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**Loss of the Rho GTPase Activating Protein
p190-B enhances hematopoietic stem cell engraftment
potential**

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Abstract

Hematopoietic stem cell (HSC) engraftment is a multi-step process involving HSC homing to bone marrow (BM), self-renewal, proliferation and differentiation to mature blood cells. The molecular mechanism underlying HSC engraftment is still poorly defined. Members of the small Rho GTPase family are critical regulator of cell migration, proliferation and differentiation in multiple cell types, including hematopoietic cells. Here, we examined the role p190-B Rho GTPase Activating Protein (GAP), a negative regulator for Rho GTPases, in HSC engraftment, using a mouse model of genetic loss of p190-B. The p190-B^{-/-} lineage-negative cells showed a 4-fold increase in RhoA activity but normal Rac and Cdc42 activities, indicating that p190-B knockout resulted in a gain of RhoA activity in the hematopoietic tissues. Serial competitive repopulation assays revealed that the loss of p190-B in HSC is associated with enhanced long-term engraftment potential compared to WT controls. Moreover, p190-B mediated this function by maintaining the regeneration of a population of quiescent LSK cells during serial transplantation that is capable of normal proliferation and normal multi-lineage differentiation. These changes are associated with a lack of upregulation of p16^{Ink4a} in HSC. Loss of p190-B also results in increased hematopoietic progenitor homing to BM compared to WT. Therefore, our study provides evidence that a member of the GAP family plays crucial roles in maintaining HSC functions after transplantation due to a combined effect of enhanced homing and maintenance of in vivo quiescence and self-renewal.

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Chapter I

Introduction

Hematopoietic stem cells (HSC) capable of self-renewal and differentiation into multiple blood lineages have been used in the form of bone marrow (BM) transplantation to treat the patients with malignancies, bone marrow failure states, and immunodeficiencies for over 30 years. Successful BM transplantation involves homing of HSC to the BM, lodging in the proper niche, expanding and differentiating to produce large numbers of mature cells - all without depletion of the stem cell pool. Considering the limiting numbers of HSCs, a significant challenge remains to harvest enough stem cells to facilitate successful engraftment. Thus, the enhancement of HSC homing and HSC expansion in vitro to ensure enough stem cells are attractive strategies to ensure an effective graft. Although remarkable advances have been made in HSC biology to date, the molecular and cellular mechanisms which regulate HSC homing, engraftment and self-renewal remain to be clarified.

Rho GTPases are guanine nucleotide binding proteins that cycle from the active GTP-bound state to the inactive GDP-bound state. As such, they have key roles in the modulation of a wide range of cellular processes including proliferation, apoptosis, cell migration, cell polarization, membrane trafficking, cytoskeleton rearrangements, and transcriptional regulation. More recently, there is growing evidence to show that Rho GTPases, such as Rac1, Rac2, Cdc42 and RhoA, play critical roles in HSC homing, proliferation and engraftment. In this study, we use a knockout mouse model to study the function of p190-B Rho GTPase Activating Protein (GAP), a negative regulator for Rho GTPases, in HSC engraftment. The ultimate goal for our study is to elucidate the specific

contributions of p190-B RhoGAP to HSC engraftment, and identify potential targets to improve HSC engraftment for clinical use in cellular and gene therapies.

1.1 Hematopoietic stem cells (HSC)

Hematopoietic Hierarchy

The current gold standard definition of an HSC is a cell capable of self-renewal and differentiation in all hematopoietic lineages in a lethally irradiated recipient. The ability of HSCs to self-renew is defined as the ability of HSC to divide and produce more HSCs. This could occur theoretically by either symmetric or asymmetric divisions. HSCs are also able to differentiate to produce all specialized blood cell types. HSCs are found in the BM of the adult. Other sources include umbilical cord blood, placenta, and peripheral blood. For experimental purposes, fetal liver, fetal spleen, and the Aorta-gonad-mesonephros (AGM) of fetal animals are also useful sources of HSCs. Distinct developmental stages of the hematopoietic hierarchy can be identified and arranged in a hierarchical tree (Figure 1.1) that begins with the long-term (LT) functional HSC. LT-HSCs produce less primitive short-term (ST) functional HSCs, and these, in turn, give rise to lineage committed progenitor cells. The lineage committed progenitor cells are directly responsible for the generation of all the blood cell types, including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets) and lymphoid lineages (T-cells, B-cells, NK-cells). A single LT-HSC, which has the highest self-renewal capacity, is both necessary and sufficient for life-long sustenance of the entire hematopoietic system in mice[2, 3], whereas ST-HSCs have restricted self-renewal potential, sustaining hematopoiesis only for a limited time in vivo [4,5]. Leukemia progenitors may acquire self-renewal capacity. The differentiated

cells are assumed to have lost the self-renewing potential completely. Based on the cell surface expression of certain antigens, HSCs and progenitors can be purified and differentiated in vitro.

Figure 1.1 Model of the hematopoietic developmental hierarchy in mouse. Modified from Passegue, et al 2003 [1]. Diagram shows the development of different blood cells from hematopoietic stem cell to mature cells. LT-HSC: Long-term hematopoietic stem cells, ST-HSC: Short-term hematopoietic stem cells, MPP: Multipotent progenitor cells, CMP: Common myeloid progenitor, CLP: Common lymphoid progenitor, MEP: Megakaryocyte Erythroid progenitor, GMP: Granulocyte macrophage progenitor.

Identification and isolation of HSC

HSCs can be prospectively identified by monoclonal antibodies directed to surface markers. The purity of the populations of HSCs isolated by fluorescence-activated cell sorting (FACS) has increased within recent years [6, 7]. In mice most commonly used FACS-purified populations of HSC/progenitor cells are $\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+$ [(LSK), cells depleted of various markers of differentiated cells are called lineage negative (Lin^-) cells]

cells [8, 9]. More recently, the expression of CD34 and Flt3 (CD135) [4, 5] has been used to further purify long-term repopulating HSCs (LSKCD34⁻ Flt3⁻) from short-term repopulating HSCs (LSKCD34⁺ Flt3⁻) and multipotent progenitors (LSKCD34⁺ Flt3⁺) [4]. Based on the quiescent (G₀) state of BM HSCs [10, 11, 12], decreased staining with the vital fluorescent dyes Hoechst 33342 (a *bis*-benzimidazole that binds to adenine–thymine-rich regions of the minor groove of DNA) and rhodamine 123 (which preferentially accumulates in active mitochondria) has long been used in flow cytometry experiments to enrich for HSCs [13, 14, 15]. Until recently, it had been generally assumed that the weak HSC staining obtained with both of these dyes was a reflection of a kinetically and metabolically quiescent cell that had condensed chromatin and very few or inactive mitochondria [16, 17]. Dual-wavelength flow cytometric analysis of cells stained with Hoechst 33342 alone by simultaneously monitoring fluorescence emission of Hoechst 33342 at approx 450 nm (“Hoechst Blue” fluorescence) and at >675 nm (“Hoechst Red” fluorescence) following ultraviolet (UV) excitation, a rare subset of mouse bone marrow cells (<0.1%) was observed that displayed low blue and red fluorescence [18]. These so-called “side population” (SP) cells, which contained the vast majority of long-term hematopoietic repopulating activity in mouse bone marrow is another method used in laboratory today to isolate HSCs.

Using SLAM proteins (a family of cell surface glycoproteins in the immunoglobulin superfamily) as cell surface markers, a population of cells with specific SLAM antigens (CD150⁺CD244⁻CD48⁻ cells) has been identified in which approximately 50% of single cells have been demonstrated to reconstitute lethally irradiated animals [19]. Purified HSCs can proliferate and differentiate with approximately unit efficiency into myelomonocytic cells, B cells, or T cells to reconstitute all blood cell types in lethally irradiated mice.

1.2 HSC engraftment

HSC engraftment is a multi-step process which shares some common features with the migration of leukocytes to inflammatory sites [34, 35, 36]. While HSC transplantation is commonly used for the treatment of genetic, immunological and malignant disease, the molecular mechanisms responsible for the homing of HSCs to the BM and the regulation of their subsequent proliferation and differentiation to produce mature functional hematopoietic cells within the BM remain to be understood in detail.

Homing of HSCs

Homing of HSCs can be defined as the set of molecular interactions that allows circulating HSCs to recognize, adhere to, and migrate across bone marrow endothelial cells, resulting in the accumulation of HSCs in the BM microenvironment (Figure 1.2). During the homing process, HSCs are postulated to initially roll on the endothelial layer via endothelial (E) and platelet (P) selectin receptors [37, 38, 39]. Activation of G-coupled chemokine receptor by stromal cell-derived factor 1 (SDF-1) leads to integrin activation, resulting in firm adhesion of stem cells to the endothelial layer. Inter cellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) [34-39] have been shown to be important for the arrest of HSCs on the vessel wall. HSCs subsequently leave the circulation by extravasation through the extracellular matrix underlining the endothelium and reach the appropriate bone marrow niche. The interactions of major β 1 integrins [very late activation antigen-4 (VLA-4), and VLA-5] with their ligand fibronectin (FN) are critical for the transendothelial migration of HSCs [40, 41, 42]. These studies suggest that cell adhesion molecules and their ligands,

extracellular matrix components, chemokines, and specialized bone marrow niches all participate in the precise regulation of the HSCs homing process.

Figure 1.2 Homing of transplanted HSC to the BM. (Modified from Peled A. et al., Blood, 2000[40].) HSCs rolling interactions on constitutively expressed endothelial (E) and platelet (P) selectins. Following rolling, CXCR4⁺ HSC are activated by SDF-1 and triggers LFA-1/ICAM-1 and VLA-4/ VCAM-1 interactions to support firm adhesion to endothelial cells. The arrested CXCR4⁺ HSCs, in response to SDF-1, will extravasate and migrate through the underlying basal lamina ECM using VLA-4 and VLA-5 integrin receptors to FN. Migrating stem cells will eventually reach the "stem cell niches," which consist of stromal cells that present the proper set of adhesion molecules, SDF-1, and growth stimulatory factors.

SDF-1: stromal cell-derived factor 1, LFA-1: lymphocyte function associated antigen 1, ICAM-1: intercellular adhesion molecule-1, VLA-4: very late activation antigen-4 (integrin $\alpha 4\beta 1$), VCAM-1: vascular cellular adhesion molecule-1, ECM: extracellular matrix, VLA-5: very late activation antigen-5 (integrin $\alpha 5\beta 1$), FN: fibronectin.

Self-renewal, proliferation/ differentiation of HSCs

Once in the niche, HSCs, which are initially in quiescent state [43], will enter the cell cycle, proliferate and differentiate to all mature blood cell lineages to reconstitute the hematopoietic system. The entry into the cell cycle of HSCs must be under stringent control, otherwise the uncontrolled proliferation of HSC and progenitors will cause hyperplastic diseases. Simultaneously, HSCs will replicate through self-renewal to prevent depletion of the stem cell pool in the BM for the maintenance of long-term hematopoiesis. The balance between self-renewal and commitment of HSCs is controlled by a complex interplay between intrinsic mechanisms and extrinsic signals from the surrounding environment or stem cell niches. Intrinsic regulators include transcription factors translocation Ets leukemia (Tel) [44], growth factor independence 1 (Gfi-1) [45], Stat3 and Stat5 [46, 47], c-myc [48], and many members of the homeobox (Hox) family of transcription factors [49, 50, 51]. Additional regulators include anti-apoptotic protein B-cell lymphoma 2 (BCL2) [52, 53], polycomb group (PcG) protein B lymphoma Mo-MLV insertion region 1 (Bmi-1)[54], and signal transducer phosphatase and tensin homolog deleted on chromosome 10 (PTEN, a negative regulator of Akt) [55, 56]. Cell cycle regulators, like the cyclin dependant kinase inhibitors p21^{Cip1/Waf1}, p18^{Ink4c}, p16^{Ink4a} and p19^{Arf}, play crucial roles in the self-renewal of HSC, and p27^{Kip1} is important for the cycling of primitive hematopoietic progenitors [57, 58, 59, 60, 61, 62]. Extrinsic signals, including Tie2- and Ang1-signaling [63], Notch ligands [64, 65], Wnts [66], Sonic hedgehog (Shh) [67], BMPs [68], and TGF- β [69] have been shown to regulate HSCs by ligand stimulation or by activation/overexpression of their internal signals. It is still poorly understood how these disparate molecular regulators are integrated to determine whether the outcome is HSC quiescence or proliferation/differentiation.

The relationship between cell cycle status and HSCs engraftment potential

HSC/P engraftment into BM has been described to be restricted to cells in G₀/G₁ phases of the cell cycle. This restriction is due, at least in part, to the fact that HSC/Ps in S/G₂+M-phase of the cell cycle exhibit reduced ability to home into BM that is associated with defective migration on fibronectin. Hence HSC/Ps in the S/G₂+M phases of the cell cycle show defective long-term repopulating potential compared to HSC/Ps in G₀/G₁-phase of the cell cycle [70, 71, 72]. These observations suggest that migration, adhesion and cell cycle transit appear to be linked during HSC/P engraftment. However, the underlying mechanisms that regulate any link between HSC/P migration and cell cycle transit are still poorly understood.

A fundamental challenge in hematopoiesis research is to understand how these programs are regulated; such understanding could be exploited for the development of HSC-based therapies. The identification of regulatory proteins that can maintain and expand HSC in vivo and in vitro without altering their multi-lineage potentiality, engraftment capacity and without inducing leukemia development would have clear clinical applications.

1.3 Small RhoGTPases and their function in HSC engraftment

Small RhoGTPases

Members of the Rho GTPase family operate as molecular switches that couple changes in the external environment to intracellular signal-transduction pathways. They can act downstream of many cell surface receptors, including integrin molecules, chemokine and cytokine receptors. Rho GTPases cycle between an active GTP-bound form and an inactive GDP-bound form (Figure 1.3). Guanine nucleotide exchange factors

(GEFs) release guanosine diphosphate (GDP) from Rho GTPases promoting the binding of GTP and activation of Rho GTPases. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho GTPases and convert GTP-bound form of Rho GTPases to inactive GDP-GTPases. GDP dissociation inhibitor (GDI) inhibits the dissociation of GDP from Rho GTPases and may sequester GDP-GTPase in the cytosol preventing the binding to cell membrane. Small GTPases act as molecular switches and play key roles in the modulation of a wide range of cellular processes including proliferation, apoptosis, cell migration, cell polarization, membrane trafficking, cytoskeleton rearrangements, and transcriptional regulation. Rho, Rac and Cdc42 are the most well studied RhoGTPases, and have been shown to be the master regulators of actin cytoskeletal activation.

Figure 1.3 Small GTPase cycle. Ras-superfamily GTPases bound to GTP are active signaling molecules. The intrinsic GTPase activity of the molecule hydrolyzes GTP to GDP, and GDP-bound forms are inactive. GTPase activating proteins (GAPs) enhance GTPase activity and favor the inactive, GDP-bound state. GTP exchange factors (GEFs) facilitate exchange of GDP with GTP and thus favor the active, GTP-bound state. GDP dissociation inhibitor (GDI) inhibits the dissociation of GDP from Rho GTPases.

RhoA coordinates proliferation and migration in non-hematopoietic cells

RhoA is a member of small RhoGTPases and has been implicated in the coordinate regulation of cell proliferation and migration in non-hematopoietic cells via focal adhesion formation [73, 74] (Figure 1.4).

Figure 1.4. RhoA coordinates migration and cell cycle transit in non-hematopoietic cells

(modified from Denicourt et al., *Genes Dev.* 2004) [75].

RhoA regulates cell migration via the turnover of focal adhesion structures. Upon stimulation, RhoA is activated by GEFs, and then GTP-bound RhoA activates ROCKs to phosphorylate one of its substrate LIMKs. By doing so, ROCK1 enhances the ability of LIMKs to phosphorylate cofilin, an actin-depolymerizing protein. This phosphorylation inhibits the activity of cofilin. Phosphorylation of LIMKs by ROCKs inhibits disassembly of actin filaments and thereby leads to an increase in stress fibers. In addition, RhoA also inhibits the expression of cyclin–CDK inhibitors $p27^{kip1}$ and $p21^{cip1}$, and affects G₁–S phase transition. $p27^{kip1}$ can relocate to the cytoplasm via Ser 10 phosphorylation, and $p21^{cip1}$ can be exported to the cytoplasm by unknown mechanism. In the cytoplasm, $p27^{kip1}$ inhibits activation of RhoA by the GEFs, and $p21^{cip1}$ can inhibit ROCK activity. Inhibition of the Rho pathway by $p21^{cip1}$ and $p27^{kip1}$ leads to reorganization of the actin stress fiber filaments and promotion of cell migration.

The roles of Rho GTPases in HSC function

Thus far, few studies have been carried out to investigate the role of Rho GTPases in HSC function. RhoA, Rac1 and Cdc42 have been reported to be ubiquitously expressed, Rac2 expression has been demonstrated to be hematopoietic-specific [76], while Rac3 has predominant expression in the developing nervous system and adult brain [77, 78]. Rac1, but not Rac2, was found to be important for the engraftment of HSC. The defective engraftment by Rac1^{-/-} HSC/Ps is probably the result of a defect in the capacity of viable Rac1^{-/-} HSC/Ps to relocate to or be retained within the appropriate hematopoietic microenvironment niche *in vivo* [79]. Rac1^{-/-} HSCs show reduced *in vitro* proliferation associated with impaired growth factor-stimulated cyclin D1 induction, whereas Rac2^{-/-} HSCs show defective growth factor rescue of apoptosis *in vitro* [79]. Mice lacking Rac1 and Rac2 showed increased mobilization of HSC from the bone marrow [80]. In the Cdc42GAP^{-/-} mouse model, constitutively increased Cdc42-GTP species cause increased hematopoietic progenitor apoptosis, disorganized actin structure, and defective engraftment without affecting the cell cycle status [81]. A study using an inducible conditional knockout mouse model unveils a role of Cdc42 in maintaining HSC quiescence and in retaining HSCs in the correct location in the BM niche by regulating the expression of a number of key cell cycle regulators (including c-Myc and p21^{Cip1/Waf1}) and cell adhesion molecules (such as β 1-integrin and N-cadherin) and the actin structure [82]. Moreover, expression of dominant negative RhoA in the bone marrow cells led to increased stem cell proliferation and engraftment, likely via decreasing p21^{Cip1/Waf1} expression and increasing cyclin D1 levels [83]. These studies suggest that specific modulation of Rho GTPases in the transplanted HSC/P could be an interesting tool to improve the engraftment efficiency.

1.4 p190-B RhoGTPase Activating protein (p190-B)

While the role of Rho GTPases in many cellular functions has begun to be understood, the role of GAPs and GEFs has been understudied. GAPs were initially viewed as signal terminators. However, increasing evidence suggests that GAPs are regulated by external cues and play pivotal roles in a number of signaling pathways.

The RhoGAP p190-B, which functions as negative regulator of Rho activity [84], is a large molecular weight protein that contains multiple protein binding motifs in addition to the GAP domain, which is responsible for accelerating the hydrolysis of GTP bound to different Rho members. In mammals, there are two GAP proteins for Rho, referred to as p190-A RhoGAP and p190-B RhoGAP. The domain structure of both p190-A and p190-B are similar and share 51% amino acid identity. The N-terminal portion of p190-A and p190-B contain several motifs characteristic of a GTPase domain, while their C-terminus contain a Rho GAP domain, which is responsible for accelerating the hydrolysis of GTP bound to different Rho members [84]. p190-B and Rho are both recruited to the plasma membrane following integrin crosslinking where p190-B functions to inhibit Rho activity by stimulating GTP-hydrolysis activity [84]. The p190 proteins are evidently potent inhibitors of Rho activity: the combined activity of both p190 RhoGAPs accounts for the majority of RhoA inhibitory activity in fibroblast lysates, with p190-B displaying five times the activity of p190-A [85]. These data suggest that p190-B may be a major down-modulator of Rho GTPase activity in vivo.

p190-B regulates multiple cellular functions, including proliferation [86, 87], gene transcription [88], integrin signaling [84], cytoskeletal reorganization [89], vesicular trafficking and development [90, 91]. Moreover, p190-B RhoGAP has been shown to act

as a scaffold protein and to interact with various chromatin-remodeling complexes through the GAP domain. The physiological function of p190-B has begun to be studied using a gene-targeted knockout mouse model. Mice lacking p190-B die immediately after birth due to defects in the central nervous system, thymus and atelectasis (failure of lung inflation) [92]. Interestingly, p190-B-deficient embryos exhibit striking deficits in cell and organism size [92]. Cells derived from embryos lacking p190-B RhoGAP exhibit excessive Rho activity, are defective for adipogenesis, but undergo myogenesis [93]. These defects result from impaired signaling through insulin/insulin-like growth factors (IGFs) and cAMP-response element binding proteins (CREB) [92, 93]. p190-B interacts with the IGF-1R signaling pathway to regulate postnatal mammary ductal morphogenesis [86, 94].

We hypothesize that RhoA is a major regulator of engraftment via cytoskeleton rearrangement. To test this hypothesis, we used mice genetically deficient in the Rho-inhibitory protein p190-B RhoGAP (p190-B), which leads to a gain of RhoA activity [92], to study the role of p190-B in hematopoietic stem cell functions. Our results demonstrate that the loss of p190-B RhoGAP results in enhanced long-term HSC engraftment assessed by serial transplantation assays, an effect that is associated with efficient maintenance of a pool of quiescent Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells and low expression of inhibitor of cyclin-dependent kinase 4A (p16^{Ink4a}) in p190-B^{-/-} HSC transplanted mice. Furthermore, loss of p190-B in HSC and progenitors (HSC/P) is also associated increased homing to bone marrow microenvironment and migration towards SDF-1 α . Therefore, our study provides evidence that a member of the GAP family plays crucial roles in maintaining HSC functions after transplantation due to a combined effect of enhanced

homing and maintenance of quiescence and self renewal of HSC in vivo. p190-B might thus be a potential target to improve the cellular and gene therapy.

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Chapter II

Title:

The role of Rho GTPase Activating Protein p190-B in hematopoietic stem cell (HSC) function

2.1 Materials and Methods:

Mice

p190-B RhoGAP^{+/-} mice (backcrossed into C57BL/6J N=10), and B6.SJL-PtcrPep3b/BoyJ (B6.BoyJ, CD45.1⁺) congenic mice were bred in-house in a pathogen-free environment. Embryonic day-14.5 fetal livers (FL) p190-B and wild type (WT) littermates were used for experiments. All studies were conducted with a protocol approved by the Animal Care Committee of Cincinnati Children's Hospital Medical Center.

Fetal liver cell isolation

p190-B RhoGAP^{+/-} mice were used to generate embryonic day-14.5 fetal livers. Gestational age of heterozygous pregnancies was determined by detection of the vaginal plug (as embryonic day 0.5 [E0.5]). Fetal livers were harvested from individual embryos. Single-cell suspensions were prepared by pushing the hepatic tissues through progressively smaller needles (23 gauge) and filtered through a 40- μ m filter.

Bone marrow collection and lineage depletion

BM cells were flushed from femora and tibiae and suspended in Hanks Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Laurenceville, GA), and 1% penicillin (10000 U/mL)/streptomycin (5000 μ g/mL; Cambrex Bio Science, East Rutherford, NJ). Mononuclear cells were isolated by

low-density centrifugation (Histopaque 1083; Sigma-Aldrich, St Louis, MO) and incubated with a cocktail of biotinylated antibodies against epitopes on differentiated leukocytes: anti-CD11b (clone M1/70), anti-B220 (clone RA3-6B2), anti-CD5 (clone 53-7.3), anti-Gr-1 (clone RB6-8C5), anti-Ter119, anti-CD8a (Clone 53-6.7; all from BD Pharmingen, San Diego, CA). Antibody-labeled Lin⁺ cells were removed using sheep anti-rat IgG magnetic immunobeads (Dynal Inc., Lake Success, NY, USA).

Rho GTPase effector domain pull-down assays

Rho GTPases (RhoA, Rac, and Cdc42) activities were examined by an effector domain, GST-fusion pull-down protocol, as previously described [1]. In brief, Lin⁻ BM cells were lysed in a buffer containing 1% Triton X-100 and incubated with the glutathione bead-bound GST-Rhotekin (for RhoA activity) or GST-PAK1 (for Cdc42 and Rac activities). The bead-immobilized, GTP-bound Rho GTPases and the Rho GTPases in the lysates were probed by immunoblotting with respective antibodies, and the relative amount of proteins bound to the beads were quantified by densitometry measurements.

Flow cytometry analysis and purification of HSC/P

Lineage staining of BM cells utilized a cocktail of biotinylated anti-mouse antibodies to anti-CD11b (M1/70), anti-B220 (RA3-6B2), anti-CD5 (53-7.3), anti-Gr-1 (RB6-8C5), anti-Ter119, and anti-CD8a (53-6.7). Lineage staining of FL used a similar cocktail of biotinylated antibodies except for CD11b. For detection and sorting we used streptavidin-APC-Cy7. Directly conjugated antibodies were anti-Sca-1-PEcy7 (clone D7; eBioscience, San Diego, CA), anti-c-kit-APC (clone 2B8), anti-CD34-PE. For congenic strain discrimination, anti-CD45.1-PE and anti-CD45.2-FITC were used. Unless specified, all Abs were from BD Pharmingen, San Diego, CA. Fluorescence-activated

cell sorting was performed on a FACSSStar^{plus} cell sorter and analysis was performed with a Facscanto (Becton Dickinson, San Jose, CA).

Colony-formation assays

Myeloid clonogenic progenitor assays was performed as previously described [2]. The colony forming unit-granulocyte and macrophage (CFU-GM), granulocyte, erythroid, monocyte, and macrophage (CFU-Mix), and burst forming unit-erythroid (BFU-E) were scored at day 7 of culture. High-proliferation-potential colony forming cell (HPP-CFC) was performed as described in detail in [1]. Colonies >1mm were scored at 14 days of culture.

Cobblestone Area-Forming Cell (CAFC) assay

The CAFC assay was performed as described previously [3]. In brief, FL cells were seeded in limiting dilution on confluent monolayers of FBMD-1 stromal cells in IMDM (Mediatech, Manassas, VA), 20% horse serum (BioWhittaker, Walkersville, MD), 10^{-4} 2-Mercaptoethanol (Fisher Scientific, Fair Lawn, NJ), 10^{-5} M hydrocortisone (Sigma, St. Louis, MO), 80 U/mL penicillin, 80 mg/mL streptomycin (both Hyclone, Logan, UT). CAFC were scored at days 7, 14, 21, 28, and 35. Frequencies of CAFCs were calculated by using maximum likelihood analysis. The frequency of CAFCs equals 1 divided by the number of cells yielding 37% negative wells, as described [4].

Separation of cell cycle subfractions by cytokine culture for sorted LSK cells

The BM cells from reconstituted mice were harvested and sorted for LSK as described above. Then, LSK cells were cultured at an initial density of 5,000 cells/ml in Dulbecco's Modified Essential Medium low glucose (Life Technologies/GIBCO BRL) containing 15% heat-inactivated fetal calf serum (HyClone Laboratories), 1% penicillin (1000

U/ml)/1% streptomycin (100 mg/ml), and 1% L-glutamine (100 mg/ml). The cytokine cocktail used was rmIL-3 (50 U/ml), rmIL-6 (50 U/ml), rhIL-11 (50 ng/ml), and SCF (50 ng/ml) as described previously [5]. The LSK cells were cultured with the aforementioned cytokines for 48 h in a humidified 5% CO₂, 37°C water-jacketed incubator. A small portion of cultured cells were subjected to cell cycle analysis by propidium iodide staining, as previously described [2].

Competitive repopulation assays and serial transplantation

For competitive repopulation of total FL cells, 0.4×10^6 E14.5 FL cells from WT or p190-B^{-/-} mice (CD45.2⁺) plus 1.6×10^6 CD45.1⁺ BM cells were injected into 8-10 lethally irradiated CD45.1⁺ congenic recipient mice per experiment. At 4 month post transplant, 1×10^6 BM cells from primary recipients were harvested and used as donors for serial transplantation.

For transplantation of LSK FL cells, 1500 FL LSK (CD45.2⁺) cells plus 1.0×10^6 FL CD45.1⁺ cells were used. For transplantation of LSK cells after in vitro culture, 800,000 CD45.2⁺ cells plus 250,000 BM CD45.1⁺ cells were used.

For transplantation of LSK cells in different phases of the cell cycle, CD45.2⁺ LSK (1500) plus 250,000 CD45.1⁺ BM cells were injected into lethally irradiated CD45.1⁺ congenic mice for competitive repopulation assays.

Reconstitution of CD45.2 and lineage differentiation was monitored in PB and BM cells.

Cell adhesion and migration assays

Adhesion of c-Kit⁺ FL cells to recombinant fibronectin (FN CH296) was performed as previously described [2]. Adherent cells were harvested and assessed by progenitor assay.

Migration of c-Kit⁺ cells was measured in transwell filters coated with 10 µg/cm² FN

CH296 towards 100ug/ml stromal cell-derived factor-1 α (SDF-1 α) for 4 hours. Migrated cells were harvested for progenitor assay. The percentage of adherent or migrated progenitors was expressed as ratio of formed colony numbers from the adherent or migrated cells compared to input cells.

CFU-homing assay

FL cells (5×10^6) were injected intravenously into lethally irradiated C57Bl/6 mice. Sixteen hours after injection, femorae, tibiae, iliac crest were harvested and assessed for progenitor assay. The bone marrow-homing efficiencies were calculated on the basis of the number of CFU colonies per bone marrow divided by the number of input, assuming that femorae, tibiae and the iliac crest contains 25% of all the mouse bone marrow [6].

Analysis of F-actin

To characterize F-actin assembly, LK cells (5×10^4) were stimulated with 100 ng/mL SDF-1 α onto FN-coated slides for 30 min at 37°C. The cells were then fixed with 2% paraformaldehyde and stained with rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR). Z series of fluorescence images were captured with a Leica DMIRB fluorescence microscope (Leica Microsystem Inc., Deerfield, IL) at 63X magnification with an ORCA-ER C4742-95 camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) driven by Openlab software (Improvision Inc., Waltham, MA). Images were analyzed by deconvolution.

Cell cycle analysis

For cell cycle status analysis, BM cells from serially transplanted animals were stained with 5 μ g/mL Hoechst 33342 (Molecular Probes, Eugene, OR) for 90 minutes at 37°C then further stained for LSK as described above. Assay for LSK cells in G0 phase of the

cell cycle, cells were first incubated with 1 mg/ml Pyronin Y (Polysciences Inc., Warrington, PA) at 37°C for 45 min [7], and then stained for LSK as described above. For in vitro bromodeoxyuridine (BrdU) incorporation, we used the APC-BrdU Flow kit (BD Pharmingen, San Diego, CA) according to manufacturer's instruction. BrdU (10µM) was added to the culture medium for 30 minutes. The cells were then fixed and stained with anti-BrdU Ab, 7AAD, and for LSK as described above.

In vitro culture of HSCs and reconstitution assay

FL LSK cells (5×10^3 cells) were cultured in Stem-Span medium (StemCell Technologies Inc.), containing 50 ng/ml stem cell factor (SCF), 50ng/ml megakaryocyte growth and development factor (MGDF, Amgen, Thousand Oaks, CA), 20 ng/ml interleukin-3 (IL-3), 50 ng/ml IL-11 (Genetic Institute Inc., Cambridge, MA) and 50 ng/ml IL-6. Unless specified, all cytokines were from Peprotech, Rocky Hill, NJ. Cells were analyzed 6 - 7 days post culture and assessed for cell cycle, apoptosis, progenitor assay and competitive repopulation assay as described above.

Analysis of HSCs division

Sorted FL LSK cells were stained with 0.5µM Carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) for 10 min at 37°C. CFSE-stained cells were cultured in Stem-Span medium as described above. Cells were stained at 24h, 36h, and 48h after culture for LSK, and the fluorescence intensity of CFSE was analyzed by FACS.

Bone marrow haemoablation

5-Fluorouracil (150 mg /kg) was administered by intra-peritoneal (IP) injection.

Differential counts and lineage reconstitution was analyzed blood obtained by retro-orbital bleed at time of injection and at weekly intervals for the next five weeks.

Gene expression analysis.

RNA was isolated using RNeasy Micro Kit (Qiagen, Valencia, CA) from CD45.2⁺ LSK cells. First-strand complementary DNA synthesis was primed with random hexamers by using the Sensiscript RT Kit (Qiagen). Real-time quantitative PCR was carried out in an ABI Prism 7700 Sequence Detector by using Taqman PCR Master Mix reagent (Applied Biosystems, Foster City, CA). To generate standard curves, cDNA derived from wild-type BM RNA was used as template in a 10-fold dilution series (0- to 1,000-fold).

Relative expression was calculated by using the standard curve method (ABI Prism 7700 Sequence Detection System, user Bulletin 2; PE Applied Biosystems). Primers and probes for p16Ink4a and beta-actin were purchased from Applied Biosystems.

Statistical analysis

Data are expressed as the mean $\pm$ SEM. Differences between groups were analyzed by means of an unpaired 2-sided *t* test. A probability value of less than 0.05 was considered significant.

2.2 Results:

p190-B-deficiency is associated with normal frequency of progenitor colonies, but increased frequency of cobblestone area-forming cell (CAFC)

Since p190-B^{-/-} embryos die before birth [8], day 14.5 WT and p190-B^{-/-} embryos were used to investigate the effect of genetic deletion of p190-B RhoGAP on hematopoietic stem and progenitor cell (HSC/P) function. p190-B^{-/-} embryos exhibited

decreased total liver cellularity compared to WT controls (Figure 2.1A) due to a specific impaired late erythropoiesis at this stage of development (unpublished data).

Phenotypical analysis of FL HSC/P by flow cytometry analysis was first examined. We detected more long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs), based on c-Kit, Sca-1 and Mac-1 expression [$\text{Lin}^- \text{Sca-1}^+ \text{c-Kit}^+ \text{Mac-1}^{\text{low}}$ cells (LSKMac-1^{low}) and LSK] in p190-B FL compared to WT while the frequency of $\text{Lin}^- \text{c-Kit}^+$ (LK, hematopoietic progenitors) was comparable to WT controls (Figure 2.1B). The clonogenic capacity and differentiation potential of myeloid and erythroid progenitors were further examined using standard colony-forming unit (CFU) assays. The number and size of multipotential (CFU-MIX), erythroid (BFU-E), and myeloid progenitors (CFU-GM) colonies from WT and p190-B^{-/-} FL cells were comparable (Figure 2.1C). The frequency of HPP-CFC (immature progenitors with self-renewal potential) was increased in p190-B^{-/-} FL cells compared to WT FL cells (24 ± 3 vs 18 ± 3 per 2×10^4 FL cells, $p < 0.01$) (Figure 2.1C). Finally, the frequency of primitive HSC/P was analyzed by the cobblestone area forming cell (CAFC) assay. p190-B^{-/-} FL cells showed two-fold increase in day-28 and day-35 CAFC frequency compared with WT controls (Figure 2.1E). Consistent with the CFU frequency assessed in methylcellulose, day-7 CAFC were similar between WT and p190-B^{-/-} FL cells. In this assay, the frequency of CAFC scored at day-7 represents the CFU-GM population, while CAFC scored at day-28 and day-35 correlates with the in vivo repopulation potential of primitive hematopoietic progenitors [9]. Given the decreased cellularity of p190-B^{-/-} FL, the total number of CFU colonies and HPP-CFC colonies (Figure 2.1D) per p190-B^{-/-} liver was decreased, while the number of primitive HSC cells remained unchanged compared to WT controls. These

functional measures of HSC/P correlated with the phenotypical analysis of FL HSC/P by flow cytometry analysis. These data indicate the HSC pool size is maintained in p190-B^{-/-} fetal livers.

The loss of p190-B enhances both short-term and long-term HSC/P engraftment potential

To determine if the increased number of HSC/P defined by in vitro assays affects functional change, we first analyzed the ability of p190-B^{-/-} FL to reconstitute the hematopoiesis of lethally irradiated congenic mice. One to four months post transplantation, mice transplanted with WT or p190-B^{-/-} FL cells exhibited 95% hematopoiesis reconstitution from the donor cells, as assessed by peripheral blood (PB) chimerism (Figure 2.2A). The total white blood cell counts, neutrophils, lymphocytes, monocytes, red blood cells and platelets measured 4 months after transplantation were comparable between the genotypes (Table 1). Flow cytometry analysis indicated similar B cell, T cell and myeloid cell lineage differentiation between mice engrafted with WT and p190-B^{-/-} FL cells (Figure 2.2B). Furthermore, analysis of BM 4 months post transplantation confirmed full hematopoietic reconstitution from the donor cells. Mice transplanted with WT and p190-B^{-/-} FL cells exhibited equivalent cellularity, frequency of LK, LSK, LSKCD34⁻ and Pyronin Y negative LSK cells, and the lineage differentiation of B cell, T cell and myeloid cell (Figure 2.2C, D, E & F). These data indicate that p190-B^{-/-} FL cells retain the ability to repopulate lethally irradiated mice with normal multi lineage differentiation potential.

Table1: Blood count for reconstituted mice

Genotype	WBC (k/ μ l)	NE (k/ μ l)	LY (k/ μ l)	MO (k/ μ l)	RBC (M/ μ l)	PLT (k/ μ l)
+/+	12.2 $\pm$ 1.9	2.4 $\pm$ 0.5	9.3 $\pm$ 1.4	0.3 $\pm$ 0.1	8.6 $\pm$ 0.4	404.8 $\pm$ 68.3
-/-	10.9 $\pm$ 0.6	2.4 $\pm$ 0.2	8.1 $\pm$ 0.5	0.3 $\pm$ 0.0	8.2 $\pm$ 0.4	504.6 $\pm$ 34.5

WBC: white blood cell, NE: neutrophils, LY: lymphocytes, MO: monocytes, RBC: red blood cells, PLT: platelets

To more vigorously assess the function of p190-B^{-/-} HSC/P, we performed a competitive repopulation assay in which 0.4 x10⁶ FL cells from WT or p190-B^{-/-} E14.5 embryos were competed against 1.6 x10⁶ BM cells from congenic in lethally irradiated recipients. WT FL showed increased PB chimerism over time and reached a plateau at 65% chimera six months post transplant. p190-B^{-/-} FL cells showed higher PB chimerism than WT controls 2 and 3 months post transplantation (Figure 2.3A) (48.76 $\pm$ 13.25% vs 37.45 $\pm$ 16.87%, p<0.05; and 58.28 $\pm$ 13.91% vs 45.54 $\pm$ 19.31%, p<0.05, respectively), and reached a plateau of engraftment earlier than WT controls. In addition, mice engrafted with p190-B^{-/-} FL cells showed increased absolute numbers of CD45.2⁺ cells in the PB 2 months (6.41 $\pm$ 2.66 K/ μ l vs 4.54 $\pm$ 2.74 K/ μ l, p<0.05), 3 months (7.85 $\pm$ 2.91 K/ μ l vs 4.51 $\pm$ 2.68 K/ μ l, p<0.05), and 4 months (7.62 $\pm$ 2.52 K/ μ l vs 5.27 $\pm$ 2.97 K/ μ l, p<0.05) post transplantation compared to WT cells (Figure 2.3A). Analysis of bone marrow 4 months post transplantation confirmed that multi-lineage differentiation and LSK reconstitution of WT and p190-B^{-/-} FL were equal. These data suggest faster

recovery of p190-B^{-/-} derived hematopoiesis compared to WT controls. Enhanced engraftment could be due to increased frequency or function of HSC in p190-B^{-/-} FL.

To investigate the self-renewal capacity of p190-B^{-/-} HSC/P, serial transplants were performed in these studies, equivalent number of FL cells were used for there successive transplants with 4 months between transplants. p190-B^{-/-} cells showed enhanced engraftment compared to WT controls in the secondary recipients and were still largely detectable in the third recipients (Figure 2.3B), while the contribution of FL WT cells to hematopoietic cells decreased with successive transplants to reach near zero in the tertiary recipients(Figure 2.3B). Importantly, bone marrow analysis revealed that the maintenance of repopulation ability of p190-B^{-/-} cells arise from the maintenance of a pool of HSC. The frequencies of CD45.2⁺ BM LSK and LK cells derived from p190-B^{-/-} LSK and LK cells was comparable in primary and secondary recipients while it decreased in secondary recipients compared to primary recipients transplanted with WT FL cells (Figure 2.3C). p190-B^{-/-} LSK and LK cells were still present in the BM of tertiary recipients, although to a lower degree than in secondary recipients (data not shown). Given that the cellularity of BM was comparable between the genotypes, the total number of LSK and LK cell per femur was higher in p190-B^{-/-} transplanted secondary recipients than in WT control (data not shown) but remained in normal range compared to a non transplanted control animal. p190-B^{-/-} HSC/P maintained normal multi-lineage differentiation into both myeloid and lymphoid cells (Table 2) during serial transplantation. In tertiary recipients, p190-B^{-/-} HSC/P transplanted mice showed lineage skewing with decreased B220⁺ cells and increased myeloid cells as seen in aged mice. These data suggest that p190-B^{-/-} HSC have a repopulating advantage over WT cells due

to enhanced ability to maintain a pool of HSC/P that retain a normal lineage differentiation capacity during serial transplantation, thus indicating enhanced self-renewal of p190-B^{-/-} HSC/P.

Table 2. Lineage distribution within CD45.2+ cells in PB and BM during serial transplantation

Transplant	Organ	Genotype	B220 ⁺ cells (%)	CD3 ⁺ cells (%)	Mac-1 ⁺ or Gr-1 ⁺ cells (%)
1 st T	PB	+/+	59.0 ± 7.2	12.9 ± 7.0	20.0 ± 6.8
		-/-	64.3 ± 9.6	11.6 ± 5.5	15.7 ± 2.1
1 st T	BM	+/+	27.5 ± 7.0	4.0 ± 2.1	68.2 ± 7.5
		-/-	26.2 ± 2.1	2.8 ± 0.5	70.2 ± 3.0
2 nd T	PB	+/+	52.9 ± 4.8	28.4 ± 5.0	18.8 ± 1.6
		-/-	51.7 ± 3.1	27.6 ± 2.7	20.2 ± 2.8
2 nd T	BM	+/+	28.6 ± 11.4	6.0 ± 4.3	61.8 ± 4.8
		-/-	23.3 ± 5.9	4.6 ± 4.2	69 ± 4.8
3 rd T	PB	-/-	42.2 ± 16.6	8.1 ± 2.9	40.8 ± 15.1
3 rd T	BM	-/-	10.2 ± 5.3	1.4 ± 0.5	86.1 ± 6.1

B (B220), T (CD3ε) and myeloid (Mac-1 and Gr-1) cells were analyzed by FACS on gated CD45.2+ PB or BM cells 4 months post transplantation. Data shown are the representative experiment from 3 independent experiments, and represent the percentage of each lineage in donor-derived cells (mean ± SD, n = 6-10).

Highly enriched p190-B^{-/-} HSC exhibited enhanced long-term reconstitution capacity

To demonstrate the cell-intrinsic nature of the increase self renewal ability of p190-B^{-/-} HSC, we next tested the function of more purified LSK from E14.5 WT and p190-B^{-/-} embryos. Serial transplantation assays were performed using equivalent numbers of immunophenotypically defined LSK cells with congenic FL cells as

competitors. In the primary recipients, the PB chimerism 4 months post transplantation was similar between WT and p190 B^{-/-} LSK cells transplanted mice. However, p190 B^{-/-} HSC/P demonstrated significant increased PB chimerism in the secondary recipients 4 months post-transplantation (Figure 2.4). These data suggest that the enhanced engraftment of HSC/P in the absence of p190-B was due to a difference in the quality of p190-B^{-/-} HSC compared to WT HSC.

p190-B-deficiency is associated with increased HSC/P homing

Given the importance of GAP proteins in the regulation of the cytoskeleton, cell adhesion and migration, increased HSC engraftment of p190-B^{-/-} cells could be attributed to enhanced HSC homing to the bone marrow microenvironment. Progenitor-homing was assessed using CFU assays after 5 x10⁶ FL cells were injected into lethally irradiated recipient mice. Bone marrow of the recipient animals were harvested 16 hours post injection and assessed by CFU assay. p190-B^{-/-} FL cells demonstrated significantly increased CFU-homing to BM compared with WT cells (Figure 2.5A).

HSC/Ps home to the BM microenvironment by migrating across the endothelium in response to stromal cell-derived factor-1 α (SDF-1 α), a ligand for the chemokine receptor CXCR4. This process can depend on interaction with extracellular matrix underlining the endothelium via α 4 β 1 [CD49d/CD29: very late antigen-4 (VLA-4)] and α 5 β 1 [CD49e/CD29: very late antigen-5 (VLA-5)] integrins and HSC/P migration towards SDF-1 is enhanced in the presence of a ligand for VLA-4 and VLA-5 integrins, fibronectin (FN), in vitro. p190-B^{-/-} c-Kit⁺ cells demonstrated increased CFU-adhesion to FN compared with WT cells (Figure 2.5B). The VLA-4 and VLA-5 integrin expression in both LSK and LK cells was equivalent between p190-B^{-/-} cells and WT controls (data

not shown). Since filamentous actin (F-actin) polarization at the leading edge leads to polarization of cell shape during migration, we further investigated the role of p190-B in F-actin reorganization upon SDF-1 α stimulation and integrin ligation. Most WT cells polarized with a single F-actin leading edge made up of lamellipodia. In contrast, p190-B^{-/-} cells showed multiple membrane protrusions containing F-actin at the leading edge associated with abnormally elongated uropod (Figure 2.5C-D). Since p190-B has been shown to be regulated by integrins, we finally examined the role of p190-B in SDF-1 α induced HSC/P migration in the presence or absence of FN using transwell migration assays. No significant differences in CFU-migration were observed between WT and p190-B^{-/-} c-Kit⁺ cells in response to SDF-1 α alone (Figure 2.5E). Interestingly, p190-B^{-/-} c-Kit⁺ cells exhibited significantly increased CFU-migration towards SDF-1 α in the presence of FN compared with WT controls (Figure 2.5E).

Together, these data suggest that the loss of p190-B enhances HSC/P homing and migration which is associated with altered F-actin assembly. These changes may contribute to the enhanced engraftment capacity of HSC/P in the absence of p190-B.

p190-B-deficiency does not affect the cell cycle status of HSC/P, but is associated with higher frequency of quiescent LSK cells during transplantation

To examine the mechanism by which the loss of p190-B enhanced serial transplantation, we next determine the potential function of p190-B in HSC/P proliferation. Thus, the cell cycle status of HSC/P during serial transplantation was analyzed. The frequency of S/G2M cells in donor (CD45.2⁺) LSK and LK populations was comparable between WT and p190B^{-/-} HSC/P transplanted primary and secondary recipients (Figure 2.6A), indicating a normal proliferation rate in the absence of p190-B.

To further examine the G0 population of HSC in transplanted mice, we stained BM cells by Pyronin Y (PY) and LSK. The serial transplantation of WT HSC secondary recipients was accompanied by a decrease in frequency and total number of PY^{neg} LSK cells. In contrast, the frequency and number of PY^{neg} LSK cells in both primary and secondary recipients of p190-B^{-/-} cell transplants were higher compared to WT controls (Figure 2.6B). These results indicate that the loss of p190-B in HSC is associated with maintenance of an increased number of quiescent HSCs during serial transplantation. To further evaluate the role of p190-B on the response of the HSC/P to proliferative stress in vivo, single dose of 5-fluorouracil (5-FU) was injected into mice reconstituted with WT or p190-B^{-/-} FL cells. 5-FU is a chemotherapeutic agent which kills rapidly dividing progenitors in the bone marrow, leading to HSC proliferation. The loss of p190-B did not alter the rate of total leukocyte, neutrophil, and hemoglobin recovery after 5-FU compared with WT controls (Figure 2.6C). Thus, although the absence of p190 expression enhances the number of quiescent LSK cells during serial transplantation, it does not alter the proliferation and differentiation of HSC/P in response to chemotherapy injures. Together, these data indicate that the loss of p190-B expression does not affect HSC/P proliferation during transplantation or after subablative chemotherapy, but is associated with maintenance of a population of quiescent cells that may represent HSC self-renewal thus contributing to enhanced HSC engraftment during serial transplantation.

p190-B^{-/-} HSC/P maintain low expression of p16^{Ink4a} during serial transplantation

Previous studies have suggested that the loss of long term function of HSC during serial transplantation can depend, at least in part, on the upregulation of the cyclin

dependant kinase inhibitor p16^{Ink4a} [10]. Upregulation of p16^{Ink4a} has also been associated with defective self-renewal during aging of HSCs and neural stem cells [11, 12, 13]. We next examined expression of p16^{Ink4a} using real-time PCR. The expression of p16^{Ink4a} in the fetal liver LSK population was not detectable, in either WT or p190-B^{-/-} FL LSK cells in spite of the fact that these cells are highly proliferative. The expression of p16^{Ink4a} increased in WT donor (CD45.2⁺) BM LSK cells during serial transplantation. Donor (CD45.2⁺) p190-B^{-/-} BM LSK cells maintained a lower level of p16^{Ink4a} expression in both primary and secondary recipients compared WT controls (Figure 2.7). These data may provide an explanation for the enhanced engraftment, and may suggest that p190-B^{-/-} HSC/P possess increased self-renewal capacity.

The loss of p190-B is associated with increased RhoA activity in hematopoietic tissues

To test whether p190-B is a specific GAP protein for RhoA, lineage-negative cells isolated from mice reconstituted by WT and p190-B^{-/-} FL cells were used to perform the effector domain pull-down assay. Deletion of p190-B did not affect the expression of the Rho GTPases RhoA, Cdc42 or Rac, nor did it affect the level of active Cdc42-GTP, Rac-GTP in Lin⁻ BM cells (Figure 2.8). However, it selectively increased the RhoA-GTP content by 4-fold (Figure 2.8), suggesting p190-B knockout causes a specific gain of RhoA activity in hematopoietic cells.

Loss of p190-B does not alter the engraftment capacity of cycling HSC

To test whether RhoA links migration and cell cycle transit in HSC/P engraftment, we examined the engraftment capacity of HSC during various phases of the cell cycle. To obtain the HSCs in the S/G₂+M phases of the cell cycle, LSK cells isolated from mice

reconstituted with fetal liver cells from each genotype were cultured in DMEM containing 15% FCS with rmIL-3, rmIL-6, rhIL-11, and SCF as described above. Cell cycle status for these cultured LSK cells were monitored by PI staining [Figure 2.9A]. Before culture, 85.9% of the WT LSK cells and 79.1% of the p190-B^{-/-} LSK cells were in G₀/G₁ phases of the cell cycle. These data indicate that the majority freshly isolated LSK cells are in G₀/G₁ phases of the cell cycle, and there is no difference between WT and p190-B^{-/-} LSK cells. After 10 hours in culture, 69.4% of the WT LSK cells and 63.6% of the p190-B^{-/-} LSK cells were still in G₀/G₁ phases of the cell cycle. These cells were used as “G₀/G₁” cells in subsequent experiments. After 30 hours in culture, 74.4% of WT LSK and 75.1% of the p190-B^{-/-} LSK were in S/G₂+M phases of the cell cycle. These cells were used as “S/G₂+M” cells in subsequent experiments.

To assess the function of p190-B^{-/-} HSC during various phases of the cell cycle, 1500 “G₀/G₁” LSK or “S/G₂+M” LSK cells from the cultured cells were combined with 250,000 freshly isolated BM cells from congenic mice were injected into lethally irradiated recipients in a competitive repopulation assay. As expected, G₀/G₁ LSK cells from WT BM provided 2-fold higher levels of PB chimerism compared with equal numbers of WT S/G₂+M cells [Figure 2.9B]. Increased PB chimerism was also observed in p190-B^{-/-} G₀/G₁ LSK transplanted mice as compared with p190-B^{-/-} S/G₂+M LSK transplanted recipients. No significant differences were observed in PB chimerism between WT and p190-B^{-/-} LSK transplanted mice 4 months post-transplantation in either the “G₀/G₁” cell group or the “S/G₂+M” cell group [Figure 2.9B]. These data

suggest that the loss of p190-B does not alter the engraftment activity of HSC/P during various phases of the cell cycle in primary transplanted mice.

The loss of p190-B expression enhances HSC/P reconstitution activity after in vitro culture

Given the above data suggesting a role for p190-B in HSC quiescence and self-renewal, we postulated that the loss of p190-B expression may preserve the repopulating capacity of HSC/P after its culture. WT and p190 B^{-/-} FL LSK cells were cultured in serum free medium with a cocktail of cytokines (SCF, IL-3, IL-6, IL-11 and MGDF) in vitro for 7 days. While freshly isolated LSK cells from WT and p190-B^{-/-} mice exhibited comparable CFU frequency, the clonogenic capacity of p190-B^{-/-} LSK after 7 days in culture was 2 fold-higher compared with WT controls (Figure 2.10A). Importantly, p190-B^{-/-} LSK cells also demonstrated higher repopulating activity in vivo compared with WT cells after culture, as measured in competitive repopulation assays (Figure 2.10B). The total number of cell generated after 7 days of culture from p190-B^{-/-} FL LSK cells was similar to that from WT controls (Figure 2.10C). Cell division of WT and p190B^{-/-} LSK cells for the first 2 days of culture, examined by CarboxyFluorescein Succinimidyl Ester (CFSE) labeling, was comparable between the genotypes (Figure 2.10D). There was no detectable difference in cell cycle progression and apoptosis of WT and p190-B^{-/-} LSK cell upon culture, as assessed by Bromodeoxyuridine (BrdU) incorporation, and annexin V and 7AAD staining, respectively (Figure 2.10E and 2.10F). These data indicate that the loss of p190-B does not affect the growth, division, cell cycle progression, and apoptosis, but increases the clonogenic capacity and engraftment of cultured FL LSK cells. These

data are consistent with the hypothesis that p190-B regulates self-renewal divisions of a population of primitive HSC. Thus, modulation of p190-B is a potential target for the purpose of maintaining HSC/P repopulating capacity during in vitro culture.

2.3 Discussion

Maintenance of the HSC pool after bone marrow transplantation is essential to ensure long term hematopoiesis. Defining the regulatory pathways controlling HSC proliferation is thus of critical importance for our basic understanding of HSC activities and for the development of HSC-based therapeutics modalities. Here, we show that loss of p190-B is associated with maintenance of long term hematopoiesis during serial transplantation. The absence of p190-B expression results in the maintenance of a pool of primitive HSC in secondary and tertiary transplant recipients. The maintenance of this population may be due to a combined effect of enhanced homing, maintenance of quiescence, and increased self-renewal. Transplantation of a sorted population of p190-B^{-/-} cells enriched on HSC indicated the cell intrinsic nature of these properties. Given that the loss of p190-B leads to increased in vitro cytokine-induced expansion of primitive progenitors that sustain engraftment and normal lineage distribution, manipulation of the p190-B pathway may be of considerable importance for therapeutic applications in the future.

Homing of the circulating HSC/P to the bone marrow is a critical first step in HSC engraftment. Homing is thought to involve a series of chemotactic and adhesive events that facilitate cells crossing the blood endothelial barrier and migrating into the bone marrow medullary cavity. Considering the role of Rho GTPase family members in cytoskeletal reorganization [14], it was not surprising to find that loss of p190-B

expression was associated with changes in F-actin organization, migration, and adhesion in HSC. p190-B-deficiency may lead to increased homing and migration due a stimulatory effect on F-actin containing cellular protrusions. Although the exact relationship between these cellular changes and homing is unclear, this enhanced homing although modest may contribute to the enhanced engraftment associated with p190-B-deficiency. Since the increased engraftment seen in primary transplant recipients was also modest, it seems unlikely that changes in homing properties of p190-B^{-/-} HSC solely account for the dramatically enhanced engraftment seen in secondary and tertiary transplant.

The maintenance of a pool of HSCs in G₀ is crucial to assure a source of HSC that can support lifelong multi-lineage hematopoiesis. We provide indirect evidence suggesting that the maintenance of long term reconstitution associated with loss of p190-B is due to the self-renewal of a pool of primitive HSC. During competitive serial transplantation, phenotypic analysis showed maintenance of p190-deficient LSK in secondary recipients while the frequency of WT LSK dramatically dropped. Notably, mice transplanted with p190-B^{-/-} HSC maintained a population of quiescent cells as assessed by Pyronine-Y staining, while this population became exhausted in WT control animals. Importantly, the total numbers of LSK and LK in mice transplanted with p190-B^{-/-} cells remained comparable to a non transplanted animal. Finally, p190-B^{-/-} HSC gave rise to normal lineage distribution in serially transplanted mice. Therefore, the maintenance of a pool of quiescent HSC during engraftment associated with loss of p190-B is not a result of an uncontrolled HSC differentiation. A series of experiments further indicate that p190-B-deficiency does not affect the recovery of blood cells after

chemotherapy insult or the proportion of HSC in S/G2 + M in primary and secondary recipients. Moreover, the loss of p190-B does not alter HSC/P divisions during in vitro culture as measured by CFSE labeling assay. Therefore, these data suggest that p190-B^{-/-} HSCs maintain self-renewal divisions to efficiently regenerate the pool of HSC upon transplantation.

Little is known about the cell-intrinsic regulatory mechanisms that control self renewal of HSC. However, a tight control of the cell cycle is likely to be required and recent studies point out a critical role of cyclin dependent kinase inhibitor of the Ink4 family during this process. Ink4 proteins are direct inhibitors of the early G1 phase. Notably, the findings that p16^{Ink4a} (hereafter p16) specifically regulates the life span of HSC during serial transplantation [10] may be of particular importance. p16 expression is low in primary transplant and gradually increases during subsequent round of transplantation. This increased expression is linked to the “exhaustion” of HSC occurring during this process, since maintenance of low levels of p16 was associated with sustained capacity to support long term hematopoietic repopulation during serial BM transplantation [10], a phenotype similar to that demonstrated here in p190-B^{-/-} HSC/P. We show that p16 is undetectable is upregulated in primary and secondary transplant recipients of WT HSC/P. However, p190-B^{-/-} HSCs maintain lower levels of p16 expression after the primary and secondary transplantation than WT HSC. These data suggest a relationship between p190-B and p16. Does p190-B control the expression of p16 ? The upregulation of p16 during serial transplantation has been shown to be regulated by the p38 mitogen-activated protein kinase (MAPK) [10]. Interestingly, the level of phosphorylated p38 MAPK is substantially reduced in p190- B^{-/-} embryonic

tissues (including FL cells) [8], thereby providing a mechanism by which p190-B may control p16 expression. Previous studies indicated that the expression of p16 is low in HSCs from young mice while it is dramatically upregulated in HSCs from aged mice [12]. Furthermore, HSCs from p16^{-/-} mice were more rapidly depleted by serial transplantation than WT HSC. In contrast, p16^{-/-} HSCs derived from aged mice were protected from rapid depletion [12]. Therefore, low expression of p16 is beneficial for HSC functions while high expression is deleterious, indicating that a tight control in p16 expression is necessary for HSC functions. While the effect of high p16 expression on HSC functions in old animals was related to change in cell cycle regulation, the effect of low expression of p16 and its relationship with the cell cycle regulation of HSC remains unclear. Indeed, the absence of p16 expression in young HSC had no effect on BrdU incorporation [12]. Thus, p190-B might also regulate self renewal other than through the cell cycle. Indeed, during ex vivo culture of p190-B^{-/-} HSC, numbers of cell division and total cell numbers were comparable to WT while a net CFU expansion and increased repopulation ability were obtained in p190-B^{-/-} HSC cultures. Therefore, p190-B might participate in the regulation of symmetric cell division enhancing the probability of self-renewal division. A propensity to execute symmetric division might also prevent the exhaustion of p190-B^{-/-} HSC pool during serial transplantation.

Initial studies have reported that homozygous deletion of p190-B results in central nervous system defects leading to perinatal lethality [8]. Furthermore, p190-B regulates cell size and adipocyte/myocyte cell fate decision during development [8]. p190-B is also critically involved in cell proliferation. These effects have been attributed

to Rho activity and its effector ROCK through modulation of the IGF-I signaling. Our study did not reveal any changes in hematopoietic cell size (data not shown), growth or cell differentiation associated with p190-B-deficiency, which may reflect the context-dependency of p190-B functions. Indeed, p190-B can be regulated by phosphorylation and dephosphorylation depending on the stimulus, (e.g. cytokines, chemokines and integrins), a process that may ultimately influence its function. Although our data demonstrated deletion of p190-B selectively increased the RhoA-GTP content without changing the level of active Rac-GTP and Cdc42-GTP in Lin-negative cells, the contribution of Rho in p190-mediated HSC self-renewal remains to be examined in detail.

Previous studies have reported that decreasing RhoA activity, by means of expression of dominant negative mutant RhoA, leads to enhanced HSC engraftment, enhanced proliferation of progenitors and decreased HSC/P adhesion and SDF-1 α directed migration in vitro [15]. In our model, higher RhoA activity through loss of p190-B is associated with enhanced engraftment, enhanced quiescence and enhanced migration but no effect on proliferation. These studies indicate that both increased and decreased activation of RhoA results in enhanced HSC long-term engraftment. However, the mechanism leading to this enhance engraftment appears distinct. There are several potential explanations for these findings. Cycling between GTP- and GDP-bound states may be required for the biological function, and shifting the level of activity predominantly to either side may block signaling pathways that emanate from the turnover. An abnormal ratio between GTP-bound and GDP-bound RhoA (high or low) may in both cases lead inappropriate activation of downstream effectors, a process that may affect distinct cellular functions. Related to this hypothesis, the role of Rho-GTPase

signaling in the particular function may be tightly controlled both spatially and temporally. Alternatively, the biological function may depend on a critical level of activity necessary throughout the cells. Previous studies have demonstrated that low-level expression of both constitutively active and dominant negative forms of RhoA disrupted the gate and fence functions of tight junctions in MDCK cells [16], both activation and inhibition of RhoA and Rac1 disrupt wound closure of bronchial epithelial cell sheets [17]. Moreover, dominant mutant studies have limitations in defining roles of individual GTPases and fine aspects of their regulation. Dominant-negative Rho GTPase mutants are believed to act by titrating guanine nucleotide exchange factors [18]. If two Rho GTPases share a common exchange factor (most exchange factors have activities toward several Rho GTPases, at least *in vitro*), then expressing a dominant-negative mutant of one GTPase could interfere with the function of another GTPase. Finally, due to the large size of p190-B, the presence of multiple protein motifs, the ability to act as a scaffold protein and the capacity to interact with various chromatin-remodeling complexes through its GAP domain, p190 may regulate some HSC functions that are not mediated by Rho activity, as recently suggested in other cell types [19]. Further understanding of the molecular pathways involved in the regulation of HSC function by p190-B will provide the means to dissect the specific role of p190-B in HSC engraftment.

Since RhoA has been shown to coordinate cell cycle transit and migration in non-hematopoietic cell, here we assessed the engraftment capacity of p190-B^{-/-} HSC during various phases of the cell cycle. To obtain synchronized HSC/P either in G₀/G₁ or in S/G₂+M phases of the cell cycle, we used a culture system to stimulate the sorted LSK cells to enter the cell cycle. Under this culture condition, majority LSK cells can enter the

cell cycle without loss of viability. Regardless of which genotype, cycling cells were deficient in engraftment potential, similar to what has been previously reported in human [20] and murine [21] systems. By F-actin staining, S/G₂+M p190-B^{-/-} LSK showed increased frequency of polarized cells compared to WT S/G₂+M cells (data not shown), which suggesting that the migration capacity may be increased in S/G₂+M p190-B^{-/-} LSK cells. Thus we expected that p190-B^{-/-} LSK in S/G₂+M could show increased engraftment capacity compared to WT controls. However, our data demonstrated that p190-B^{-/-} LSK in S/G₂+M showed similar engraftment activity compared to WT controls, suggesting that the loss of p190-B does not alter the engraftment activity of HSC/P during various phases of the cell cycle in primary transplanted mice.

The role of p190-B in the maintenance of self-renewal of HSC suggests that p190-B is a potential target for manipulation for the purpose of HSC expansion in vitro. The expansion of rigorously defined HSC in vitro has proven a great challenge. Understanding the mechanisms of self-renewal of HSCs will facilitate the amplification of HSCs in culture while maintaining stem cell function in cell and gene therapies. The loss of p190-B enhances HSC long-term engraftment potential in secondary and tertiary transplanted recipients with normal lineage differentiation profiles and normal proliferation rate. Furthermore, we show that p190-B-deficiency improves the maintenance of HSC functions upon exposure to cytokines in vitro. Importantly, the development of leukemia was not been observed during our studies in mice transplanted with p190-B^{-/-} HSC/Ps. Therefore, our study provides first evidence that a member of the GAP family plays crucial and unique roles in HSC functions that primarily relate to the regulation of self-renewal. The cell intrinsic nature of the effects of p190-B loss on

enhanced HSCs self-renewal also provides a powerful experimental handle for exploration of mechanisms of self-renewal.

2.4 Figure Legends:

Figure 2.1 p190-B^{-/-} FL cells show normal progenitor frequency, but increased HSC frequency compared to WT FL cells

(A) Fetal liver cellularity of WT and p190-B^{-/-} E14.5 embryos. Data shown are means $\pm$ SD per liver (n=24).

(B) Frequency of Lin⁻Sca-1⁺c-Kit⁺Mac-1^{low} cells (LSKMac-1^{low} cells), LSK cells, and LK cells of E14.5 fetal liver cells. Histograms show mean $\pm$ SD, n=24.

(C&D) Myeloid and high-proliferative-potential progenitors quantified by methylcellulose culture with SCF, IL-3, G-CSF and EPO for 7 days or with SCF, IL-3, IL-1a, GM-CSF and M-CSF for 14 days, respectively. Data are frequency of colonies (C), or total number of colonies per liver (D) (Mean $\pm$ SD, n=9-12, from 3 independent experiments).

(E) Primitive progenitor cells evaluated using the CAFC assay. Total fetal liver cells were cultured with FBMD-1 cells for 35 days. CAFC frequency was determined on the day noted on the *x*-axis. Data represent mean number $\pm$ SEM (** p<0 .01, n = 3 independent experiments, n = 2–3 embryos per group in each experiment).

Figure 2.2 p190-B^{-/-} FL cells are able to reconstitute the hematopoiesis in lethally irradiated mice

(A) Reconstitution of FL cells. 2×10^6 total FL cells from E14.5 embryos were injected into lethally irradiated Ly5.2 mice. Histogram represents the percentage of donor-derived cells in the PB at the indicated time points after transplantation (mean $\pm$ SD, n=5).

(B & C) Lineage reconstitution in PB and BM for reconstituted mice. Donor contribution to B (B220), T (CD3 ϵ) and myeloid (Mac-1 and Gr-1) cells were analyzed by FACS on gated CD45.2⁺ PB (B) and BM (C) cells at 4 months post transplantation. Histogram represents the percentage of each lineage in donor-derived cells (mean $\pm$ SD, n=5).

(D) BM cellularity of reconstituted mice at 4 months post transplantation. Histogram represents the BM cellularity in reconstituted mice as contained in 2 femoral, 2 tibiae and pelvis (mean $\pm$ SD, n=5).

(E) Frequency of LSKCD34⁻, LSK and LK cells in the BM of reconstituted mice. BM cells from reconstituted mice at 4 months post transplantation were stained with anti-CD45.2, c-Kit, Sca-1, CD34, and a cocktail of lineage Abs, CD34⁻ cells in the LSK fraction were detected by FACS. Histogram represents the percentage of LSKCD34⁻, LSK cells and LK cells (mean $\pm$ SD, n=5).

(F) Frequency of quiescent LSK cells in the BM of reconstituted mice. BM cells from reconstituted mice were isolated 4 months after transplantation, and stained with anti-CD45.2, c-Kit, Sca-1, a cocktail of lineage Abs, and Pyronin Y dye for FACS analysis. Histogram represents the percentage of Pyronin Y negative cells in donor-derived LSK cells (mean $\pm$ SD, n=5).

Figure 2.3 Enhanced long term engraftment capacity of p190-B^{-/-} fetal liver cells

WT and p190-B^{-/-} E14.5 FL cells (0.4×10^6 CD45.2⁺ FL) plus 1.6×10^6 BM competitor (CD45.1⁺) cells were injected into lethally irradiated recipient mice.

(A) The number of CD45.2 vs CD45.1 cells in the PB was determined between one to six months. Histograms show the percentage of CD45.2⁺ cells (left panel) and the absolute number of CD45.2⁺ cells (right panel) in the PB at the indicated time points after transplantation (mean $\pm$ SD, *p<0.05, n=24).

(B) Serial transplantation of WT and p190-B^{-/-} FL cells. Average percentage of CD45.2⁺ cells in PB (left panel) and BM (right panel) at 4 months post transplant of primary, secondary and tertiary recipients. Mean $\pm$ SD, n=6-10, one representative experiment from 3 independent experiments.

(C) Frequency of LSK (left panel), and LK cells (right panel) in CD45.2⁺ BM cells of primary and secondary recipients at 4 months post transplantation. Mean $\pm$ SD, n=6-10, one representative experiment from 3 independent experiments.

(D) Flow cytometry analysis of LSK frequency in CD45.2⁺ BM cells from secondary recipient. Data shown are the frequencies of LSK cells (mean $\pm$ SD, n=6, * p<0.01).

Figure 2.4 Highly enriched p190-B^{-/-} HSCs (LSK FL cells) exhibit enhanced reconstitution capacity compared to WT cells

Competitive repopulation assay of E 14.5 LSK FL cells. LSK FL cells (1500) plus 1.0 x 10⁶ FL competitor cells were injected into lethally irradiated recipients. Histograms show the percentage of CD45.2⁺ in the PB 4 months post primary and secondary transplantation (mean $\pm$ SD, n=6).

Figure 2.5 Increased homing of p190-B^{-/-} HSC/P to BM

(A) Homing of HSC/P to bone marrow. FL cells (5 x 10⁶) from E14.5 embryos were injected into lethally irradiated mice. Data shows the percentage of progenitors (CFU) in

the BM divided by CFU activity of input cells (mean $\pm$ SEM, n=3 independent experiments, n=5 recipient mice / genotype)

(B) Adhesion of HSC/Ps to FN. c-Kit⁺ FL cells were allowed to adhere for 1 h at 37°C to FN. Adherent cells were collected for CFU assays. Data represents the percentage of adherent progenitors (CFU) divided by CFU activity of input c-Kit⁺ FL cells (mean $\pm$ SEM, n=3 independent experiments).

(C & D) F-actin assembly of HSC/Ps. c-Kit⁺Ter119⁻ FL cells were stimulated with SDF-1 α on FN-coated slides for 30 minutes. The adherent cells were stained with rhodamine labeled phalloidin. Representative images from WT and p190-B^{-/-} HSC/Ps (C). Data represents the percentage of FL HSC/Ps with multiple membrane protrusions (D) (mean $\pm$ SEM, n=3).

(E) Migration of HSC/Ps. C-Kit⁺ FL cells were allowed to migrate for 4 hours at 37°C towards SDF-1 α in transwell through a membrane coated with or without FN. The migrated cells were then collected for CFU assay. Data represents the percentage of migrated progenitors (CFU) divided by CFU activity of input c-Kit⁺ FL cells (mean $\pm$ SEM, n=4 independent experiments).

Figure 2.6 Maintenance of HSC quiescence in p190-B^{-/-} HSC/P transplanted mice during serial transplantation

(A) Cell cycle status of WT and p190-B LSK and LK cells. BM cells of primary and secondary recipients were stained for LSK and Hoechst 33342 dye. Histograms represent the percentage of CD45.2⁺ LSK (left panel) or LK (right panel) cells in S/G2M phases of the cell cycle (mean $\pm$ SD, n = 4).

(B) Quiescence of LSK cells. BM cells from primary and secondary transplant recipients were stained for LSK and Pyronin Y (PY) dye. Data represent the percentage of PY⁻ CD45.2⁺ LSK cells (mean $\pm$ SD, n = 6 to 8).

(C) The effect of p190-B-deficiency on the recovery of hematopoiesis in vivo under conditions of stress. Kinetics of white blood cell (WBC), neutrophils and hemoglobin recovery following a single dose of 5-fluorouracil (mean $\pm$ SEM, n=3 independent experiments, n=5 mice per group and experiment).

Figure 2.7 Maintenance of low expression of p16^{Ink4a} in HSC from p190-B^{-/-} HSC/P transplanted mice during serial transplantation

p16^{Ink4a} expression of FL LSK cells and donor-derived BM LSK cells during serial transplantation. FL LSK cells and CD45.2⁺ BM LSK cells from primary and secondary transplant recipients 4 months post transplantation were purified by FACS sorting, and used for RNA preparation. The relative mRNA expression level of p16^{Ink4a} was examined by quantitative real-time PCR and normalized to β -actin expression. Data represent the ratio of p16^{Ink4a} transcript level over β -actin (mean $\pm$ SD, n=3).

Figure 2.8 The effects of genetic deletion of p190-B on Rho GTPase activities in lineage-negative hematopoietic cells

Lineage negative (Lin⁻) isolated from mice transplanted with WT and p190-B^{-/-} FL cells were subjected to effector domain pull-down assays, and the activities of Cdc42, Rac (detected by GST-PAK1), and RhoA (detected by GST-Rhotekin) were examined and compared in the anti-RhoA, Rac, or Cdc42 immunoblots. Blotting of the respective total-cell lysates was carried out in parallel. Relative amounts of GTP-bound form of the

GTPases were quantified by densitometry measurements and normalized to those of the WT cells.

Figure 2.9 Engraftment of Lin⁻Sca-1⁺c-Kit⁺ (LSK) cycling and noncycling cells.

(A) Cytokine culture for sorted LSK cells. LSK cells were cultured at an initial density of 5,000 cells/ml in DMEM containing 15% FCS with rmIL-3, rmIL-6, rhIL-11, and SCF. Cell cycle status of cultured LSK cells were monitored by propidium iodide staining. Data shown are the representative flow profiles of PI staining for LSK cells at 0h, 10h and 30h during the culture.

(B) Competitive repopulation assays. Cultured CD45.2⁺ LSK (1500) plus 250,000 CD45.1⁺ BM cells were injected into lethally irradiated CD45.1⁺ congenic mice for competitive repopulation assay. Histogram represent the percentage of CD45.2 cells in the PB at the indicated time points after transplantation (mean $\pm$ SD, n=2 independent experiments, n = 5 for WT G₀/G₁ mice, n = 7 for WT S/G₂+M mice, n = 6 for p190-B^{-/-} G₀/G₁ mice, n = 9 for p190-B^{-/-} S/G₂+M mice).

Figure 2.10 Engraftment activity of HSC/Ps after in vitro culture

(A) CFU capacity of FL LSK cells before (750 cells per plate) and after (8000 cells per plate) culture. Data represent number of colonies/input cells on day 0 and day6 during the culture. Data represent mean $\pm$ SEM (n=3 independent experiments).

(B) CFU expansion of cultured FL LSK cells expressed by fold increase (mean $\pm$ SD, n=3, one representative experiment from 3 independent experiments) or by relative fold increase normalized to WT controls (mean $\pm$ SEM, n=3 independent experiments).

(C) Competitive repopulation assay of cultured FL LSK cells. Cultured cells (800,000) plus 250,000 competitor BM cells were injected into lethally irradiated mice. Histogram

represents the percentage of donor (CD45.2⁺) cells in the PB at the indicated time points after transplantation (mean $\pm$ SD, *p<0.05, n=5).

(D) Cell division analysis of cultured FL LSK cells. FL LSK cells were labeled with fluorescent dye CFSE and cultured in serum free medium with SCF, IL-3, IL-6, IL-11 and MGDF for 2 days. Cells were collected at the indicated time points and the number of LSK cells were determined and analyzed. Data shown are the representative flow profiles of fluorescent intensity of CFSE⁺LSK cells at 0h, 24h, 36h and 48h during culture.

(E-G) Cellgrowth, cell cycle and apoptosis analysis of cultured FL LSK cells. Fetal liver LSK cells were cultured as above for 7 days. E. Cell count (mean $\pm$ SEM, n=6 independent experiments). F. Cell cycle analysis. Cells were labeled with BrdU *in vitro* for 30 minutes, and stained for LSK. Data represent the percentage of BrdU positive cell in LK or LSK cells (mean $\pm$ SD, n=3). G. Apoptosis analysis. Cells were stained with Annexin V and 7-AAD and for LSK. Histogram represents the percentage of Annexin V positive cells in LSK cells (mean $\pm$ SD, n=3).

2.5 Figures

Figure 2.1

Figure 2.2

Figure 2.3

Figure 2.4

Figure 2.5

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Figure 2.6

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Figure 2.7

Figure 2.8

Figure 2.9

A. Cytokine culture for sorted LSK cells:

B.

Figure 2.10

Figure 2.10

2.6 References:

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Chapter III

Summary and future studies

3.1 Summary

Here we utilized p190-B RhoGAP KO mice to study the function of p190-B RhoGAP in HSC engraftment. Serial transplantation assay in competitive settings revealed enhanced engraftment capacity in p190-B^{-/-} HSC transplanted secondary and tertiary recipients compared to WT controls, this was also evident when sorted, phenotypically defined HSCs (LSK cells) were used. These data strongly suggest that genetic-deletion of p190-B results in intrinsically enhanced HSCs long-term repopulating activity. The enhanced long-term engraftment capacity of p190-B^{-/-} HSC accompanied by higher frequency of PY^{negative} quiescent LSK pool upon secondary and tertiary transplantation supports a role of p190-B in HSC self-renewal. These changes are associated with a lack of upregulation of p16^{Ink4a} in HSC (Figure 3.1A). Moreover p190-B^{-/-} FL cells is associated with increased homing compared to WT controls (Figure 3.1B). These data suggest that the enhanced engraftment of capacity of p190-B^{-/-} HSC may be due to a combined effect of enhanced homing, maintenance of quiescence, and increased self-renewal. Further, cultured p190-B^{-/-} HSC maintained higher engraftment activity compared to WT controls, which suggests that p190-B could a potential target to improve the expansion of HSC in vitro for clinical application. However the molecular mechanisms which are involved in the regulation of HSC homing, engraftment and self-renewal by p190-B remain to be studied in the future.

Figure 3.1 Proposed model of the role of p190-B in the regulation of HSC homing and engraftment.

3.2 Future studies

Understanding how p190-B is integrated into the complex signaling networks that control fundamentally important processes in stem cell biology ranging from regulation of homing and engraftment, to self-renewal, and the control of proliferation/differentiation could provide novel approaches for clinical applications.

To examine whether p190-B favors the symmetric self-renewal division of HSC

The enhanced long-term engraftment capacity of p190-B^{-/-} HSC accompanied by higher frequency of LSK cells and higher frequency of PY^{negative} quiescent LSK pool upon secondary and tertiary transplantation supports a role of p190-B in HSC self-renewal. Moreover, the increased quiescent HSC pool in the p190-B^{-/-} HSCs transplanted mice is not due to a G₀/G₁ phase block for HSCs or a prevention of cell division of HSCs. These data suggest that p190-B may favor symmetric self-renewal division. Clonal assay for HSC will be performed in the future studies to quantitatively examine the self-renewal capacity of HSCs in the absence of p190-B. In vivo self-renewal of HSCs will be studied by marking HSCs with retroviral integration [1, 2, 3]. In vitro HSC self-renewal will also be investigated by single cell culture and transplantation system [4]. In this assay, single HSC will be sorted and cultured in serum-free medium with cytokines (SCF and TPO). When a single cell gave rise to two daughter cells by day 2 of culture, one of the daughter cells was transferred into a new well by micromanipulation. Two cells derived from each paired daughter cell at day 3 of culture will be individually transplanted into an irradiated mouse. Thereby which daughter cell retains repopulating activity will be evaluated. These in vivo and in vitro self-renewal assays for HSC will allow us to assess the self-renewal capacity of HSC in the absence of p190-B.

To examine how p190-B regulates HSC self-renewal

Our knowledge about the regulation of the self-renewal process in HSC is limited. A combination of cell-intrinsic and external regulatory mechanisms has been suggested to control HSC self-renewal. In our studies, we speculate that the loss of p190-B down regulates the activity of p38 MAPK, and this may lead to the lower expression of p16^{Ink4a} in p190-B^{-/-} HSCs and result the enhancement of long-term engraftment capacity. In *Atm*^{-/-} mice, elevation of reactive oxygen species (ROS) levels induces HSC-specific phosphorylation of p38 MAPK, leads to the upregulation of p16^{Ink4a} accompanied by a defect in the maintenance of HSC quiescence [5]. The ROS level and the activity of p38 MAPK in the BM LSK cells from WT and p190-B^{-/-} HSCs transplanted mice will be examined by flow cytometry to test whether the lower expression of p16^{Ink4a} in p190-B^{-/-} HSCs is due to the lower ROS level and/or lower p38 MAPK activity. Overexpression of p16^{Ink4a} in p190-B^{-/-} HSCs will be performed to test whether the increased expression of p16^{Ink4a} reverses the enhanced long-term HSC engraftment phenotype in the absence of p190-B. Previous studies showed that the expression of p19^{Arf}, p15^{Ink4b} and p18^{Ink4c} were also upregulated in a p38 MAPK-dependent manner in *Atm*^{-/-} mice [5]. In our studies, we only check the expression of p16^{Ink4a} by real-time PCR. Microarray analysis using CD45.2⁺ BM LSK cells from WT and p190-B^{-/-} HSCs transplanted mice will be performed to further dissect the genetic pathways involved in the regulation of HSC self-renewal by p190-B.

To investigate whether p190-B regulates HSC function via RhoA downstream effectors

The p190-B is evidently potent inhibitors of Rho activity: the combined activity of both p190-A and p190-B accounts for the majority of RhoA inhibitory activity in

fibroblast lysates, with p190B displaying five times the activity of p190A [6].

Interestingly, p190-B is known to regulate both Rho and Rac GTPases [7, 8], and the specificity of p190-B can be switched in vitro by certain phospholipids [9]. Our data demonstrated that deletion of p190-B does not affect the expression of the Rho GTPases RhoA, Cdc42 or Rac, nor does it affect the level of active Cdc42-GTP, Rac-GTP in Lin-BM cells. However, it selectively increased the RhoA-GTP, suggesting p190-B knockout causes a specific gain of RhoA activity in hematopoietic cells. To examine whether p190-B regulates HSC function via RhoA downstream effectors, the knockdown of expression of RhoA by shRNA in p190-B^{-/-} HSCs will be performed. Homing and engraftment assay will be further used to test whether the decreased expression of RhoA in p190-B^{-/-} HSCs reverses the enhanced homing and engraftment of HSCs in the absence of p190-B.

RhoA regulates diverse cellular processes including cytoskeletal reorganization, transcription and transformation. In the case of mammalian RhoA, a number of potential effector molecules have been identified which interact preferentially with the GTP-bound form of RhoA. RhoA effectors include two families of protein kinases, the ROCKs (also known as ROKs) [10] and PKN-related kinases (the PRK family) [11]. In addition, RhoA can interact with the myosin binding subunit of MLC phosphatase [12]; putative adaptor proteins such as Rhotekin, Rhophilin and Citron [13, 14, 15], two mouse Diaphanous homologues, p140mDia and mDia2 [16], and Kinectin [17]. Biochemical studies have provided strong evidence that the ROCKs, MLC phosphatase and possibly mDia participate in RhoA-induced cytoskeletal reorganization. RhoA-induced signalling is also implicated in gene transcription, cell-cycle progression, and cell transformation. Functional RhoA is required for serum- and LPA-induced activation of the transcription

factor SRF, and activated forms of the protein can activate SRF in the absence of external stimuli [18]. Functional RhoA is required for the G₁-S transition in Swiss 3T3 cells, and microinjection of activated RhoA protein can induce quiescent Swiss 3T3 cells to enter the cell cycle [19, 20]. Our knowledge about how these and likely other unknown effectors downstream of RhoA are involved in the regulation of HSC function is limited. To dissect their relevance in HSC regulation will be another part of future studies.

To study whether p190-B is a target for HSC expansion in vitro

Elucidating the molecular machinery that governs self-renewal of HSC could lead to new therapeutic interventions that would include their in vitro expansion under conditions where HSCs are limited in numbers. To achieve significant numbers of HSCs in culture will enhance the success of a range of transplant-based therapies for malignant and genetic disorders. However, the search for factors that can stimulate HSC self-renewal has proven difficult. The role of p190-B in the quiescence maintenance and self-renewal of HSC suggests that p190-B is a potential target for HSC expansion in vitro. The loss of p190-B enhances HSC long-term engraftment potential in secondary and tertiary transplanted recipients with normal lineage differentiation profiles and normal proliferation rate. No leukemia was found in p190-B^{-/-} HSC transplanted mice. These data suggest that the loss of p190-B does not lead to transformation of HSC, or skew lineage commitment in hematopoietic differentiation, but increases the self-renewal capacity of HSC in the absence of p190-B. Knockdown p190-B expression by lentivirus-mediated shRNA or inhibition of p190-B function by specific pharmaceutical inhibitors could be two attractive strategies to improve HSCs expansion in vitro. These strategies

will be applied to test whether p190-B-deficiency enhances human HSC expansion in vitro, which could result a direct clinical application for cellular and gene therapy.

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