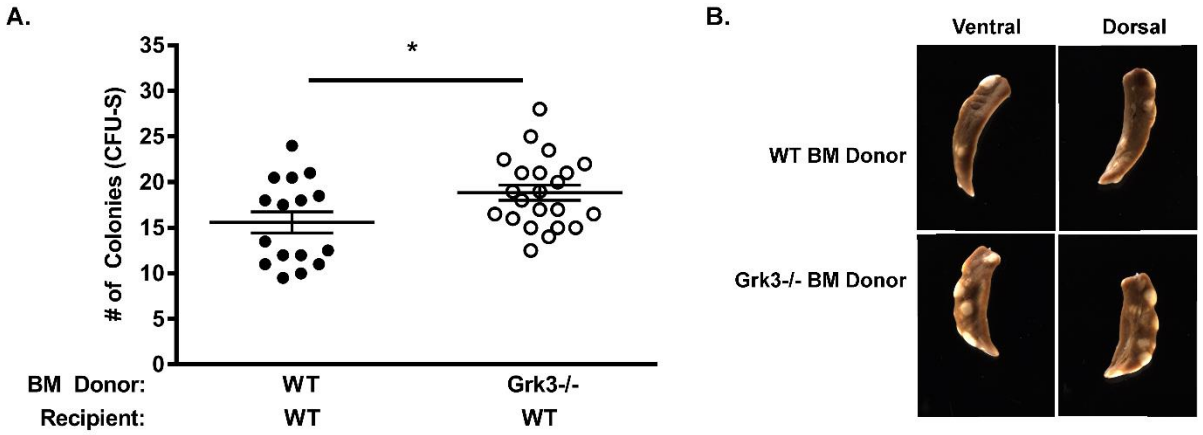


Figure 3.5. Transplantation of *Grk3-/-* bone marrow cells increases hematopoietic progenitor colony counts on the spleen. (A) Colony forming unit-spleen (CFU-S) assay day 8 harvest selectively measures hematopoietic progenitor cell homing to the spleen and proliferation. Each dot represents the number of colonies on the spleen of one mouse. **(B)** Representative images demonstrating that each colony or "white nodule" arose from a single self-renewal cell. Data represent mean ± SEM, n=16 (WT) and n=22 (*Grk3-/-*). * $P \leq 0.05$

Therefore, we utilized the well established *ex vivo* colony forming assay to assess myeloid colony formation (CFU-GM) in the presence of CXCR4 agonist CXCL12 (concentration curve available in **Appendix 10A**) and CXCR4 antagonist AMD3100. Our results demonstrate *Grk3-/-* granulocytic and monocytic colony counts were increased upon CXCL12 stimulation and decreased to WT levels upon treatment with AMD3100 **(Figure 3.6)**, suggesting the enhanced numbers of *Grk3-/-* myeloid progenitors is mediated, at least in part, through regulation of CXCL12/CXCR4.

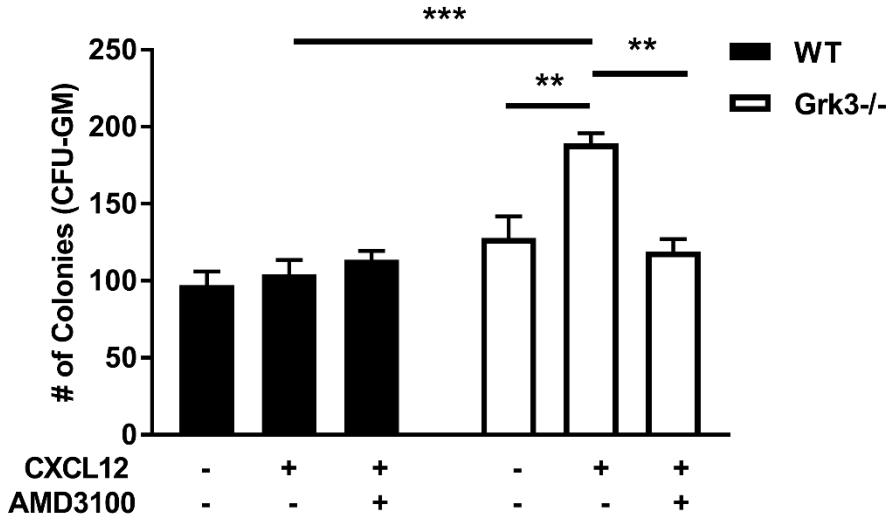


Figure 3.6. CXCL12 stimulation increases hematopoietic granulocytic and monocytic-GM progenitor colony formation (CFU-GM) of *Grk3-/-* bone marrow cells. Cells treated with ligand for CXCR4 (CXCL12, 50 ηg/mL) or both CXCL12 and antagonist for CXCR4 (AMD3100, 1,000 ηg/mL). Data represent mean ± SEM, n=3. ** $P \leq 0.01$ *** $P \leq 0.001$

CXCL12 stimulation increases hematopoietic lymphoid pre-B cell colony formation (CFU-Pre-B) of *Grk3-/-* bone marrow cells.

Next, we tested whether GRK3 regulation of CXC12/CXCR4 is responsible for the enhanced lymphoid progenitor numbers by utilizing the well established *ex vivo* colony

forming assay to assess pre-B lymphoid colony formation (CFU-Pre-B) in the presence of CXCR4 agonist CXCL12 (concentration curve available in **Appendix 10B**) and CXCR4 antagonist AMD3100. Our results revealed *Grk3-/-* pre-B colony counts were slightly increased upon CXCL12 stimulation and decreased with treatment of AMD3100, albeit not to a WT level **(Figure 3.7)**, suggesting the enhanced numbers of *Grk3-/-* pre-B lymphoid progenitors may also be mediated, at least in part, through CXCL12/CXCR4 stimulation. We also observed CXCR4 antagonist treatment with AMD3100 inhibited the size (clonal proliferation) of the WT and *Grk3-/-* CFU-Pre-B colonies, though at comparable rates (**Appendix 11**). Interestingly, we have also previously shown *Grk3-/-* mice have increased numbers of mature B cells in the blood (**Figure 3.1E**), suggesting GRK3-deficiency may potentiate B cell lineage development; a process often delayed after HCT.

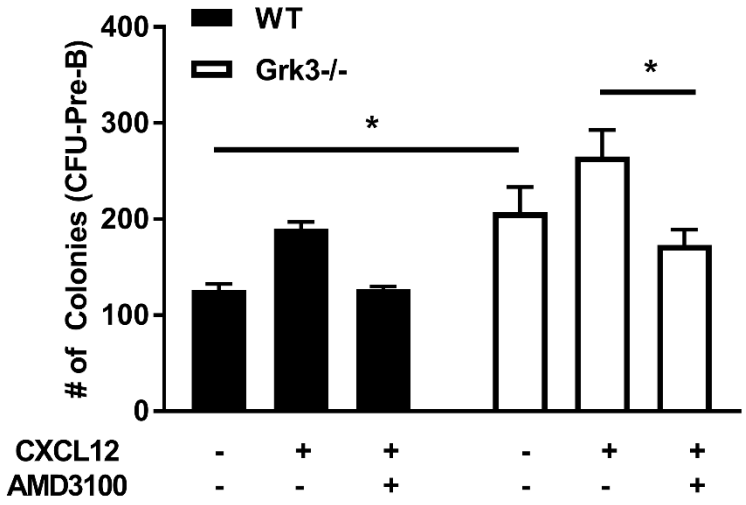


Figure 3.7. CXCL12 stimulation increases hematopoietic lymphoid pre-B cell colony formation (CFU-Pre-B) of *Grk3-/-* bone marrow cells. Cells treated with ligand for CXCR4 (CXCL12, 50 ηg/mL) or both CXCL12 and antagonist for CXCR4 (AMD3100, 1,000 ηg/mL). Data represent mean ± SEM, n=4. * $P \leq 0.05$

Discussion

HCT is the transfer of a stem cell graft to a patient whom has undergone myeloablative conditioning, i.e.: destruction of their immune system via irradiation. The stem cell graft (from bone marrow, peripheral blood or umbilical cord blood sources) contains HSCs that reconstitutes the patients' immune system. Although this clinical approach is used to treat various hematological malignancies and non-malignant diseases, HCT is also associated with morbidity and mortality due to toxicities, graft-versus-host disease, and infection [143]. HCT patients experience low levels of red blood cells, white blood cells, and platelets, termed *pancytopenia*, that lasts days to weeks after transplantation. Neutrophils are the first immune subset to recover within 2-4 weeks, followed by monocytes, natural killer cells, platelets, and RBCs, respectively. Interestingly, lymphocyte recovery can take months to years with initial recovery of CD8 T cells within 2-8 months, followed by B cells and then CD4 T cells. Thus, HCT patients are susceptible to opportunistic infections for at least one-year post-HCT due to the delay in immune reconstitution [143].

It is well established that immune reconstitution and successful patient HCT outcomes are contingent upon the number and quality of transplanted HSCs, which need to migrate to, engraft in, and repopulate the bone marrow [136]. Therefore, identifying therapeutic strategies that may increase stem cell numbers, migration, or proliferation, may enhance cellular engraftment and thus, immune reconstitution.

The G protein-coupled receptor (GPCR) CXCL12/CXCR4 pathway is the most essential chemotactic signaling mechanism involved in HSPCs [136, 144-146]. Bone marrow niche cells secrete CXCL12, which facilitate HSPC homing to the bone marrow via CXCR4; however, after myeloablative conditioning, CXCL12 expression is extremely low due to the

proteolytic environment. Therefore, there have been attempts to target and enhance CXCL12/CXCR4 chemotaxis, such as through *ex vivo* treatment of HSPCs with heat or prostaglandin E_2 (PGE2) that both increase CXCR4 expression, and PGE2 also enhances HSPC proliferation [147, 148]. Such studies have demonstrated manipulation of CXCL12/CXCR4 may enhance the functional quality of HSPCs.

Our previous studies have demonstrated GRK3, an intracellular kinase that phosphorylates GPCRs to desensitize the receptor for internalization, specifically regulates CXCL12/CXCR4 cellular functions on mature hematopoietic cells [55]. Furthermore, we observed mice deficient in GRK3 have a hypercellular bone marrow with increased HSPCs and oligopotent progenitors, as well as increased leukocytes in the blood ([55], **Figure 3.1, Figure 3.3**), suggesting GRK3 deficiency may enhance HSPC function *in vivo*.

Here, we demonstrate transplantation of *Grk3-/-* LSK HSPCs increases hematopoietic stem cell colonies on the spleen and transplantation of *Grk3-/-* bone marrow increases hematopoietic progenitor colonies on the spleen (*colony forming unit-spleen*, CFU-S) (**Figure 3.4 and 3.5)**. Due to the positive correlation between the stem cell graft CFU colony counts and the success rate of immune reconstitution, our preliminary findings suggest GRK3 deficiency may positively influence HCT through enhanced immune reconstitution. Similar findings were evident when HSPCs were treated with valporic acid, a histone deacetylase inhibitor, which also induced increased spleen colony formations and demonstrated enhanced HSPC engraftment, which is believed to be mediated through increased surface expression of CXCR4 and cellular proliferation [149]. Likewise, our previous study demonstrated hematopoietic cells deficient in GRK3 had sustained CXCR4 surface expression and enhanced migration to CXCL12 [55]. Therefore, while we

acknowledge GRK3 deficiency enhances spleen colony numbers, we recognize we have not addressed whether this may be due to enhanced homing and/or *in vivo* proliferation, which will need to be further evaluated.

Since CXCL12/CXCR4 is an essential signaling mechanism involved in HSPC function, we tested whether the increased *Grk3-/-* bone marrow numbers were due to the CXCL12/CXCR4 pathway. Using *ex vivo* CFU assays, we assessed number and types of oligopotent progenitor cells within our WT and *Grk3-/-* bone marrow. Our results suggest *Grk3-/-* bone marrow enhances hematopoietic myeloid (granulocytic and monocytic-GM progenitors) (**Figure 3.6)** and lymphoid (pre-B progenitors) (**Figure 3.7)** colony counts mediated via CXCL12/CXCR4 stimulation. Interestingly, we have also previously shown *Grk3-/-* mice have increased numbers of mature B cells in the blood (**Figure 3.1E**), suggesting GRK3 deficiency may potentiate B cell lineage development; a process often delayed after HCT.

Lastly, to test whether there may be potential differentiation-bias among WT or *Grk3-/-* bone marrow cells, all bone marrow subset percentages were also evaluated (**Appendix 9**), which showed comparable percentages among WT and *Grk3-/-* mice, suggesting no lineage skewing, just overall increased cell numbers. Further, we tested potential differentiation-bias *in vitro* by culturing LSK HSPCs in lineage-specific CFU culture conditions, though again, progenitor colony counts were comparable between WT and *Grk3-/-* (**Appendix 12**), suggesting no differentiation-bias among WT and *Grk3-/-* HSPCs.

Conclusion

In summary, our preliminary work thus far implicates the importance of GRK3 on HSPC function. Specifically, *Grk3-/-* hematopoietic cells result in increased HSPC CFU numbers. Further, we have demonstrated the increase of *Grk3-/-* HSPC numbers may be mediated through CXCL12/CXCR4. Due to the positive correlation between the CFU content of a stem cell graft and the success rate of immune reconstitution after HCT, we propose GRK3 deficiency may positively influence HCT, though further investigations are warranted.

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

Overview

The clinical utilization of hematopoietic cell transplantation (HCT) beginning in the 1950s combined with the scientific discoveries by Till, McCulloch, Friedenstein, and Schofield aided our initial understanding of the importance of the bone marrow microenvironment and the essential molecular and cellular interactions to support hematopoiesis. Subsequent cellular studies over the past 60 years have defined the intricacy of the niche, in large part due to the advancements in gene targeting and knockout murine models through homologous recombination techniques developed by Drs. Oliver Smithies, Mario Capecchi, and Martin Evans (Nobel Prize in Physiology and Medicine in 2007) [150]. We now have a clearer understanding of the defined regions within the niche, key inhabitants of each region, and their influence on directing hematopoiesis. Further molecular understanding of GPCR interactions have also led to therapies that improve HCT as a clinical therapy. While knowledge in the bone marrow/hematopoietic niche field has progressed over the last few decades, intracellular signaling and regulation of the complex cellular interactions within the niche is still evolving.

This dissertation initiated investigations to determine whether GRK3, an intracellular protein kinase that regulates GPCR signaling, modulates the function of BmMSCs and HSPCs since both cell subsets express relevant GPCRs involved in hematopoiesis. Our work suggests GRK3 impacts both BmMSC (Chapter 2) and HSPC (Chapter 3) function, and here,

I discuss the implications of this work and propose future experimental directions of each project, including both short-term and long-term goals.

Chapter 2

Field relevance.

BmMSCs are known to play an essential physiological role in the support of hematopoietic cell development, and yet interestingly, BmMSCs are not currently utilized in the United States to treat any human diseases. While promising pre-clinical HCT studies have shown BmMSCs transplanted with HSCs enhance hematopoietic cell engraftment [151], there has only been one clinical trial completed (and one recently registered as of October of this year) investigating the use of mesenchymal stem cells to aid immune reconstitution and engraftment in HCT. One interpretation of this observation is there is a significant knowledge gap in intracellular signaling, function, and regulatory mechanisms of BmMSCs that needs to be addressed before BmMSCs can advance as a cellular therapy for humans. Our work elucidates further understanding of how BmMSC function may be regulated through molecular mechanisms that enhance BmMSC cell functionality, specifically the importance of GRK3 regulation of S1P-activated S1PR. We show deficiency of GRK3 in the presence of S1P increases BmMSC proliferation and CXCL12 production, as well as further enhances differentiation into osteoblast precursors that support hematopoietic development as described in Chapter 1. Thus, the data presented in Chapter 2 serve as a strong foundation to test whether GRK3 deficiency in BmMSC has direct effects on hematopoiesis, which would be the next necessary steps toward clinical studies in HCT.

Conclusion and future directions.

BmMSCs isolated from GRK3-deficient (*Grk3-/-*) mice have enhanced proliferation, osteogenic differentiation *ex vivo*, and higher levels of CXCL12 compared to wildtype (WT) BmMSCs in identical culture conditions and passages. Given the vital role of BmMSCs and CXCL12 in supporting hematopoietic development, we found these initial findings interesting since they correlated with a previous observation that *Grk3-/-* mice have increased hematopoietic cell numbers isolated from the bone marrow; however, this dissertation research focused on understanding how GRK3 regulated the function of BmMSCs by identifying the signaling mechanism(s) through which it may be acting. Therefore, a future goal would be to determine whether *Grk3-/-* BmMSCs indeed directly impacts hematopoiesis. Our short-term goal is to test this by measuring HSC/HSPC numbers in an *ex vivo* co-culture system of WT HSC/HSPCs incubated with either *Grk3-/-* BmMSCs (or WT BmMSCs as control). The HSC/HSPCs will form proliferative clusters, termed *colonies*, on the stromal BmMSCs. Both colonies and total HSC/HSPCs can be enumerated after co-incubation to determine whether *Grk3-/-* BmMSCs provide a proliferative advantage for the neighboring HSC/HSPC. Our long-term goal is to directly test whether *Grk3-/-* BmMSCs impact hematopoiesis *in vivo* by utilizing a conditional knockout model $Grk3^{flox}$ $Prx1^{Cre}$, which will delete GRK3 from the mesenchymal lineage; however, this mouse model does not currently exist and will need to be generated.

The mechanism by which GRK3 deficiency elicits higher levels of CXCL12 compared to WT BmMSCs in identical culture conditions and passages has yet to be elucidated, and serves as another future direction. Due to the cross-talk mechanisms previously described by Golan et al. whereby S1P stimulation induces CXCL12 secretion

from BmMSCs [83, 84], we tested whether SKI treatment (reduction of S1P) could reduce CXCL12 levels in *Grk3-/-* BmMSC culture supernatants; however, our findings suggest at early timepoints (Day 1 and Day 2) *Grk3-/-* BmMSC CXCL12 levels were not reduced upon SKI treatment. Interestingly, CXCL12 levels were reduced at later timepoints upon SKI treatment, though this coincides with similar timepoints when SKI reduced proliferation, suggesting a reduction in cell number may be a contributing factor to the reduction of CXCL12 with SKI treatment (**Appendix 13**). Therefore, a short-term goal is to test whether higher levels of CXCL12 is possibly independent of S1P/S1PR and is simply due to increased cell number. To address this, we plan to harvest supernatants for CXCL12 quantification *and* perform cell counts per well. This new approach of dividing CXCL12 by cell number will create a ratio of CXCL12 produced per BmMSC for a more direct analysis.

Interestingly, while GRK3 deficiency elicited an enhanced osteogenic phenotype *ex vivo*, we did not see an enhanced trabecular bone volume fraction *in vivo*. However, this did not come as a surprise, since there is support to suggest perivascular niche BmMSCs that aid hematopoietic development may be functionally different than BmMSCs that migrate to the bone surface and differentiate into osteoblasts [45]. Thus, GRK3 deficiency in niche BmMSCs may have a more pronounced effect on hematopoiesis as opposed to mature bone development *in vivo*. Alternatively, since our studies utilized a conventional knockout murine model, it is possible that other cells in the bone marrow niche may be affected by GRK3 deficiency and act as a compensatory cellular interaction, such as osteoclasts that have prevalent GRK3 expression [121]. This can also be tested by utilizing our proposed conditional GRK3 knockout mouse model ($Grk3^{flox}$ $Prx1^{Cre}$), which would limit GRK3 deficiency to only the mesenchymal lineage.

Our mechanistic studies revealed that both *Grk3-/-* BmMSC proliferation and osteogenic differentiation were reduced to WT level upon reduction of S1P, and *Grk3-/-* BmMSCs have sustained ERK1/2 signaling upon stimulation of S1PR with S1P in comparison to WT BmMSCs. This supports our conclusion that S1P-ligand stimulation and downstream MAPK activation is essential for our observed *Grk3-/-* phenotype. However, due to limitations with antagonist treatments (i.e.: cellular toxicity of vehicle), we have yet to define mechanisms of action on specific BmMSC-expressed S1PR subtypes (i.e.: S1PR1, S1PR2, S1PR3). Future short-term goals will utilize shRNA technology targeting specific receptor subtypes S1PR1-3 to test what specific BmMSC-expressed S1PR subtype may be responsible for the enhanced functionality. shRNA technology has already been successful in reducing *Grk3* expression in these primary BmMSCs, so we do not anticipate technical difficulties achieving knocked-down expression; however, a potential pitfall could be that knocking-down important S1PR subtypes could affect cell viability. If so, alternatively, we can test whether over-expression of particular S1PR subtypes in WT BmMSCs during functional assays will elicit a similar "GRK3-deficient-like" phenotype. Identifying the S1PR responsible for the enhanced *Grk3-/-* BmMSC phenotype will provide further understanding of the intracellular signaling, function, and regulation to potentially aid future therapeutic investigations.

In summary, our future directions aim to (1) test whether *Grk3-/-* BmMSCs enhance hematopoiesis through both *ex vivo* and *in vivo* analyses, (2) define the mechanism by which GRK3 deficiency elicits higher levels of CXCL12, and (3) identify the S1PR subtype that elicits enhanced proliferation and osteoblast precursor differentiation.

Chapter 3

Field relevance.

HCT-related mortality has been reduced by almost 10% from the 1990s to the 2000s [152]; however, there is still a high risk of complications in the early weeks to months following HCT. The HCT recipient is immunocompromised until HSCs engraft and differentiate into the hematopoietic lineages necessary for establishment of the new immune system, termed *immune reconstitution*. Stem cell graft failure is a complication evident in 5-6 % of allogeneic transplants, where patients experience sustained *pancytopenia*—reduction of red blood cells, white blood cells, and platelets [153] due to insufficient quality and numbers of transplanted HSCs [136, 154] and/or immune-related complications, such as infections and graft-versus-host disease. Interestingly, the rate of engraftment positively correlates with the numbers of HSCs transplanted [154], suggesting immune reconstitution after HCT may benefit from increased stem cell numbers, as well as improved quality of stem cell function, such as enhanced homing and proliferation. Our work described within Chapter 3 suggests GRK3 deficiency not only increases numbers of multipotent HSPCs *in vivo*, but also increases numbers of oligopotent progenitors and importantly, mature lymphoid cells—the last hematopoietic lineage to develop in the transplanted recipient. Furthermore, GRK3 deficiency increases *in vivo* and *ex vivo* colony forming units (CFUs), mediated at least in part through CXCL12/CXCR4 stimulation. Taken together, given the positive correlation between HSC numbers and CFU colony counts within the stem cell graft and the success rate of engraftment and immune reconstitution after HCT, respectively, we propose the work within Chapter 3 provides a foundation for future directions to test

mechanistically how GRK3 deficiency improves stem cell function and determine whether such enhanced functions improve engraftment and immune reconstitution after HCT.

Conclusion and future directions.

Grk3-/- mice have a hypercellular bone marrow with increased HSPCs and oligopotent progenitors, as well as increased leukocytes in the blood, suggesting GRK3 deficiency enhances hematopoiesis. However, several different cell types can contribute to this phenotype in a conventional knockout murine model. As such, Chapter 2 investigated the role of GRK3 deficiency on BmMSC function, and Chapter 3 focused on the role of GRK3 deficiency on HSPC function.

To investigate whether GRK3 deficiency in HSC/HSPCs enhances hematopoietic cell function within the *in vivo* transplantation setting, we utilized a short-term transplantation assay, termed *colony forming unit-spleen* (CFU-S) assay. In the CFU-S assay, transplanted HSPCs with self-renewal capabilities form clonal colonies (CFUs) on the spleen that can be enumerated to measure extramedullary hematopoiesis. Interestingly, transplanted *Grk3-/-* Lin^{-} $Sca1^{+}$ $c\text{-}Kit^{+}$ (LSK) multipotent HSPCs had increased CFUs on the explanted spleen at day 12 in comparison to WT controls. Next, to determine whether oligopotent progenitors from *Grk3-/-* mice had increased functionality *in vivo*, we transplanted whole bone marrow and showed *Grk3-/-* colony counts on the explanted spleen at day 8 were also increased in comparison to WT controls.

While both *Grk3-/-* HSPC and whole bone marrow transplantation showed increased CFUs, the mechanism remains to be elucidated and will be the focus of future directions. It is possible the increased CFU numbers are due to enhanced hematopoietic cell proliferation,

homing abilities, or both. Therefore, as part of our short-term goals, we will first test whether GRK3 deficiency enhances HSPC proliferation *in vivo* through bromodeoxyuridine (BrdU) injections, which incorporates into the DNA during S-phase of the cell cycle and can be analyzed via flow cytometry. We will next test HSPC homing by utilizing allelic variants of CD45 in mice (CD45.1 and CD45.2) to distinguish between recipient (CD45.1) and donor (CD45.2) cells during analysis. We will test WT and *Grk3-/-* HSPC homing by transplanting equal numbers of *Grk3-/-* HSPCs or WT HSPCs (CD45.2) into B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) recipient mice and harvesting the spleen at day 12 for flow cytometry analyses.

We showed in Chapter 3 that *Grk3-/-* hematopoietic myeloid and lymphoid colony counts *ex vivo* increased with CXCR4 ligand stimulation (CXCL12), and *Grk3-/-* CFU-GM colony counts reduced to WT levels with CXCR4 antagonist treatment (AMD3100). Thus, our *ex vivo* CFU data suggest GRK3 regulates bone marrow hematopoietic growth of progenitor cells, at least in part, through CXCL12/CXCR4 stimulation. However, our recent finding that GRK3 regulates the S1P/S1PR pathway on BmMSCs (Chapter 2) suggests we must now consider and test whether S1P/S1PR may also be a player in our observed *Grk3-/-* HSPC phenotype since S1PRs are expressed on hematopoietic cells, including HSPCs, and are important for cell function [136]. Therefore, investigating GRK3 regulation of S1P/S1PR signaling on HSPCs will be the focus of future directions.

Lastly, since it is our long-term goal to investigate the therapeutic potential of GRK3 for immune reconstitution and engraftment, we plan to perform long-term transplantation studies using a similar experimental design as the homing assay with CD45.1/CD45.2 allelic variants that distinguish between recipient (CD45.1) and donor (CD45.2) cells during immune reconstitution analysis. CD45.1 recipients will be transplanted with either CD45.2

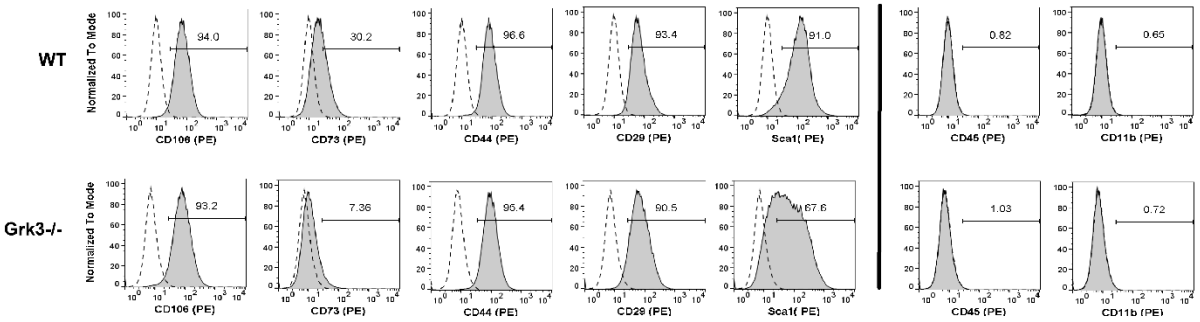
WT or *Grk3-/-* donor HSPCs. Experimental end-points will assess whether *Grk3-/-* HSPCs enhance cellular engraftment and immune reconstitution, in comparision to transplanted WT HSPCs, with the evaluation of mature leukocyte lineage repopulation (immune reconstitution) within the bone marrow, spleen, lymph nodes, and peripheral blood, and whether recipient host survival may be enhanced.

In summary, our future directions will (1) test whether proliferation and/or homing is responsible for the increased number of *Grk3-/-* HSPCs *in vivo*, (2) investigate GRK3 regulation of S1P/S1PR signaling in HSPCs, and (3) determine whether transplanted *Grk3-/-* HSPCs improve engraftment, hematopoiesis, and survival in HCT.

Final Thoughts

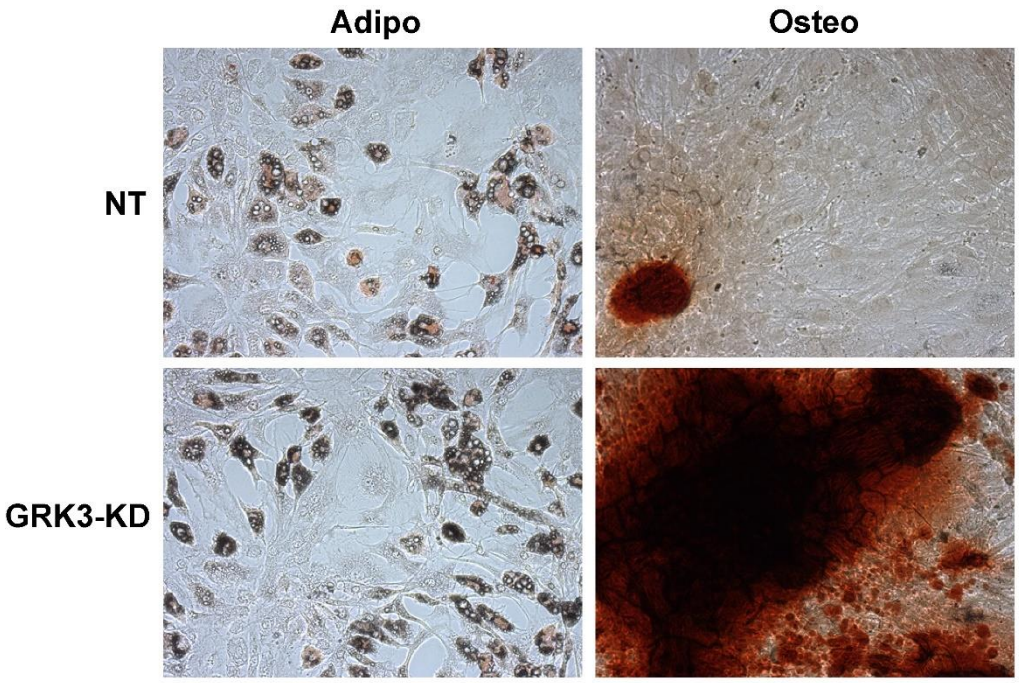
The intracellular signaling, regulatory mechanisms, and overall functionality of bone marrow niche cells are under active investigation and continually being defined within the field of hematopoiesis and stem cell research. The goal of this dissertation work is to provide further insight into the intracellular regulatory mechanisms and function of bone marrow niche cells in hopes to contribute to the overall understanding of the highly regulated, intricate cellular and molecular network within the bone marrow. Herein, we describe a newly elucidated pathway of regulation on two niche cells, BmMSCs and HSPCs. Our data suggest *G protein-coupled receptor kinase 3* (GRK3) functions as a negative regulator of G protein-coupled receptors (GPCRs) on both stem cell subtypes and can modulate stem cell function.

APPENDIX 1: MOUSE MESENCHYMAL STEM CELL MARKER PANEL



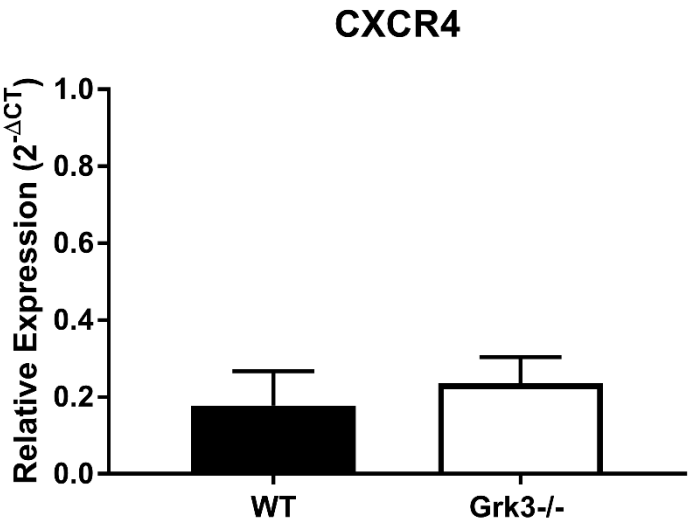
Appendix 1. Mouse mesenchymal stem cell marker panel. WT (top) and *Grk3-/-* (bottom) BmMSCs expression of positive markers CD106, CD73, CD44, CD29, Sca1 and negative hematopoietic markers CD45 and CD11b (macrophage). Data plotted as percent (normalized to mode).

APPENDIX 2: DIFFERENTIATION OF GRK3-KNOCKDOWN BmMSCS



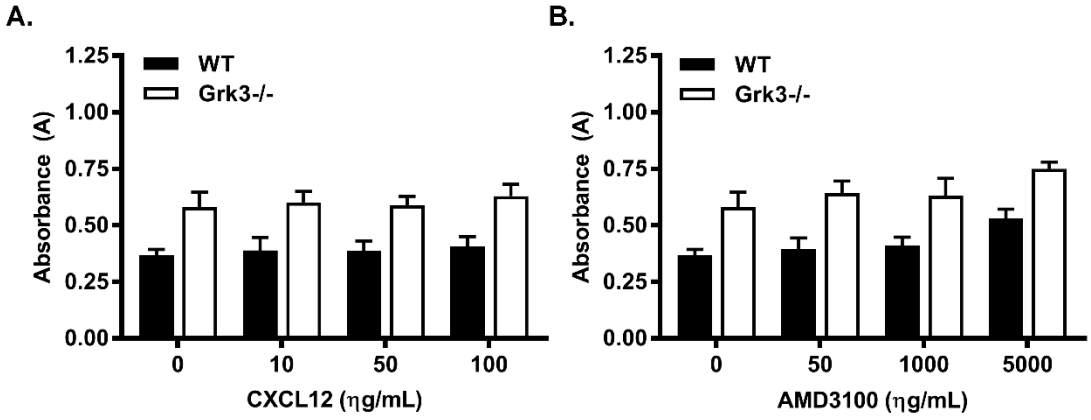
Appendix 2. GRK3-knockdown (GRK3-KD) BmMSCs have enhanced osteogenic differentiation in comparison to Non-target (NT) control BmMSCs. shRNA Non-target (NT) control and GRK3-knockdown (GRK3-KD) BmMSCs have comparable adipogenic differentiation (Oil Red O stain); however, GRK3-KD BmMSCs show enhanced osteogenic differentiation (Alizarin Red stain), similar to BmMSCs isolated from *Grk3-/-* mice. Images captured at 10X magnification using the Olympus 1X-81 microscope and MetaMorph software.

APPENDIX 3: CXCR4 mRNA EXPRESSION ON BmMSCS



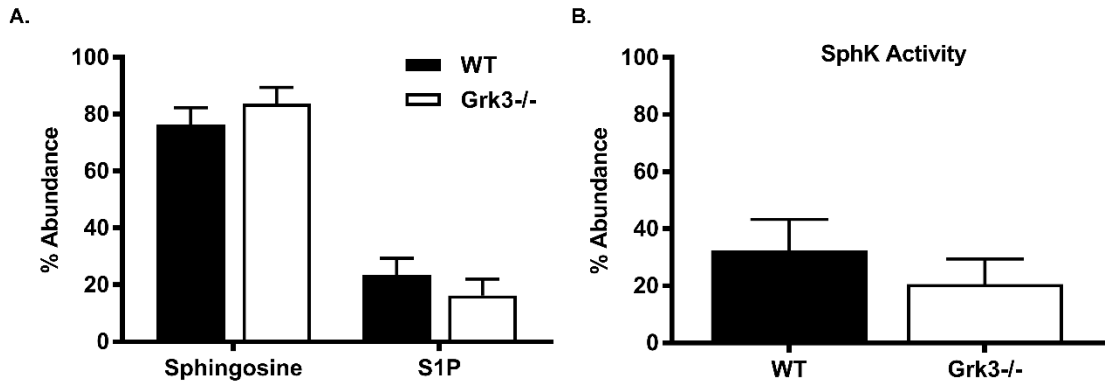
Appendix 3. WT and *Grk3*-/- BmMSCs express low levels of CXCR4 mRNA. Quantification of CXCR4 relative expression ($2^{-\Delta CT}$) normalized to housekeeping gene IDUA. Data represent mean ± SEM, n=5.

APPENDIX 4: BmMSC PROLIFERATION WITH CXCL12 AND AMD3100



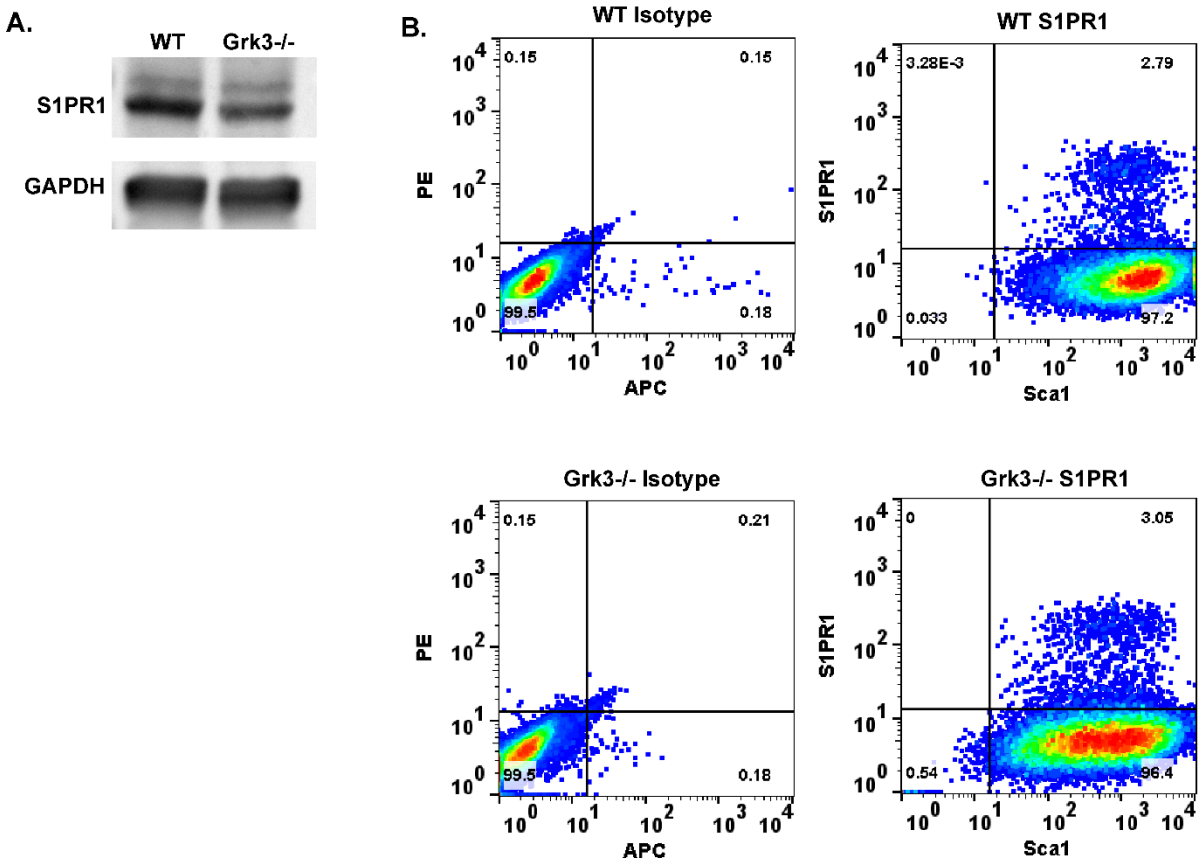
Appendix 4. BmMSC proliferation was not affected by CXCL12 stimulation or CXCR4 signaling inhibition with AMD3100. (A) Cellular proliferation was not enhanced in the presence of CXCL12, a CXCR4 agonist, at various concentrations, and **(B)** cellular proliferation was not reduced in the presence of AMD3100, a CXCR4 antagonist at various concentrations. Data represent mean ± SEM, n=3.

APPENDIX 5: BmMSC SPHINGOSINE KINASE ACTIVITY



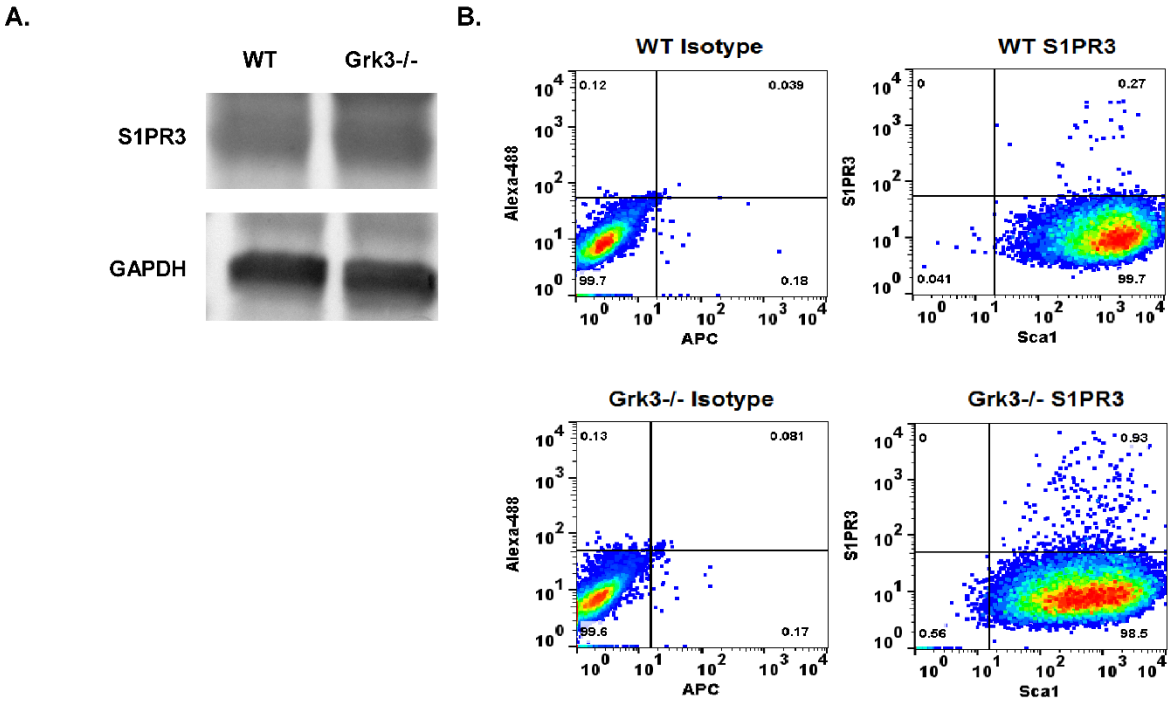
Appendix 5. WT and *Grk3-/-* BmMSCs have comparable sphingosine kinase activity. WT and *Grk3-/-* BmMSCs were treated with fluorescein-labeled sphingosine to assess the BmMSC ability to convert sphingosine to active ligand sphingosine-1-phosphate (S1P) through sphingosine kinase (SphK) activity. WT and *Grk3-/-* BmMSC converted sphingosine into S1P (i.e.: SphK activity) at comparable levels. Data represent mean ± SEM, n=3.

APPENDIX 6: BmMSC EXPRESSION OF S1PR1



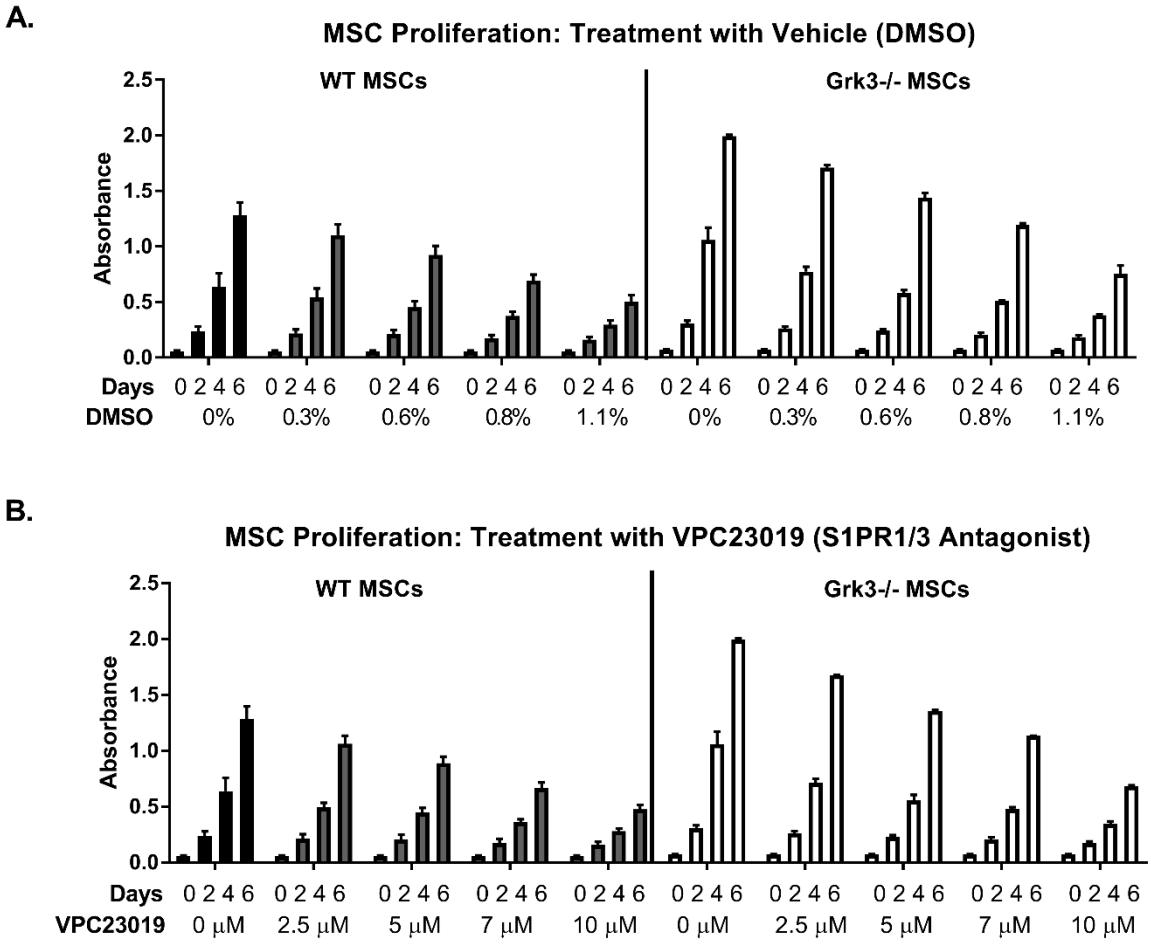
Appendix 6. WT and *Grk3-/-* BmMSCs express S1PR1. (A) Immunoblot detection of S1PR1 from both WT and *Grk3-/-* BmMSC lysates, and **(B)** flow cytometry detection of surface S1PR1 (PE-conjugated) on Sca1+ (APC-conjugated) WT and *Grk3-/-* BmMSCs.

APPENDIX 7: BmMSC EXPRESSION OF S1PR3



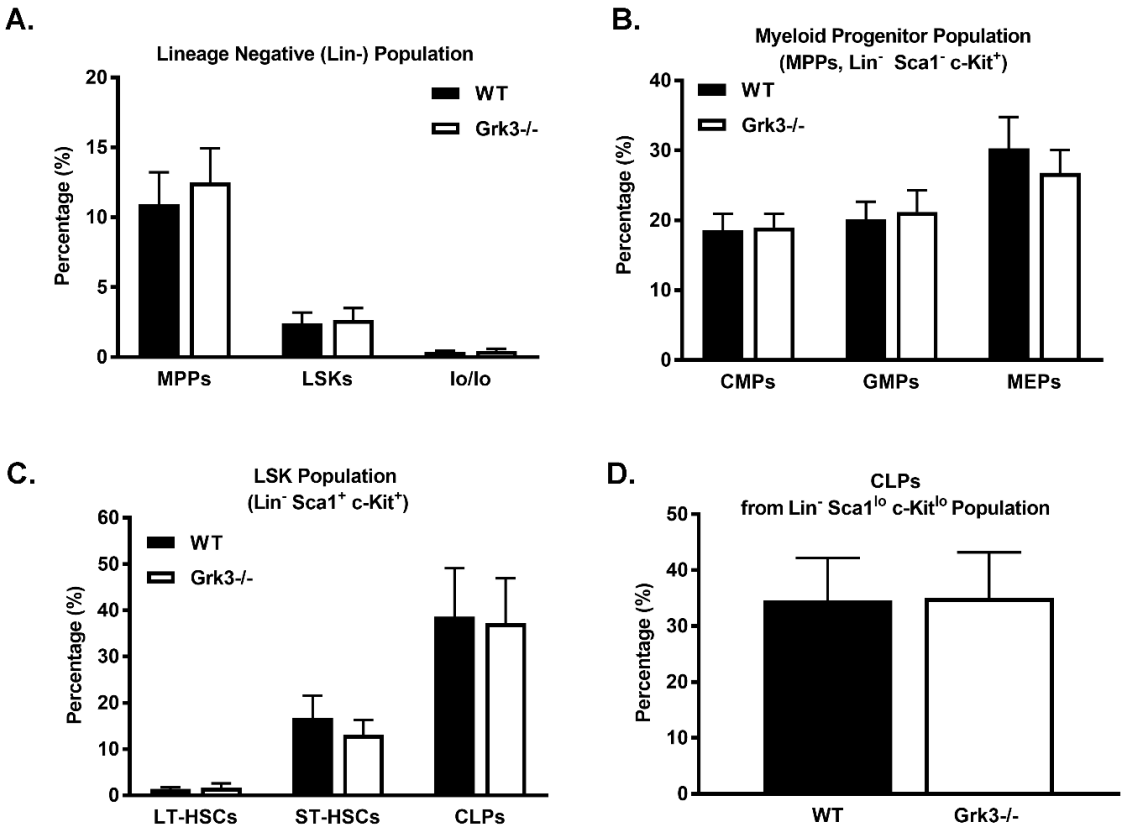
Appendix 7. WT and *Grk3-/-* BmMSCs express S1PR3. (A) Immunoblot detection of S1PR3 from both WT and *Grk3-/-* BmMSC lysates, and **(B)** flow cytometry detection of surface S1PR3 (Atto-488-conjugated) on Sca1+ (APC-conjugated) WT and *Grk3-/-* BmMSCs.

APPENDIX 8: DMSO VEHICLE TOXICITY WITH COMPARABLE DOSING AS VPC23019



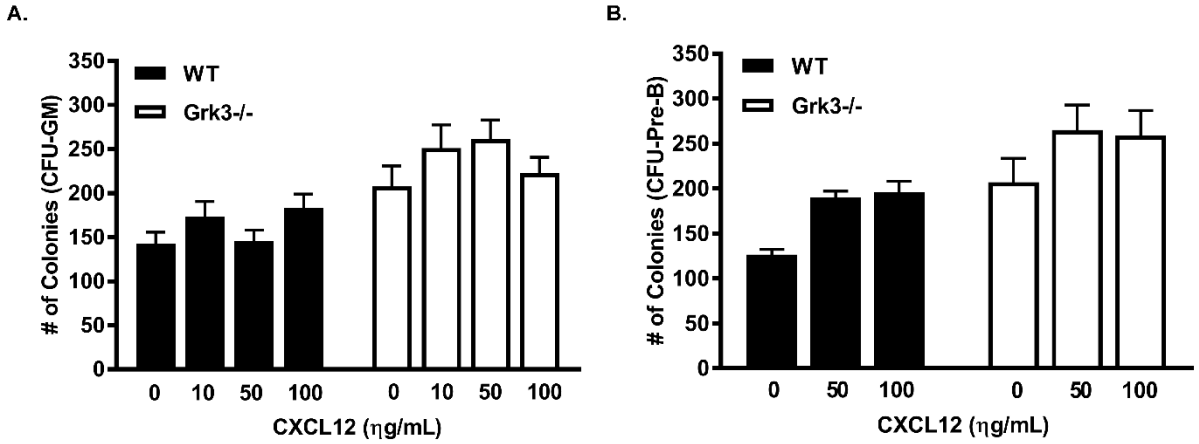
Appendix 8. DMSO vehicle toxicity evident at comparable dosing as VPC23019. BmMSC proliferation determined by increased formazan dye production from viable cells over time using CCK-8 proliferation assay. BmMSCs treated with **(A)** DMSO vehicle control or **(B)** VPC23019 at comparable dosing. Data represent mean ± SEM, n=2.

APPENDIX 9: BONE MARROW STEM AND PROGENITOR FLOW ANALYSES



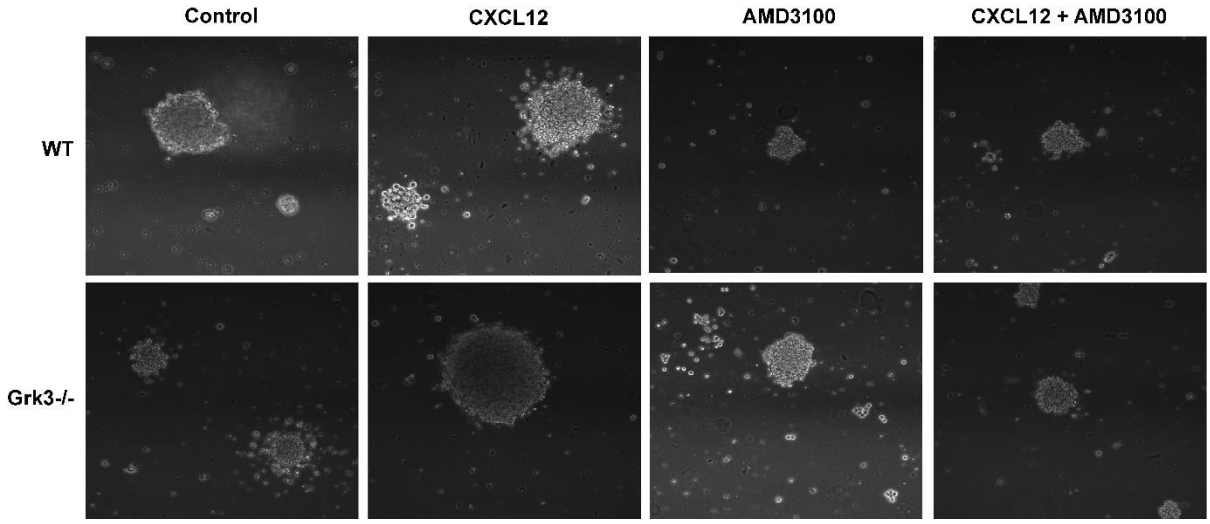
Appendix 9. WT and *Grk3-/-* hematopoietic stem and progenitor percent populations are comparable within the bone marrow. WT and *Grk3-/-* bone marrow analyzed by flow cytometry. **(A)** Lineage negative (Lin^-) gating can be further subcategorized into MPPs ($Sca1^-$ $c\text{-}Kit^+$), LSKs ($Sca1^+$ $c\text{-}Kit^+$), and $Sca1^{lo}$ $c\text{-}Kit^{lo}$ populations. **(B)** MPP gating can be further subcategorized into myeloid progenitors: common myeloid progenitors (CMP), granulocytic monocytic progenitors (GMP), and myeloid erythroid progenitors (MEP), **(C)** LSK gating can be further subcategroized into long-term HSCs (LT-HSC), short-term HSCs (ST-HSC), and common lymphoid progenitors (CLP). **(D)** $Sca1^{lo}$ $c\text{-}Kit^{lo}$ gating can be further subcategroized into $Sca1^{lo}$ $c\text{-}Kit^{lo}$ CLPs. Data represent mean ± SEM, n=8.

APPENDIX 10: OPTIMIZATION OF CXCL12 CONCENTRATION



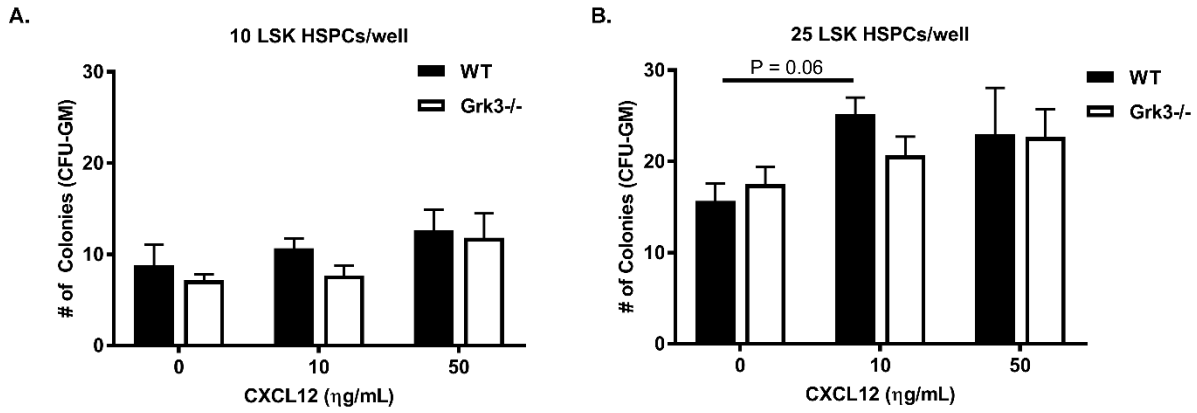
Appendix 10. Dose evaluation of CXCL12 for CFU assays. (A) CFU-GM CXCL12 dose curve with peak response at 50 ηg/mL for *Grk3-/-* cultures. Data represent mean ± SEM, n=7 (control), n= 5 (10 ηg/mL), n=4 (50 ηg/mL), n=5 (100 ηg/mL). **(B)** CFU-Pre-B CXCL12 dose curve with comparable response at 50 and 100 ηg/mL with a slight peak response at 50 ηg/mL for *Grk3-/-* cultures. Data represent mean ± SEM, n=4.

APPENDIX 11: AMD3100 INHIBITS COLONY PROLIFERATION



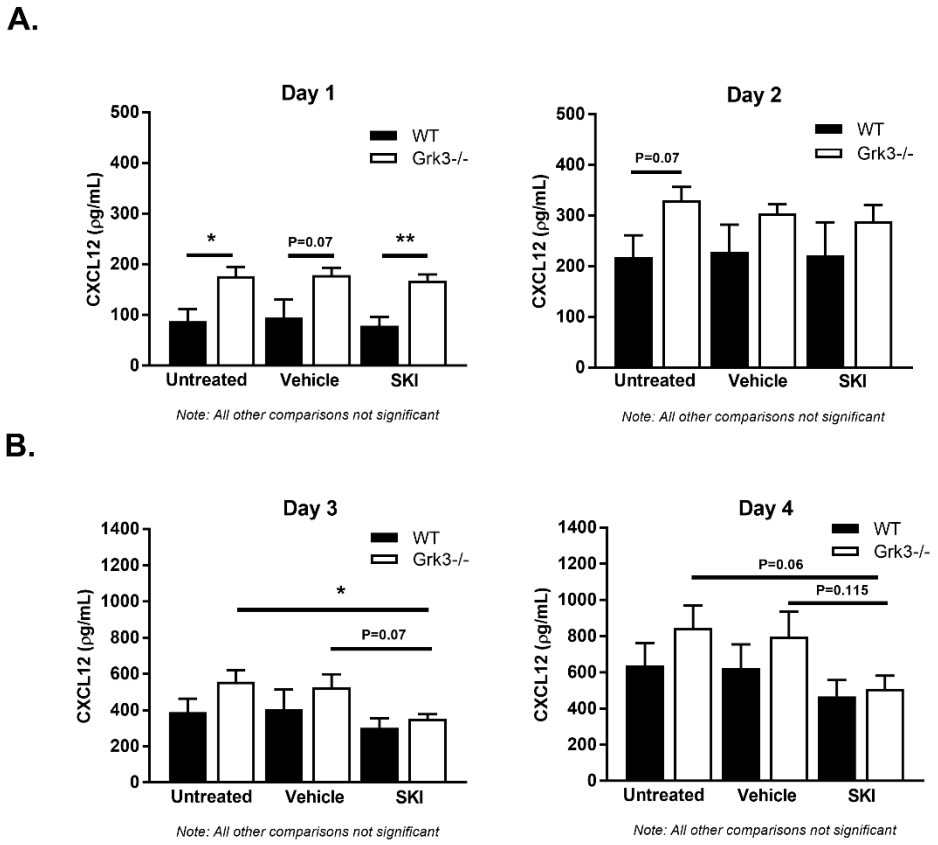
Appendix 11. CXCR4 antagonist treatment (AMD3100) inhibits WT and *Grk3-/-* CFU-Pre-B colony proliferation. CFU-Pre-B cultures were treated with CXCR4 ligand (CXCL12, 50 ηg/mL), CXCR4 antagonist (AMD3100, 1,000 ηg/mL), or both. Representative images at 15X magnification.

APPENDIX 12: *Grk3-/-* LSK HSPC DIFFERENTIATION



Appendix 12. *Grk3-/-* LSK HSPCs do not more readily differentiate into myeloid (granulocytic and monocytic-GM) progenitors *ex vivo*. Assay optimization utilizing **(A)** 10 LSK HSPCs/well or **(B)** 25 LSK HSPCs/well for CFU-GM assay. Data represent mean ± SEM, n=3.

APPENDIX 13: CXCL12 LEVELS OF BmMSCS TREATED WITH SKI



Appendix 13. Sphingosine kinase inhibitor (SKI)-treatment does not reduce BmMSC CXCL12 levels at early timepoints. Quantification of CXCL12 protein concentration from supernatant of untreated BmMSCs and treated BmMSCs with 5 μM SKI or vehicle (DMSO) at early timepoints **(A)** day 1 and day 2, and later timepoints **(B)** day 3 and day 4. Lower limit of detection at 46.9 ρg/mL. Data represent mean ± SEM, n=4. *P≤0.05 **P≤0.01

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