

IN VIVO HEMATOPOIETIC CELL ENGRAFTMENT IS MODULATED BY  
DPPIV/CD26 INHIBITION AND RHEB2 OVEREXPRESSION

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## ABSTRACT

Timothy Brandon Campbell

### IN VIVO HEMATOPOIETIC CELL ENGRAFTMENT IS MODULATED BY DPPIV/CD26 INHIBITION AND RHEB2 OVEREXPRESSION

Hematopoietic cell transplantation (HCT) is an important modality used to treat patients with hematologic diseases and malignancies. A better understanding of the biological processes controlling hematopoietic cell functions such as migration/homing, proliferation and self-renewal is required for improving HCT therapies. This study focused on the role of two biologically relevant proteins, dipeptidylpeptidase IV (DPPIV/CD26) and Ras homologue enriched in brain 2 (Rheb2), in modulating hematopoietic cell engraftment. The first goal of this study was to determine the role of the protein DPPIV/CD26 in modulating the engraftment of human umbilical cord blood (hUCB) CD34<sup>+</sup> stem/progenitor cells using a NOD/SCID mouse xenograft model, and based upon previous work demonstrating a role for this enzyme in Stromal-Derived Factor-1/CXCL12 mediated migration and homing. Related to this first goal, pretreatment with an inhibitor of DPPIV/CD26 peptidase activity increased engraftment of hUCB CD34<sup>+</sup> cells *in vivo* in recipient Non Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice while not disturbing their differentiation potential following transplantation. These results support using DPPIV/CD26 inhibition as a strategy for enhancing the efficacy of cord blood transplantation. The second goal was to determine, by overexpression, the role of the Rheb2 in affecting the balance between proliferation and *in vivo* repopulating activity of mouse hematopoietic cells. Rheb2 is

known to activate the mammalian target of rapamycin (mTOR) pathway, a pathway important in hematopoiesis. Rheb2 overexpression increased the proliferation and mTOR signaling of two hematopoietic cell lines, 32D and BaF3, in response to delayed IL-3 addition. In primary mouse hematopoietic cells, Rheb2 overexpression enhanced the proliferation and expansion of hematopoietic progenitor cells (HPCs) and phenotypic hematopoietic stem cells (HSCs) *in vitro*. In addition, HPC survival was enhanced by Rheb2 overexpression. Using *in vivo* competitive repopulation assays, Rheb2 overexpression transiently expanded immature HPC/HSC populations shortly after transplantation, but reduced the engraftment of total transduced cells. These findings support previous work showing that signaling proteins able to enhance the proliferative status of hematopoietic stem cells often cause exhaustion of self-renewal and repopulating ability. These studies of hematopoietic engraftment modulated by both of these molecules provide information which may be important to future work on HCT.

Hal E. Broxmeyer Ph.D., Chair

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## LIST OF ABBREVIATIONS

$\alpha$ MEM	Alpha modified Eagle's medium
APC	Allophycocyanin
ATCC	American type culture collection
BFU-E	Burst-forming unit- erythroid
BM	Bone marrow
BSA	Bovine serum albumin
CDKI	Cyclin dependent kinase inhibitors
cDNA	complementary deoxyribonucleic acid
CFU-GEMM	Colony-forming unit-granulocyte erythroid macrophage megakaryocyte
CFU-GM	Colony-forming unit-granulocyte macrophage
c-kit	SCF receptor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
Cy	Cyclophosphamide
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPPIV/CD26	Dipeptidyl peptidase IV
ECM	Extracellular matrix
EDTA	Ethylene Diamine Triacetic Acid
eGFP	Enhanced green fluorescent protein
eIF-4E	Eukaryotic initiation factor 4E
EPO	Erythropoietin
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
5-FU	5-Fluorouracil
GAP	GTPase activating protein

GCP-2	Granulocyte chemotactic protein-2
G-CSF	Granulocyte-colony stimulating factor
GDP	Guanine diphosphate
GEF	GTP exchange factor
GIP	Glucose-dependent insulintropic peptide
GLP-1	Glucagon-like peptide-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanine triphosphate
GVHD	Graft versus host disease
HCT	Hematopoietic cell transplantation
HLA	Human leukocyte antigen
Hox	Homeo box
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
hUCB	Human umbilical cord blood
IFN- $\gamma$	Interferon-gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP-10	IFN- $\gamma$ -inducible protein-10
I-TAC	IFN-inducible T-cell $\alpha$ -chemoattractant
IUHCT	<i>In utero</i> hematopoietic cell transplantation
KSL	C-kit <sup>+</sup> Sca-1 <sup>+</sup> Lin <sup>-</sup>
Lin	Lineage
LS	Lineage <sup>-</sup> Sca-1 <sup>+</sup>
LT-HSC	Long-term hematopoietic stem cell
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
mBM	Mouse bone marrow
M-CSF	Macrophage colony-stimulating factor

MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
Mig	Monokine induced by IFN- $\gamma$
MIP-1 $\alpha$	Macrophage inflammatory protein-1 $\alpha$
MIP-1 $\beta$	Macrophage inflammatory protein-1 $\beta$
MNC	Mononuclear cells
MPB	mobilized peripheral blood
MPP	Multi-potent progenitor
MSCV	Murine stem cell virus
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NS	Not significant
PB	Peripheral blood
PBS	Phosphate buffered saline
PKD1	Phosphoinositol-dependent protein kinase 1
PE	Phycoerythrin
PeCy5.5	Phycoerythrin Cy5.5
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylene difluoride
PWMSCM	Pokeweed mitogen spleen conditioned medium
RANTES	Regulated on activation, normal T cell expressed and secreted
RE	Restriction endonuclease
Rheb	Ras homologue enriched in brain-2
RPMI	Roswell park memorial institute medium
Sca-1	Stem cell antigen-1/Ly-6A/E

SCF	Stem cell factor, aka Steel factor
SCID	Severe combined immunodeficiency
SDF-1	Stromal derived factor-1
SDS	Sodium dodecyl sulfate
SFT	Stem cell factor + Flt3L + Thrombopoietin
SHP-2	SH2 domain-containing tyrosine phosphatase 2
shRNA	Short-hairpin ribonucleic acid
S6K1	Ribosomal S6 protein kinase 1
STAT	Signal transducer and activator of transcription
ST-HSC	Short-term hematopoietic stem cell
Tat	Transactivator protein
TBI	Total body irradiation
TG	Tris-glycine
TPO	Thrombopoietin
TSC	Tuberous sclerosis complex

## INTRODUCTION

Hematopoiesis is a highly coordinated physiological process by which immature hematopoietic stem cells (HSCs) differentiate to form mature lineages of the blood, as well as undergo self-renewal to prevent exhaustion and depletion. Hematopoiesis has generally been thought to occur in a hierarchical manner in which HSCs differentiate to form immature hematopoietic progenitor cells (HPCs)(Bellantuono 2004), and undergo further expansion and differentiation to form mature HPCs (Bellantuono 2004). Mature HPCs can further expand and differentiate into each terminal lineage of the blood system, including all myeloid and lymphoid derivatives. Self-renewal potential, measured by the ability of the HSCs to long-term repopulate transplant recipients, is contained mainly within the immature HSC populations. Elucidating the mechanisms involved in controlling HSC repopulation and self-renewal has been a focus of hematopoietic research for many years with immense clinical implications. Before discussing ways in which this study has approached this issue, an overview of hematopoiesis and transplantation biology will be discussed.

In the mouse system, HSCs are typically subclassified as long-term and short-term repopulating HSCs (LT-HSC and ST-HSC) (Bellantuono 2004; Morrison and Weissman 1994; Christensen and Weissman 2001; Shizuru et al. 2005). ST-HSCs can repopulate a transplant recipient in a transient manner, unable to maintain stable long-term repopulation (Morrison and Weissman 1994; Shizuru et al. 2005; Christensen and Weissman 2001). LT-HSC by contrast, are responsible for long-term repopulation of transplant recipients which can last the entire life of the animal as well as repopulate secondary and tertiary recipients (Bellantuono 2004; Morrison and Weissman 1994; Christensen and Weissman 2001). Only the LT-HSCs, ST-HSCs and to a minor extent, the MPPs, contain multi-lineage repopulating ability. These populations are distinguished

based on phenotypic and functional characteristics. Both of these populations lack lineage antigens which identify mature B cells, T cells, neutrophils, macrophages, and erythrocytes as indicated by the Lin<sup>-</sup> designation and express cell-surface molecules including c-kit (stem cell factor receptor) and stem cell antigen -1 (Sca-1). The presence or absence of other cell-surface antigens such as Flk-2 and Thy1.1 can be used to further phenotypically distinguish these populations (Bellantuono 2004; Christensen and Weissman 2001). Other hematopoietic populations can be distinguished by surface markers and functional assays. These include the multi-potent progenitors (MPP) which are immediate downstream derivatives of the HSC cells and are able to differentiate to both myeloid and lymphoid lineages; however, they lack measurable self-renewal ability and cannot maintain long-term engraftment in a transplant recipient (Bellantuono 2004; Shizuru et al. 2005). These cells further differentiate to common myeloid and common lymphoid progenitors (CMPs and CLPs) which can differentiate to form all the cells of either the myeloid or lymphoid lineages but, for the most part, are restricted to either one of these two lineages and do not crossover (Shizuru et al. 2005; Akashi et al. 2000; Kondo et al. 1997). Finally, the CMPs and CLPs differentiate to more restricted, mature progenitors such as CFU-GM (granulocyte-macrophage progenitor), BFU-E (erythroid progenitor) and others. All of these progenitors have substantial ability to expand and differentiate towards their mature, daughter cell types, e.g. neutrophils or B cells, and lack self-renewal ability. With differentiation of these progenitors, the expression of lineage antigens increases. Techniques such as semi-solid colony-forming assays have been used as gold-standards for assessing progenitor cell number and behavior. A single progenitor cell can expand and differentiate to form a mature colony in semi-solid medium containing hematopoietic growth factors, which can be assessed based on cell morphology and other characteristics.

In the human system, the classification of subpopulations of stem and progenitor cells is less clear. CD34 positivity has long been accepted as a marker for stem cell populations in bone marrow and umbilical cord blood (UCB). It is recognized, however, that the CD34<sup>+</sup> population is a heterogeneous mixture of immature stem and progenitor cells (Bhatia et al. 1997b; Link et al. 1996; Hao et al. 1995), and thus other markers have been proposed for use in isolating more defined populations. The absence of the myeloid cell-surface antigen, CD38, in combination with CD34 positivity can identify more immature HSC/HPCs. CD34<sup>+</sup>CD38<sup>-</sup> cells are more immature HSC/HPCs able to better repopulate immunodeficient NOD/SCID  $\beta$ 2-microglobulin-null mice (Ishikawa et al. 2003). Similar to mouse hematopoiesis, various combinations of cell-surface markers and functional assays are used in the human system to define subsets of progenitor cells, including CMPs and CLPs as well as others (Hoebeke et al. 2007; Chen et al. 2007).

Preservation and fidelity of self-renewal and differentiation in the HSC pool are essential for maintaining homeostatic conditions and preventing leukemic disease (Passegue and Weisman 2005; Lessard et al. 2004). The entire field of transplantation is dependent on this balance, and understanding how these events are regulated has immense clinical value. Over 50 years ago, researchers showed it was possible to protect mice and other animals from death after lethal radiation by transplanting bone marrow cells (Lorenz et al. 1951; Diaconescu and Storb 2005). These early experiments started the field of hematopoietic cell transplantation (HCT). This therapy has been used to treat and/or cure a variety of diseases such as immunodeficiencies, aplastic anemia and myeloproliferative diseases including various leukemias (Diaconescu and Storb 2005). Advancements in this therapy have been tremendous in the last few decades. First, better ways of conditioning the recipient have resulted in better engraftment of stem cells



and better overall survival. Some of these ways include the use of cyclophosphamide (Cy) and its combination with fractionated doses of total body irradiation (TBI) or with other myelosuppressive agents such as busulfan (Thomas et al. 1982; Deeg et al. 1986; Santos et al. 1983). Second, better understanding of immunology, matching of HLA antigens, and better immunosuppressive therapies have led to less rejection and graft-versus-host disease (GVHD), which has dramatically increased the post-transplant survival of patients as well as enabled allogeneic transplantation from unrelated bone marrow donors (Hansen et al. 1998; Diaconescu and Storb 2005). One of the most important ways in which HCT has been improved is the source of the transplantable donor cells. Two populations of transplantable HSC grafts have been used increasingly in the past 20 years. The first is composed of HSCs collected in the peripheral blood after mobilization out of the bone marrow (mobilized peripheral blood, MPB). The second is composed of HSCs collected from human umbilical cord blood (hUCB) after a baby's birth.

The most common mobilizing agent used clinically is granulocyte colony-stimulating factor (G-CSF) (Cashen et al. 2007; Pelus and Fukuda 2008; Nervi et al. 2006). G-CSF mobilized grafts are superior to BM grafts in post-transplantation time to stable engraftment and survival of the recipient, even though GVHD is similar between the two sources (Diaconescu and Storb 2005). The mechanism of action of G-CSF in mobilizing HSCs is complicated and not fully understood. The interplay of adhesion molecules, matrix metalloproteases and other molecules are likely involved in this process (Lapidot and Petit 2002). Other mobilizing agents have been tested in both mouse and human systems. Molecules such as the CXCR2 ligand Gro beta (Pelus and Fukuda 2006; Fukuda et al. 2007) and the CXCR4 antagonist AMD3100 (Broxmeyer et al. 2005; Broxmeyer et al. 2007) are effective at mobilizing HSCs. AMD3100 antagonizes the

stromal-derived factor 1/CXCL12 (SDF-1/CXCL12) chemokine, CXCR4 chemokine receptor interaction, and its use as a potent mobilizing agent illustrates the importance of this axis in hematopoietic stem cell movement between the bone marrow and peripheral tissues (Broxmeyer et al. 2005; Broxmeyer et al. 2007).

In addition to mobilized peripheral blood, hUCB contains transplantable hematopoietic stem cells capable of restoring the blood system of myeloablated recipients (Broxmeyer 2006; Gluckman et al. 1989). There are advantages of hUCB over other sources of HSCs, including less GVHD, lower risk of virally-contaminated grafts and no risk to the donor (Broxmeyer 2006; Lewis 2002).

The work advancements summarized thus far, better conditioning regimens, better HLA matching and using alternative sources such as MPB and hUCB, have improved HCT. There still remain significant roadblocks in the application of HCT for the treatment of many hematologic diseases. There are still many recipients who cannot get a fully matched donor graft for HCT, many of whom have to resort to using haploidentical grafts (Diaconescu and Storb 2005; Hurley et al. 2003), and this number is higher for minorities such as African Americans (Diaconescu and Storb 2005). In addition, there is still significant morbidity and to an extent mortality, resulting from HCT, mostly related to engraftment failures, delayed engraftment, GVHD or issues resulting from immunosuppression (Shizuru et al. 2005). Also, there are risks such as infection and splenomegaly to donors of BM and MPB grafts. For hUCB transplantation, many problems result from the fact that HSCs are limited in number from this source, restricting this therapy primarily to pediatric patients (Broxmeyer 2006; Lewis 2002).

These clinical problems imply that the mechanism controlling engraftment of HSCs in a transplant recipient is not fully understood. Currently, there are many areas of

hematopoietic research directed at understanding HSC functions such as self-renewal, differentiation, homing/migration and engraftment and applying this information to the clinical problems still associated with HCT. Two branches of research addressing these problems include (1) modulating HSC homing/engraftment by affecting the SDF-1-CXCR4 axis and (2) modulating growth signaling pathways leading to expansion of HSCs *ex vivo*. Previous work done in each of these areas will be summarized in more detail in order to give adequate background for the current study.

Enhancement of homing/engraftment of HSCs by modulating the SDF-1-CXCR4 axis:  
one strategy to improve HCT efficacy

If homing and engraftment can be enhanced, the time to recovery of hematopoietic lineages can be reduced resulting in less engraftment failures and better overall survival, especially in hUCB transplantation (Broxmeyer 2006; Lewis 2002). A better understanding of factors which affect migration and homing of HSCs is important in solving these problems and expanding HSC clinical applications.

Homing is defined as the ability of HSCs, once injected into the peripheral circulation, to move out of the PB and into the BM stroma where niches conducive to HSC expansion and differentiation exist. This process is thought to occur shortly after transplantation, often within 24 hours and before the cells have had a chance to divide (Lapidot et al. 2005). Understanding how HSCs migrate *in vitro* and *in vivo* has direct clinical relevance, since once transplanted, the cells need to leave the PB and enter delicate niches in the BM stroma (Chute 2006; Lapidot et al. 2005). In addition, stable long-term engraftment is dependent upon adequate homing of the HSCs into their niches (Chute 2006; Lapidot et al. 2005). Enhancing this migration from the PB into the BM is proposed as a way to increase the overall engraftment of the cells, leading to fewer failures and

better survival (Chute 2006; Christopherson et al. 2004). This strategy is especially applicable in hUCB transplantation where low numbers of HSCs contained in the graft often limit this therapy to younger recipients. Enhancing the homing and engraftment efficiency of hUCB cells could expand this therapy for use in older or larger patients (Christopherson et al. 2002; Christopherson et al. 2004; Campbell et al. 2007b).

Migration and homing of HSCs are processes dependent upon extracellular signals from growth factors, adhesion molecule/ligand interactions and chemokines (Broxmeyer 2008; Laird et al. 2008; Yaniv et al. 2006; Lapidot et al. 2005; Cancelas et al. 2006).

Hematopoietic chemokines such as SCF produced by stromal cells affect homing of transplanted cells, by mechanisms including the upregulation of extracellular matrix adhesion molecules (Lapidot et al. 2005; Hart et al. 2004). Adhesion molecules, such as members of the integrin and selectin families, are known to play important roles in movement of cells out of the blood vessel and into the BM stroma, and inhibition of these interactions leads to impaired *in vivo* homing and engraftment (Hidalgo et al. 2002; van der Loo et al. 1998; Chute 2006; Kollet et al. 2001)

For our current studies, we focused on the important role of chemokines which are small protein cytokines responsible for stimulating *in vitro* migration and *in vivo* homing of various cell types. These proteins act on cell surface receptors, in many cases of the G-protein linked type, and stimulate directed movement of the cell towards a gradient. In the body, these protein mediators are responsible for physiological processes such as lymphocyte movement into lymph nodes, inflammatory cell recruitment at sites of inflammation, anchorage of HSCs in the BM stroma and metastasis of many different types of cancer cells from the original tumor to distant tissue beds (Zlotnik and Yoshie 2000; Baggiolini 1998; Sallusto et al. 1998; Broxmeyer 2001; Broxmeyer 2008; Murphy

1994). The chemokine stromal-derived factor 1 (SDF-1)/CXCL12 and its receptor CXCR4 play crucial roles in these processes, including allowing human HSCs to get to their BM destination (Peled et al. 1999; Lapidot et al. 2005; Cottler-Fox et al. 2003). Human and mouse phenotypic HSCs express the receptor CXCR4 and migrate towards an SDF-1 gradient *in vitro* (Aiuti et al. 1997; Kim and Broxmeyer 1998; Wright et al. 2002; Rosu-Myles et al. 2000; Christopherson et al. 2002; Christopherson et al. 2004). Inhibition of the SDF-1-CXCR4 interaction by antibodies to CXCR4 decreases engraftment of immunodeficient mice by human CD34<sup>+</sup> cells; conversely, upregulation of CXCR4 on the CD34<sup>+</sup> cells by cytokines stimulation or transduction enhances *in vivo* engraftment (Peled et al. 1999; Kahn et al. 2004; Brenner et al. 2004).

Homing and engraftment are complex processes, and it is clear that the SDF-1 CXCR4 axis is not the only player involved; however, modulation of this axis does dramatically affect HSC movement and behavior.

#### Modulation of SDF-1 – CXCR4 axis by the CD26/dipeptidylpeptidase IV (DPPIV)

##### Enzyme

*In vivo*, many chemokines do not exist as a pool of full-length proteins. Substantial evidence has shown that chemokines can be processed, especially at their N-terminus, by proteases and peptidases (Baggiolini 1998; Crump et al. 1997; Clark-Lewis et al. 1995). This results in a mixed pool of cleaved and full-length molecules. The enzyme dipeptidylpeptidase IV/CD26 can cleave the N-terminus from many chemokine substrates, including regulated on activation, normal T cell expressed and secreted (RANTES/CCL5); granulocyte chemotactic protein-2 (GCP-2/CXCL6); macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ /CCL4); macrophage-derived chemokine (MDC/CCL22); the CXCR3 ligands monokine induced by IFN- $\gamma$  (Mig/CXCL9), IFN- $\gamma$ -

inducible protein-10 (IP-10/CXCL10) and IFN-inducible T-cell  $\alpha$ -chemoattractant (I-TAC/CXCL11); eotaxin/CCL11; macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ /LD78 $\beta$ /CCL3-L1) and stromal derived factor-1 (SDF-1/CXCL12) (Oravecz et al. 1997; Proost et al. 1998a; Struyf et al. 1998a; Guan et al. 2004; Guan et al. 2002; Proost et al. 1999; Struyf et al. 1998b; Struyf et al. 1999; Proost et al. 2001; Ludwig et al. 2002; Proost et al. 2000; Struyf et al. 2001; Proost et al. 1998b; Shioda et al. 1998; Ohtsuki et al. 1998). In most cases, the cleavage of an individual chemokine by CD26 produces a form that has altered functional activity, summarized in Table 1.

<b>CD26-Truncated Chemokine</b>	<b>Functional Changes</b>
RANTES/CCL5 (aa 3-68)	Change in receptor selectivity Decreased CCR1 activity Decreased CCR3 activity Equal or Increased CCR5 activity
MIP1 $\beta$ /CCL4 (aa 3-69)	Change in receptor specificity Addition of CCR1 and CCR2b activity No change in cognate CCR5 activity
MDC/CCL22 (aa 3-69 and aa 5-69)	Decreased activation of CCR4 Possible activity via unidentified receptor on monocytes
Eotaxin/CCL11 (aa 3-74)	Minimal decrease in CCR3 binding Loss of CCR3 activation
MIP1 $\alpha$ /LD78 $\beta$ /CCL3-L1 (aa 3-70)	Increased activity through receptors CCR5 and CCR1 Decreased activity through CCR3
GCP-2/CXCL6 (aa 3-77)	No changes in intracellular calcium release from neutrophils
IP-10/CXCL10 (aa 3-77)	CXCR3 binding and activation are significantly reduced
I-TAC/CXCL11 (aa 3-73)	Binding to CXCR3 is modestly reduced CXCR3 intracellular calcium release is significantly reduced CXCR3 internalization is modestly reduced
Mig/CXCL9 (aa 3-103)	No significant decrease in CXCR3 binding nor intracellular calcium release
SDF-1/CXCL12 (aa 3-68)	Substantially reduced activation of CXCR4 Binding to CXCR4 is modestly reduced

**Table 1: CD26/DPPIV effect on select chemokine functions.** Shown are various chemokines known to be cleaved by CD26 and the resulting changes in activity and/or receptor binding.



The role of CD26 in the physiological cleavage of SDF-1/CXCL12 was established by a series of studies published by various researchers (Proost et al. 1998b; Shioda et al. 1998; Ohtsuki et al. 1998). These groups examined the effect of CD26-mediated SDF-1/CXCL12 cleavage on chemotaxis and CXCR4 activation in both cell lines and primary peripheral blood cells. They demonstrated that CD26 could indeed cleave SDF-1/CXCL12 under physiological conditions in transfected cell lines overexpressing CD26; and recombinant soluble CD26 could cleave SDF-1/CXCL12 *in vitro*. CD26/DPPIV action produced SDF-1/CXCL12 (aa 3-68), a truncated molecule which lost its ability to induce chemotaxis. In intracellular calcium release assays the truncated SDF-1/CXCL12 (aa 3-68) had some ability to desensitize subsequent signals from full-length SDF-1/CXCL12, but this was at significantly higher concentrations of the cleaved form compared to full-length (Proost et al. 1998b). This desensitization can be explained by a mechanism in which SDF-1/CXCL12 (3-68) cannot signal through the receptor but can bind, albeit less efficiently, to CXCR4, and is supported by detailed structure-functional experiments showing that the N-terminus of SDF-1/CXCL12 is important for CXCR4 receptor binding and activation, with activation being highly dependent on the first two residues (Crump et al. 1997). In fact, loss of the first two residues, resulting in the form SDF-1/CXCL12 (aa 3-68), abolishes CXCR4 activation but only modestly impairs CXCR4 binding (about a 10-fold decreased binding affinity) (Crump et al. 1997). Presumably, the antagonism of CXCL12 (aa 3-68) can be explained by a steric hindrance mechanism. An alternative explanation for the desensitizing effect of cleaved SDF-1 (aa 3-68) could be an ability to downmodulate CXCR4 by activating non-canonical signaling pathways not measured by the calcium release functional assays used in these studies, a phenomenon supported by studies of at least one other chemokine (Ludwig et al. 2002).

Given the importance SDF-1- CXCR4 interactions in HCT, the physiological consequences of CD26 mediated SDF-1 cleavage on mouse and human HSC chemotaxis, mobilization and *in vivo* engraftment/repopulation using various CD26 inhibitors and CD26-knockout mice was studied. Specific CD26 inhibitors Diprotin A and Val-Pyr were used in these studies, in addition to the CD26 <sup>-/-</sup> knockout mouse (Christopherson et al. 2004). Kent Christopherson II and colleagues in the Broxmeyer laboratory showed that immature HPCs/HSCs from both human cord blood (CB) and mouse bone marrow (BM) expressed CD26 on their surface (Christopherson et al. 2002; Christopherson et al. 2003; Christopherson et al. 2004). About 8% of hUCB CD34<sup>+</sup> cells were positive for surface expression of CD26 (Christopherson et al. 2002). CD26 was present on the surface of 70% of phenotypic c-kit<sup>+</sup> sca-1<sup>+</sup> lin<sup>-</sup> (KSL) mouse HSCs (Christopherson et al. 2003). The CD26 expressed on HSCs was functional as measured by an *in vitro* enzymatic assay utilizing a chromogenic reaction (Christopherson et al. 2003; Christopherson et al. 2002; Christopherson et al. 2004; Christopherson II et al. 2007).

In studies on mouse HSCs/HPCs, Diprotin A pre-treatment enhanced SDF-1/CXCL12 chemotaxis *in vitro* (Christopherson et al. 2003; Christopherson et al. 2004). In addition, purified truncated SDF-1/CXCL12 (aa 3-68) was unable to stimulate chemotaxis, and further, pre-incubation of these cells with the truncated form antagonized chemotaxis to full-length SDF-1/CXCL12 (Christopherson et al. 2003). This suggests that the cleaved SDF-1 binds to CXCR4 and blocks activation by the full-length molecule.

In addition to *in vitro* chemotaxis, CD26 was found to have a role in modulating G-CSF mediated mobilization *in vivo*, a process involving the SDF-1 – CXCR4 axis (Lapidot and Petit 2002; Broxmeyer et al. 2005). Two different strains of mice (C57BL/6 and DBA/2) were treated with the CD26 inhibitor Diprotin A or Val-Pyr during G-CSF induced

mobilization and the extent of mobilization of HPCs was measured by colony-forming assays using peripheral blood cells. Both CD26 inhibitors reduced the numbers of G-CSF mobilized HPCs in the periphery in both strains of mice (Christopherson et al. 2003). In further studies using CD26 knockout mice, the absence of CD26 substantially decreased normal G-CSF induced mobilization when compared to wild-type mice (Christopherson et al. 2006). The mechanism of CD26 in mobilization is not fully elucidated, but presumably the inhibition of CD26 could strengthen anchoring of HSCs/HPCs by the SDF-1 – CXCR4 axis in the BM stroma, thus reducing their tendency to move out into the PB in response to G-CSF induced mobilization (Christopherson et al. 2003).

An important role for CD26 in modulating mouse HSC *in vivo* repopulation was further established by inhibition of CD26 on donor cells or using CD26<sup>-/-</sup> donor cells in congenic competitive repopulation assays. These assays involve mixing donor and competitor cells from congenic mouse strains differing at the CD45 locus, a marker present on most hematopoietic cells, and transplanting these cells into lethally irradiated congenic recipients (often expressing the same CD45 marker as competitor cells). The CD45 markers can be distinguished and followed monthly in the PB by using flow cytometry. This *in vivo* assay is considered to be the gold-standard for assessing HSC function. Pre-treating donor HSC/HPCs with the CD26 inhibitors Diprotin A or Val-Pyr for short periods before transplantation significantly and dramatically increased their short-term homing and competitive repopulation of primary and secondary recipients, suggesting that the engraftment of HSCs was enhanced by this treatment (Christopherson et al. 2004). Secondary transplantation of BM cells pre-treated with Diprotin A likely results from a higher number of HSCs in the primary transplant recipients and not from any long-term effect of Diprotin A, since this is a reversible inhibitor. Furthermore, CD26<sup>-/-</sup>

mouse donor HSCs were transplanted and found to have increased homing and engraftment compared to wild-type (Christopherson et al. 2004). The effect of CD26 inhibition on engraftment and repopulation of donor cells was seen under conditions of limiting cell numbers; that is, in these mouse models CD26 inhibition can lower the threshold needed to get adequate repopulation and survival of lethally irradiated recipient mice (Christopherson et al. 2004). The CD26 inhibitors used in these studies are short-lasting in duration, about 4 hours in pre-treated hematopoietic cells (Christopherson et al. 2004), but the net effect of their use is to increase long-term engraftment of donor cells. This could be explained by a mechanism in which the immediate effect of CD26 inhibition is to increase the efficiency of homing of HSCs to the BM of recipients, akin to transplanting higher numbers of HSCs. Since long-term engraftment is reliant upon numbers of HSCs able to home and take residence in the BM, this short-term treatment would have consequences on stable engraftment. Other processes such as adhesion or retention in the BM could be affected by this treatment; however, we believe homing is the most logical mechanism involved (Christopherson et al. 2004).

The effect seen on engraftment in mouse transplants has been reproduced in two studies by independent laboratories. The first group showed that inhibition of CD26 could enhance engraftment of limiting numbers of virally-transduced hematopoietic cells expressing a recombinant allogeneic MHC class I molecule (Tian et al. 2006). This group showed that the enhanced level of engraftment seen in animals transplanted with Diprotin A pre-treated cells correlated with decreased rejection of an allogeneic skin graft (Tian et al. 2006). The second independent group showed that CD26 inhibition significantly increased homing and engraftment in the context of non-ablative, allogeneic *in utero* hematopoietic-cell transplantation (IUHCT). Specifically, they were able to show

that short-term homing (4 hours or 48 hours) of transplanted whole BM and purified HSCs pre-treated with Diprotin A was enhanced, a higher percentage of mice with detectable engraftment was found, the level of stable engraftment was increased up to 6 months post-transplant, and the Diprotin A treated cells could dramatically outcompete non-treated congenic donor cells in competitive IUHCT experiments (Peranteau et al. 2006).

These results strongly support a model where inhibition of CD26 activity enhances the ability of donor HSCs to transplant conditioned recipients. This treatment could have clinical relevance in many different transplant situations; however, it could have an immediate impact in the field of hUCB transplantation where, as mentioned previously, donor HSCs are of small number and limit the therapy to pediatric patients. There is evidence that CD26 inhibition enhances chemotaxis/migration of hUCB CD34<sup>+</sup> cells in a similar fashion as mouse HSCs, but no evidence that inhibition of CD26 on CD34<sup>+</sup> cells has a similar effect on *in vivo* engraftment as in the mouse system (Christopherson et al. 2002). Extending on previous mouse transplantation studies by determining the role of CD26 in *in vivo* engraftment of hUCB CD34<sup>+</sup> HSCs is one of the goals of my thesis project.

#### Strategies to expand HSCs *ex vivo* and enhance HCT

Another way in which researchers are attempting to solve the problems associated with HCT and apply this therapy to a broader patient population is by using strategies aimed at expanding hematopoietic stem and progenitor cells *ex vivo* while still maintaining their full functional repertoire. Cells expanded in this fashion could be transduced with viral or other types of vectors in order to complement a genetic defect, as in the case of Fanconi Anemia, or could be used to transplant many recipients from one donor. Additionally,

being able to expand HSCs *ex vivo* could be accompanied with treatments aimed at suppressing reactive T cell activity or enhancing anti-tumor specific T cell activity, strategies which would help decrease GVHD or increase graft versus tumor response, respectively.

All of these goals rely upon a more complete understanding of which molecular pathways modulate proliferation, quiescence, differentiation, survival and metabolism in normal HSCs and HPCs and how these pathways interact with and influence each other. We will give an overview of selected molecules that recently have been used to expand HSCs in pre-clinical studies and discuss our interest in the role of a novel Ras homologue, Rheb2, and its downstream signaling pathway, mTOR, in HSC expansion.

#### *Cytokines and HSC expansion*

The use of cytokines, including stem cell factor (SCF), thrombopoietin (TPO), Flt3L, IL-3, IL-6 and others can expand immature mouse and human hematopoietic cells *ex vivo* (Gammaitoni et al. 2003; Bodine et al. 1989; Bodine et al. 1992; Petzer et al. 1996; Bhatia et al. 1997a; Heike and Nakahata 2002). However, their ability to expand long-term repopulating cells is not clear, as many of these early studies relied on spleen colony-forming assays and long-term cultures to assess stem cell function, both of which more appropriately measure short-term stem cells or multipotent progenitors. Furthermore, there is no clear clinical data showing an advantage in terms of repopulation in expanding human HSCs before transplantation (Heike and Nakahata 2002).

#### *Notch and HSC expansion*

More recent studies on the use of extracellular signaling molecules to expand HSCs have focused on the developmentally relevant membrane-bound Notch. The receptor

Notch-1 activation by the ligands Jagged-1 or Delta-1 expressed by BM stromal cells including osteoblasts expanded immature human and mouse hematopoietic cells *in vivo* (Karanu et al. 2000; Calvi et al. 2003; Ohishi et al. 2002). In select studies, transducing mouse HSCs with vectors overexpressing Notch-1 led to expansion of repopulating HSCs (Stier et al. 2002). However, Notch-1 hyperactivation impaired the ability of mouse HSCs to differentiate normally as skewing of myeloid and lymphoid lineages, with a preference for lymphoid cells, was found in these studies (Stier et al. 2002; Ohishi et al. 2002). Better regulation of Notch-1 activity is necessary before clinical use of these molecules is possible.

#### *Transcription factors and HSC expansion*

Analysis of the transcription factor HoxB4 has shed light on another intracellular pathway important in the expansion of HSCs. HoxB4 is a member of the Hox family of transcription factors expressed in HSCs (Sorrentino 2004). Studies performed using mouse HSCs overexpressing HoxB4 found expansion of HSCs *in vivo* in mouse transplant assays as well as *in vitro* under liquid culture conditions (Sauvageau et al. 1995; Antonchuk et al. 2002). Additional studies using a Tat-HoxB4 fusion protein found that mouse HSC expansion could be accomplished without the need of a viral vector, bypassing the biological and safety concerns associated with viral gene therapy (Krosi et al. 2003). Problems associated with HoxB4 induced expansion include the possibility of leukemic transformation, observed using other Hox family members, and impairment of normal blood cell differentiation (Perkins and Cory 1993; Thorsteinsdottir et al. 1997; Brun et al. 2003; Schiedlmeier et al. 2003).

### *Wnt and HSC expansion*

The Wnt- $\beta$  catenin pathway has recently been studied for its role in expanding HSCs (Sorrentino 2004). Soluble Wnt proteins activate the cell-surface receptor frizzled which can then, through a series of intracellular mediators, inhibit the degradation of  $\beta$  catenin allowing it to translocate to the nucleus and act as a transcription factor for a host of genes involved in cell proliferation, differentiation and other processes. The  $\beta$  catenin pathways is often altered in malignancies, including leukemia (Sorrentino 2004). In studies on both mouse and human hematopoietic cells, researchers found that addition of soluble Wnt proteins expanded HSCs and/or HPCs in liquid cultures (Willert et al. 2003; Austin et al. 1997; Van Den Berg et al. 1998; Murdoch et al. 2003). Candidate Wnt proteins used in these studies were Wnt-1, Wnt-5a, Wnt-10b and Wnt3a for mouse cells, and Wnt-5a, Wnt-2b and Wnt-10b for human cells. At least two of these Wnt proteins, Wnt3a for mice and Wnt5a for humans had some ability to expand cells capable of *in vivo* repopulation of recipient animals (Willert et al. 2003; Murdoch et al. 2003). Although soluble Wnt proteins can expand HSCs *ex vivo*, there is evidence that sustained Wnt activation leads to a loss of HSC repopulating activity, potentially a major concern in any therapy aimed at treating humans (Fleming et al. 2008).

### *Ras family members and HSC expansion*

The ras family of GTPase intracellular signaling proteins contains members which play important roles in HSC functions and malignant transformation. These GTPase molecules and their associated signaling pathways remain interesting targets for modulating HSC expansion and repopulating ability. We have focused on a novel ras related molecule and activator of the mammalian target of rapamycin (mTOR) pathway, Rheb2, for our current studies on hematopoietic cell growth and repopulation.



Ras intracellular GTPase proteins act as molecular switches inside cells, alternating between GTP-bound active and GDP-bound inactive forms in response to signals from growth factors, including hematopoietic cytokines such as SCF (Linnekin 1999). Ras family members such as K-ras and N-ras are well-known for their role as oncoproteins in human leukemias and proliferative blood disorders, in most cases resulting from mutation of wild-type protein leading to constitutively GTP-bound ras (Scheele et al. 2000; Van Meter et al. 2007). In normal mouse or human hematopoietic stem and progenitor cells, expression of mutant, hyperactive K-ras or N-ras causes hyperproliferation, myeloid skewing, including myeloproliferative disease in mice, and alteration of many intracellular signaling pathways, while loss of normal K-ras leads to defects in mouse fetal liver erythropoiesis (Braun et al. 2004; Van Meter et al. 2007; Shen et al. 2004; Tuveson et al. 2004; Khalaf et al. 2005; MacKenzie et al. 1999). Loss of neurofibromin 1 (NF1), the ras GAP protein, leads to hyperactive ras signaling and altered growth of hematopoietic cells, including hypersensitivity to hematopoietic growth factors (Bollag et al. 1996). Other ras family members have important roles in hematopoiesis. Members of the Rho family, such as rhoH, rac1 and rac2, have profound roles in hematopoietic cell migration and chemotaxis. Loss of rac1 and rac2 leads to defective mouse hematopoietic cell migration, while loss of rhoH leads to enhanced migration through a mechanism mediated by rac1 (Cancelas et al. 2006; Chae et al. 2008; Gu et al. 2003).

*Rheb2: a potential target in HSC expansion*

The role of a novel ras subfamily, Ras homologue enriched in brain (Rheb), in mammalian hematopoiesis has not been extensively studied. Interestingly, Rheb2, one member of the Rheb family, has been found in a subtractive cDNA library to be preferentially expressed in immature mouse HSCs compared to mature cells (Ivanova et

al. 2002), indicating that this molecule may play a role in HSC functions such as self-renewal and/or differentiation. Human Rheb proteins are ubiquitously expressed and are elevated in many types of tumors (Basso et al. 2005; Yuan et al. 2005). Like most Ras proteins, Rheb proteins are small (about 20kDa) GTPase molecules which can cycle between active GTP-bound and inactive GDP-bound forms and act primarily in cells as upstream activators of the mammalian target of rapamycin (mTOR) pathway, a pathway involved in cell growth, proliferation and survival of various cell types in organisms as diverse as yeast and man (Wullschleger et al. 2006; Fingar and Blenis 2004; Tee et al. 2005).

#### *Rheb2 and mTOR signaling*

Both Rheb family members, Rheb1 and Rheb2, activate the mTOR serine/threonine kinase by mechanisms not fully known, but which rely on binding of active GTP-bound Rheb to mTOR (Long et al. 2005b; Long et al. 2005a). The mTOR pathway is involved in different aspects of HSC biology. Many of the molecules discussed above as capable of HSC expansion, including growth factor cytokines (Flt3Ligand, SCF, TPO, and IL-3), Wnt proteins and Notch proteins are able to activate the mTOR pathway, indicating the involvement of this pathway mediating responses to these factors (Chan et al. 2007; Mungamuri et al. 2006; Wieman et al. 2007; Choo et al. 2006; Inoki et al. 2006). In normal mouse megakaryocytes, mTOR activation by thrombopoietin is needed for end-stage differentiation of platelets, measured by expression of phenotypic cell surface antigens (Raslova et al. 2006). mTOR also mediates IL-3 dependent proliferation in the mouse myeloid progenitor cell line 32D (Cruz et al. 2005). In studies on various hematologic tumors and malignancies, the use of rapamycin or rapamycin analogues, often in combination with other treatments, increased cell death, indicating that mTOR is important for their survival (Bertrand et al. 2005; Xu et al. 2005a; Xu et al. 2005b; Follo

et al. 2007; Hammerman et al. 2005). Finally, mTOR hyperactivation in the context of the tumor suppressor phosphatase PTEN conditional knockout mouse was important for leukemic transformation, and for expansion of normal HSC numbers followed by their exhaustion (Yilmaz et al. 2006; Zhang et al. 2006).

*Rheb proteins lie upstream of mTOR in the PI3K-Akt signaling pathway*

Rheb proteins, like most small GTPase molecules, are directly regulated by two major classes of proteins: the GTP exchange factors (GEFs) and the GTPase activating proteins (GAPs). GEFs increase the GTP loading of GTPase molecules, thereby activating them, while GAPs accelerate their intrinsic GTPase activity accelerating catalysis of GTP to GDP and inactivating protein. Recent evidence from drosophila suggests that the protein translationally controlled tumor protein (TCTP) is the putative Rheb GEF (Hsu et al. 2007). Interestingly, the Rheb GAP is the tuberous sclerosis complex (TSC), a heterodimer of TSC1 and TSC2 individual proteins (Wullschleger et al. 2006; Astrinidis and Henske 2005). The function of TSC is impaired in patients afflicted with the clinical syndrome tuberous sclerosis (TS), an autosomal dominant, primarily pediatric genetic disease characterized by benign hamartomas, i.e. benign solid tumors, in various locations throughout the body including the brain causing mental retardation and other sequelae depending on tumor location (Astrinidis and Henske 2005). Rheb proteins respond to upstream signals coming mainly from growth factors acting through the PI3K-PIP3-PDK1-Akt pathway. Various growth factors activate cell surface receptors such as tyrosine kinase receptors leading to activation of PI3K which converts PIP2 to PIP3, resulting in activation of PDK1 and finally Akt (Morgensztern and McLeod 2005; Cruz et al. 2005; Desponts et al. 2006; Shaheen 2005; Steelman et al. 2008). Akt is an intracellular kinase capable of interacting with and phosphorylating various downstream effectors. As one of its downstream effectors, TSC2 is phosphorylated by Akt, leading to

inactivation of the TSC GAP activity (Wullschleger et al. 2006; Astrinidis and Henske 2005). This leads to a greater proportion of GTP-bound active Rheb, capable of activating mTOR downstream (Li et al. 2004a; Li et al. 2004b; Astrinidis and Henske 2005).

mTOR forms two distinct complexes in cells: mTOR complex 1 and complex 2 (mTORC1 and mTORC2) which differ in their components, sensitivity to rapamycin and downstream effectors (Wullschleger et al. 2006). The mTOR kinase is common to both complexes in mammalian cells. mTOR bound with raptor and mLST8 forms mTORC1, which is sensitive to rapamycin inhibition and activates downstream targets primarily involved in enhancing cellular translation, including ribosomal S6 protein kinase 1 (S6K1) and 4E-binding protein (4E-BP) (Wullschleger et al. 2006). Phosphorylation of S6K1 enhances general translation by increasing the synthesis of ribosomal proteins. Phosphorylation of 4E-BP relieves inhibition on initiation factor 4E (eIF-4E) which can then initiate translation (Wullschleger et al. 2006). mTORC1 is involved in other cellular processes, including inhibition of autophagy and enhancing the function of transcription factors mainly involved in stress responses (Wullschleger et al. 2006). There is recent evidence suggesting that mTORC1 activates STAT1, STAT3 and PPAR $\gamma$  transcription factors (Wullschleger et al. 2006). mTOR bound with rictor, hSIN1 and mLST8 forms mTORC2, a complex which is insensitive to rapamycin and activates proteins such as Rho involved in actin cytoskeletal dynamics (Wullschleger et al. 2006). Negative and positive feedback loops acting on the Akt pathway from mTORC1 and mTORC2, respectively, are now beginning to be appreciated for their roles in modulating mTOR signaling (Wullschleger et al. 2006). The majority of Rheb effects are attributed to its ability to activate mTORC1, and a recent study found no activity of Rheb1 on mTORC2 (Huang et al. 2008). Rheb2 has not been studied for activity on mTORC2. There is also

evidence to suggest that Rheb1 can have negative effects on signaling through the Raf-MAP kinase pathway (Karbowniczek et al. 2006; Im et al. 2002). This function has not been described for Rheb2 and the functional significance of this activity is not clear.

## AIMS

Strategies to enhance HCT include modulation of homing/engraftment by altering the SDF-1-CXCR4 axis *in vivo* and manipulation of signaling proteins in cells responsible for hematopoietic cell expansion and repopulating ability. We chose to investigate these strategies by focusing on two aims.

AIM 1: Determine the role of CD26 inhibition in increasing human umbilical cord blood CD34<sup>+</sup> cell engraftment using a xenogeneic mouse transplantation model, and furthermore, assess the effect of CD26 inhibition on multilineage reconstitution *in vivo*.

AIM 2: Determine the role of the novel Ras-related molecule Rheb2 in expansion of mouse hematopoietic cells and engraftment of mouse HSCs using congenic murine transplantation models.

## MATERIALS & METHODS

### Part I

#### *Human umbilical cord blood CD34<sup>+</sup> cell isolation*

Human CB was obtained from the umbilicus after full term births at Wishard Hospital, Indianapolis, IN. Cord blood was diluted 1:1 with Phosphate buffered saline (PBS) (Lonza, Switzerland) and mononuclear cells were isolated by Ficoll-Paque™ Plus (Amersham Biosciences, Piscataway NJ, USA) separation as previously described (Broxmeyer 2006). CD34<sup>+</sup> cells were isolated by positive magnetic selection with Miltenyi MACS magnetic activated cell sorting kit (Miltenyi Biotec, Germany). The CD34<sup>+</sup> purity was analyzed by flow cytometric staining with anti-human CD34 antibodies (BD Pharmingen, Franklin Lakes, NJ or Dako Cytomation, Denmark). The cell purity was greater than 90% in experiments 1 and 2, and less than 40% in experiment 3.

#### *Transplantation of NOD/SCID mice*

NOD/SCID mice (8-10 wk old) were obtained from the Indiana University School of Medicine xenograph core facility and were exposed to a sub lethal dose of 300 cGy total body irradiation 4 hours before transplantation. The CD34<sup>+</sup> cells, prepared as described above, were washed once with PBS and resuspended in PBS for transplantation by tail-vein injection. All animals were fed food pellets containing doxycycline 1 week before transplantation and maintained on this diet approximately 4 weeks after transplantation.

#### *Staining of mouse bone marrow cells for engraftment*

Mouse BM cells were isolated from both femurs using aseptic procedures.  $1 \times 10^6$  cells were resuspended in FACS staining buffer (SB) (PBS + 2% FBS + 0.5 mM EDTA) and were stained with either anti-human CD45-PE (BD Pharmingen) or isotype control-PE

antibodies (BD Pharmingen), incubated for 30 minutes in the dark at 4°C, washed twice with PBS containing 2% BSA, and resuspended in PBS containing 1% para-formaldehyde for analysis by flow cytometry. About 40,000 to 50,000 events were collected for each sample.

For staining with multiple hematopoietic markers,  $5 \times 10^5$  cells were resuspended in SB and stained with anti-human CD38-FITC, anti-human CD34-PE and anti-human CD45-APC antibodies (BD Pharmingen), or with anti-human CD19 PE, anti-human CD33 FITC and anti-human CD45 APC antibodies (BD Pharmingen). Isotype control stainings were done with isotypic FITC, PE and APC conjugated antibodies (BD Pharmingen). Cells were washed twice with PBS containing 2% BSA and resuspended in PBS with 1% para-formaldehyde. 20000 events were collected for each sample.

#### *Staining of human CB subsets for CD26 expression*

CB mononuclear cells were isolated by Ficoll-Paque™ Plus separation, and CD34<sup>+</sup> cells isolated using Miltenyi MACS kit were resuspended in SB and stained with anti-human CD34, anti-human CD38 and anti-human CD26 antibodies (BD Pharmingen, Caltag and Dako Cytomation). Control stainings were performed with isotype antibodies (BD Pharmingen and Dako Cytomation). Cells were washed twice with PBS containing 2% BSA and resuspended in PBS containing 1% para-formaldehyde. At least 8000 events were collected for each sample.



## Part II

### *Cloning of Rheb2-Mieg3 retroviral vector*

The full cDNA of mouse Rheb2 was acquired from ATCC (BC016521; MGC 27835) in the vector pCMV-Sport6. Primers were designed to amplify the Rheb2 cDNA containing a *Stu*I RE site and Flag-tag at the 5' end and an *Xho*I RE site at the 3' end, with Rheb2 flanking sequences in order to amplify the product. The Rheb2 cDNA was PCR amplified using PuRe Taq PCR beads (Amersham Biosciences, Piscataway NJ, USA). This PCR product was cloned into the vector pCR2.1 using the kit TA cloning kit (Invitrogen). Correct cloning of the product was verified by *Stu*I and *Xho*I RE digests, as well as DNA sequencing (Indiana University School of Medicine (IUSM) DNA sequencing core facility, Indianapolis IN, USA). The Rheb2 cDNA was cut out of this vector by *Stu*I and *Xho*I RE digestion and ligated into linearized Mieg3 retroviral vector obtained from Dr. Wen Tao in our laboratory using DNA ligase enzyme (Invitrogen) following the manufacturer's protocol. Accurate cloning of Rheb2 cDNA, including alignment in Mieg3 vector and accurate sequence was verified by *Stu*I and *Xho*I RE digestion as well as DNA sequencing (IUSM DNA sequencing core facility). Production of full-length Rheb2 protein by this vector was confirmed by Western blot of NIH-3T3, BaF3 and 32D cell lysates as explained below. Mieg3 empty vector was used as a control in all experiments.

### *Retroviral Transduction and Supernatant production*

The murine stem cell virus (MSCV) retroviral vector Mieg3 is a bicistronic vector that expresses enhanced green fluorescent protein (eGFP) controlled by the internal ribosome entry site (IRES) and has been previously used to transduce mouse hematopoietic cells (Tao et al. 2004). Retroviral supernatants were prepared by transfection of 8  $\mu$ g per 10 cm<sup>2</sup> culture dish Mieg3 (empty vector) or Rheb2-Mieg3

plasmid DNA into Phoenix Ecotropic 293T cells (Nolan Lab, Stanford CA, USA) using lipofectamine (Invitrogen, Carlsbad CA, USA). Medium was changed after 12-16 hours to fresh DMEM + 10% FBS (Hyclone, Logan UT, USA). Retroviral supernatants were collected 24 and 48 hours later, filtered using 0.45 µm syringe filters, aliquoted for titering and stored at -80°C.

#### *Retroviral Titers on NIH 3T3 cells*

Trypsinized NIH 3T3 cells were plated in individual wells of a 6-well plate at a concentration of 30,000 cells per 3 mls of IMDM + 10% FBS. The following day, either 20 or 100 µl of retroviral supernatants was added to individual wells in 1.5 mls complete IMDM + 5 µg/ml polybrene transduction reagent (Chemicon International, Billerica, MA USA). In addition, the cell number per well was calculated at this time by trypsinizing 3 wells of NIH 3T3 cells, counting the total cell number by trypan blue exclusion and dividing by 3 to get the cell number per well. Medium was changed after 24 hours to fresh complete IMDM, and cells were harvested by trypsin 24 hours later. The percent GFP<sup>+</sup> cells was determined by FACS, and the titers were calculated by multiplying the proportion of GFP<sup>+</sup> cells by the total number of cells at time of infection and dividing that number by the volume of retroviral supernatant added (either 0.02 or 0.1 ml) to get the number of viral particles per ml.

#### *Transduction of BaF3 and 32D cell lines*

BaF3 cells were maintained in RPMI + 10% FBS + 0.25 ng/ml mouse IL-3 (R&D Systems, Minneapolis, MN). 32D cells were maintained in RPMI + 10% FBS + 10 ng/ml mouse IL-3 (R&D Systems).

For BaF3 and 32D cell transduction, cells were resuspended in Mieg3 or Rheb2-Mieg3 supernatants containing mouse IL-3 (0.25 ng/ml for BaF3; 10 ng/ml for 32D) and plated on retronectin coated wells of a 24-well plate pre-loaded with Mieg3 or Rheb2-Mieg3 supernatants. Transductions were repeated the following day. The cells were FACS sorted for GFP expression and expanded in medium containing IL-3 to create stable cell lines.

*Western blot for detection of full-length Rheb2 protein*

NIH-3T3 Mieg3 and Rheb2-Mieg3 cellular lysates were harvested by resuspension of cells in SDS-lysis buffer followed by sonication twice for 3 minutes at 0°C and boiling at 95°C for 5 minutes, and underwent SDS-polyacrylamide gel electrophoresis using tris-glycine (TG) gels (Invitrogen). Proteins were transferred to PVDF membranes and blotted with anti-Flag antibody (Sigma). Cellular lysates from BaF3 and 32D Mieg3 or Rheb2-Mieg3 cells were harvested in M-PER buffer (Pierce, Rockford IL, USA) containing 1:100 dilutions of protease and phosphatase inhibitors (Calbiochem, Darmstadt Germany) according to manufacturer's protocol and were blotted with an anti-Rheb2 antibody (Abnova, Taipei, Taiwan).

For shRNA knock-down of Rheb2 protein, pSUPER.Retro.GFP vectors (Oligoengine, Seattle WA, USA) containing specific anti-Rheb2 DNA sequence encoding an shRNA molecule were obtained from Dr. Lawrence Quilliam's laboratory. The specific anti-Rheb2 sequence from this vector as well as a scrambled shRNA sequence were cloned into the retroviral vector pSIREN.RetroQ-ZsGreen (Clontech) by ligation of annealed primers containing the shRNA coding sequence using DNA ligase (Invitrogen). Accurate cloning of these vectors was confirmed by DNA sequencing (IUSM DNA sequencing core facility). The scrambled and specific anti-Rheb2 vectors were transfected into

parental and Rheb2-overexpressing 32D cells using nucleofection (Amaxa, Gaithersburg MD, USA) according to the manufacturer's protocol. Cell protein lysates were harvested using M-PER (Pierce) 24 hours after transfection. The percent GFP<sup>+</sup> cells in parental 32D samples was used to estimate the transfection efficiency. Lysates were blotted with anti-Rheb2 antibody (Abnova) to determine the extent of shRNA knock-down of Rheb2 protein.

#### *Primary mouse BM cell transduction*

For all primary mouse hematopoietic cell experiments, mouse bone marrow (mBM) was isolated from femurs of wild-type C57/Bl6 mice using aseptic procedures. BM cells were resuspended in  $\alpha$ MEM medium and layered on top of a lympholyte M gradient (Cedarlane, Burlington NC, USA) according to manufacturer's protocol in order to separate MNCs. mBM MNCs were prestimulated for 48 hours at 37°C in  $\alpha$ MEM medium containing 20% FBS and the following growth factors each at 100 ng/ml each: mouse stem cell factor, human thrombopoietin and human Flt3 ligand (SFT) (Biovision, Mountain View CA, USA). The prestimulated cells were resuspended in either Mieg3 or Rheb2-Mieg3 supernatants containing SFT (100 ng/ml) and plated on 8  $\mu$ g/cm<sup>2</sup> retronectin (Takara, Shiga Japan) coated 6-well plates pre-loaded with retroviral supernatants as described previously (Tao et al. 2004). After a 4.5 hour incubation, the supernatants were replaced with fresh  $\alpha$ MEM + SFT (100 ng/ml). Transductions were repeated the next day. To isolate GFP<sup>+</sup> cells, cells were Fluorescence Activated Cell Sorter (FACS) separated on the following day (total of 4 days after isolating from the mice).

### *Mouse Myeloid Colony Assays*

mBM MNCs were transduced and GFP<sup>+</sup> cells were sorted FACS. The cells were resuspended in IMDM after sorting and plated at various cell concentrations in semi-solid methylcellulose colony assays. These assays have been previously described (Broxmeyer et al. 2003a; Broxmeyer et al. 2003b). Briefly, GFP<sup>+</sup> cells were plated in 1% methylcellulose containing 30% FBS (Hyclone), 0.1 mM Hemin, 5% pokeweed mitogen spleen conditioned medium (PWMSM), 1U/ml Erythropoietin (Amgen, Thousand Oaks, CA USA) and 50 ng/ml mouse Stem cell factor (muSCF). Colonies were scored after 6-8 day incubation in a low oxygen tension (5% CO<sub>2</sub>, 5% O<sub>2</sub>), humidified incubator. For expansion cultures, GFP<sup>+</sup> cells were plated in complete medium containing SFT (50 ng/ml each); cells were removed from the liquid cultures at 0, 48 hours and 1 week and plated in methylcellulose colony assays. For delayed growth factor colony assays, GFP<sup>+</sup> cells were plated in methylcellulose containing FBS with or without 100 ng/ml human SDF-1/CXCL12 (Biovision), but without other growth factors. The combination of hemin, PWMSM, EPO and muSCF was added to the plates at 0, 24 and 48 hours after plating.

#### *Delayed Growth-factor addition proliferation of mouse hematopoietic cell lines and Western Blot for mTOR activity*

For delayed IL-3 proliferation assays, 32D or BaF3 cells were washed twice to remove residual IL-3 from the cultures and plated at  $1 \times 10^5$  cells per ml in 24-well culture plates. The cells were starved of IL-3 at 37°C for 8 or 12 hours after which IL-3 was added to the individual wells at various concentrations. The cells were counted by trypan blue exclusion after expansion. To determine mTOR activity, 32D or BaF3 cells were starved of IL-3 for 8 or 12 hours, after which PBS (Control) or IL-3 (10 ng/ml or 0.25 ng/ml) was

added for 20 or 40 minutes, cells were washed once with PBS, and cell protein lysates were harvested using M-PER lysis buffer (Pierce) containing 1:100 dilutions of phosphatase and protease inhibitors (Calbiochem, Darmstadt, Germany) according to manufacturer protocols. Proteins were separated by SDS-polyacrylamide gel electrophoresis using TG gels (Invitrogen) and blotted with anti-phosphorylated ribosomal S6 antibody (Ser235/236, Cell Signaling Technology, Danvers MA, USA). Anti-Beta actin or anti-total ribosomal S6 protein (Cell Signaling Technology) were used as loading controls (Sigma, St. Louis MO, USA).

#### *In vitro expansion of KSL cells*

Transduced unsorted mouse BM MNCs were cultured in IMDM + 10% FBS + SFT (50 ng/ml) beginning on the fourth day after isolation from C57/Bl6 mice. The cells plated in duplicate wells at approximately  $2 \times 10^6$  cells per well. Additionally, either 50 nM rapamycin (Cell Signaling Technologies) or vehicle control (methanol) was added to the duplicate cultures at this time. The cells were incubated for 4 days at 37°C and all wells were split 1:1 with fresh medium containing SFT containing either methanol or rapamycin once during this 4 day culture. On the fourth day of culture, cells were harvested and resuspended in complete IMDM without growth factors. The DNA binding dye Hoechst 33342 (Invitrogen) was added to the cells to give a final concentration of 1 µg/ml and incubated at 37°C for 30 minutes. After 30 minutes, the cells were resuspended in complete IMDM containing anti-c-kit PE, anti-sca-1 PeCy5.5 and anti-lineage APC antibodies (BD) or isotype control antibodies (BD) and incubated at 4°C for 20 minutes. The cells were washed once with complete IMDM, resuspended in complete IMDM and analyzed by FACS.

### *In vivo homing and Competitive Repopulation Transplants*

For *in vivo* short-term homing,  $1 \times 10^6$  transduced unsorted mBM MNCs from C57/Bl6 (CD45.2<sup>+</sup>) mice were injected by tail-vein into lethally-irradiated recipient BoyJ (CD45.1<sup>+</sup>) mice. BM from bilateral femurs was harvested after 24 hours and analyzed by FACS for CD45.1-PE and CD45.2-APC markers (BD).

Competitive repopulation assays were performed as previously described (Broxmeyer et al. 2005). For short-term repopulation,  $4 \times 10^5$  transduced unsorted mBM MNCs from C57/Bl6 mice (CD45.2<sup>+</sup>) were mixed with  $4 \times 10^5$  BoyJ (CD45.1<sup>+</sup>) BM cells and injected by tail-vein into C57/Bl6/BoyJ F1 double positive recipients (CD45.1<sup>+</sup>, CD45.2<sup>+</sup>) which had been total body irradiated with 9.5 Gray (950 Rad). At 1, 2 and 4 weeks after transplantation, peripheral blood was harvested by tail-vein bleeding into heparinized microcapillary tubes (Fisher Scientific, Pittsburg PA, USA), RBCs were lysed by incubating with 2.5 mls of RBC lysis buffer (0.155 M NH<sub>4</sub>Cl, 0.01 M KHCO<sub>3</sub>, 0.1 mM EDTA in H<sub>2</sub>O and filter sterilized), washed once with PBS + 2% BSA and resuspended in staining buffer containing 1:10 dilution of Fc receptor blocking agents (either mouse gamma globulin, Jackson Immunoresearch or mouse Fc receptor blocker, Miltenyi Biotec). PB cells were then stained with anti-CD45.2 APC and anti-CD45.1 PE antibodies (BD) or isotype control antibodies (BD) for FACS analysis. PB from BoyJ and C57/Bl6 animals were used as controls. BM cells were aseptically harvested at 1, 2 and 4 weeks after transplantation, resuspended in SB containing Fc receptor blocking agents and stained with anti-CD45.1-PE and anti-CD45.2-APC (BD) antibodies or isotype control antibodies (BD) for FACS analysis. BM was further stained with anti-CD117-PE (c-kit), anti-Ly-6A/E-Pe-Cy5.5 (Sca1) and anti-Lineage-APC (Lin) antigens (BD and Caltag, Carlsbad CA, USA) or the corresponding isotype control antibodies (BD and Caltag) for FACS analysis. Absolute numbers of GFP+LS cells were calculated by taking

the number of GFP<sup>+</sup>LS events divided by the total number of gated events multiplied by the total number of BM mononuclear cells isolated from both femurs and tibia from each recipient counted using a Coulter counter.

For Long-term competitive repopulation, either  $4 \times 10^5$  (1:1 group) or  $1.6 \times 10^5$  (0.4:1 group) transduced mBM MNCs from C57/Bl6 (CD45.2<sup>+</sup>) mice were mixed with  $4 \times 10^5$  BM cells from BoyJ (CD45.1<sup>+</sup>) mice; these cells were injected into irradiated recipient BoyJ (CD45.1<sup>+</sup>) mice. PB was harvested at 1, 2.5, 3.5 and 5 months after transplantation and stained with anti-CD45.2 APC and anti-CD45.1 PE antibodies (BD) or isotype control antibodies (BD) as described above.

#### *Secondary non-competitive transplantation*

BM cells were harvested from primary BoyJ transplant recipients at 5 months after transplantation, resuspended in PBS and injected by tail-vein into secondary BoyJ mice irradiated with 9.5 Gray TBI.  $2 \times 10^6$  cells from each primary animal were transplanted into each of four recipient secondary animals. PB was harvested from the secondary animals at 1 and 2 months after transplantation and analyzed for CD45.1 and CD45.2 chimerism as explained above.

#### *Multilineage differentiation by FACS staining*

PB cells from 1 month primary competitive transplant recipient BoyJ animals were resuspended in SB containing Fc receptor blocking agent and stained with anti-CD11b APC, anti-CD3 APC, anti-GR-1 PE and anti-B220 PeCy7 (BD) antibodies and analyzed by FACS.



### *Analysis of data and statistical analysis*

Flow data were analyzed using either WinMDI (The Scripps Research Institute, La Jolla CA, USA) or FCS Express V3 software (De Novo Software, Ontario, Canada). Statistical tests were performed using Student's t-test with  $p < 0.05$  significance cut-off.

## RESULTS

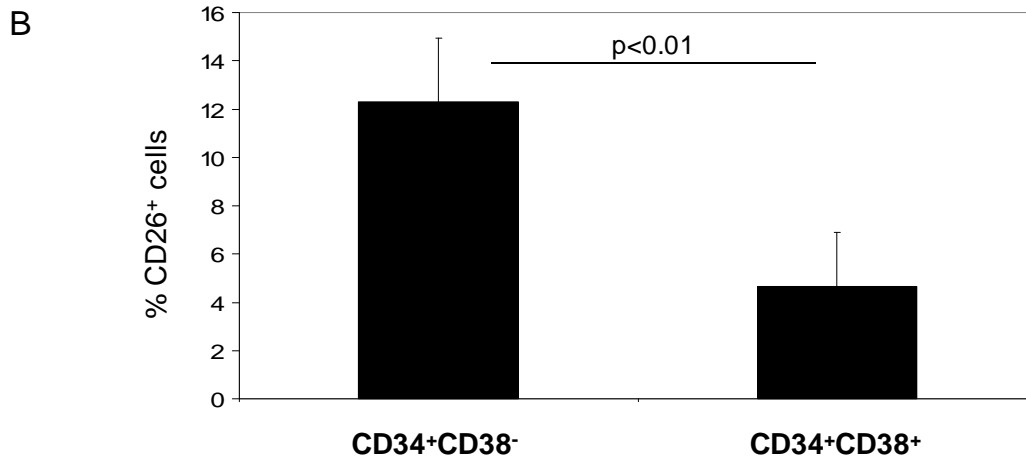
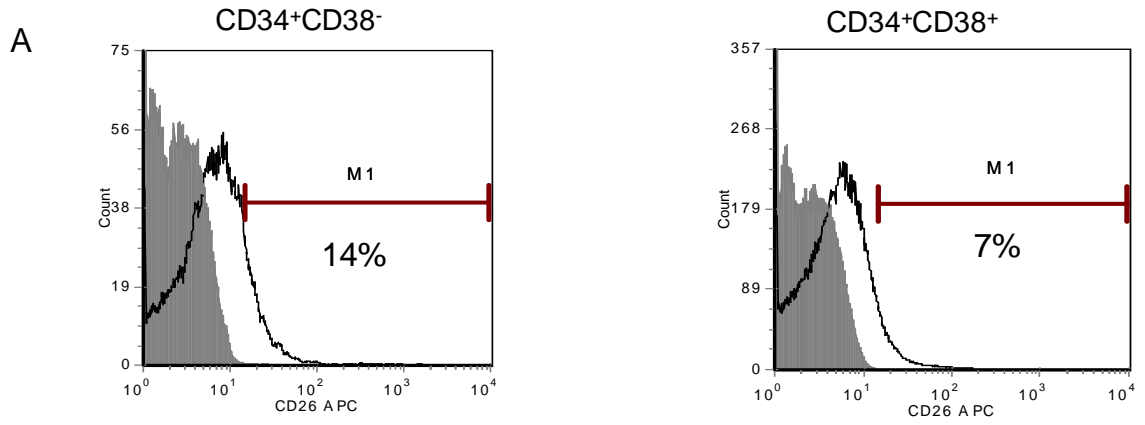
### Part 1: Inhibition of the surface peptidase CD26/DPPIV enhanced hUCB CD34<sup>+</sup> *in vivo* engraftment of immunodeficient NOD/SCID mice

#### *CD26 was preferentially expressed by immature CD34<sup>+</sup>CD38<sup>-</sup> hUCB cells*

Our laboratory established a role for CD26/DPPIV in modulation of mouse HSC engraftment *in vivo* (Christopherson et al. 2004). This current study sought to expand on these results by examining whether CD26/DPPIV could modulate *in vivo* engraftment of human UCB CD34<sup>+</sup> cells, a population purified for HSCs and HPCs, using a xenogeneic NOD/SCID mouse transplantation model. First, we determined CD26/DPPIV expression, using FACS, in subsets of the CD34<sup>+</sup> population. The CD34<sup>+</sup>CD38<sup>-</sup> population is enriched for HSCs compared to the CD34<sup>+</sup>CD38<sup>+</sup> population; therefore, we compared CD26 expression in these two subsets. Approximately 10% of CD34<sup>+</sup> cells are CD38<sup>-</sup>. 12 % ± 2.6 S.D. of CD34<sup>+</sup>CD38<sup>-</sup> hUCB cells expressed CD26/DPPIV, compared to 5 % ± 2.2 S.D. of CD34<sup>+</sup>CD38<sup>+</sup> cells, a significant difference (Figure 1, p<0.01), suggesting preferential expression in immature cells. These data are similar to mouse BM cell data, showing a very high percentage of phenotypic HSCs express CD26/DPPIV (Christopherson et al. 2003).

#### *Pre-treatment of hUCB CD34<sup>+</sup> cells with the CD26/DPPIV inhibitor Diprotin A enhanced their engraftment of NOD/SCID mice*

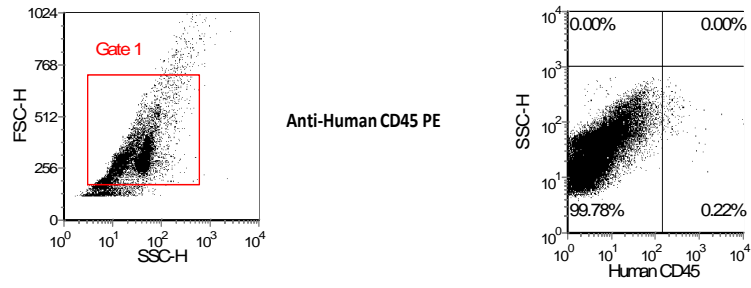
The NOD/SCID xenogeneic immunodeficient mouse model is used to analyze human hematopoietic cell engraftment *in vivo* (Bhatia et al. 1997b). This mouse model is an improvement over previous immunodeficient strains such as SCID in that the level of human cell engraftment able to be attained is higher due to decreased immune cell function (Lapidot et al. 1997). Diprotin A is an inhibitor of CD26/DPPIV used in



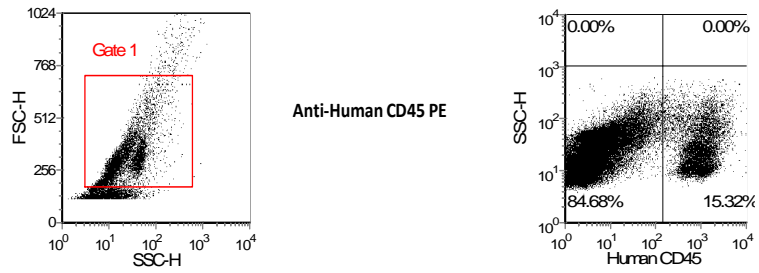
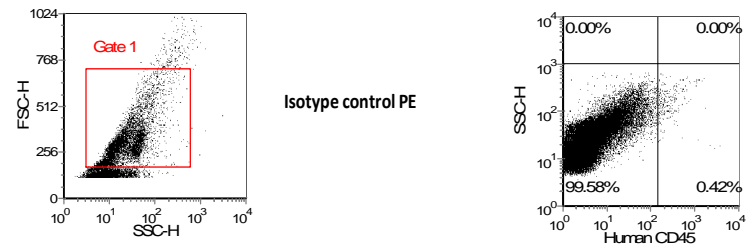
**Figure 1: Expression of CD26 in CD34<sup>+</sup> subsets.** CD34<sup>+</sup> cells were isolated from CB using magnetic cell sorting and were stained with anti-human CD34, CD38 and CD26 antibodies. A: Representative histograms are shown for one CB unit. The shaded line is isotype control staining, while the open line is the anti-CD26 staining. B: The graph shows pooled data from five separate CB units analyzed.

previous experiments to inhibit the peptidase activity (Christopherson et al. 2004). Initial experiments were performed to determine the effect of Diprotin A on the engraftment of human CD34<sup>+</sup> CB cells in sub-lethally (300 Rad) irradiated NOD/SCID mice. We used a short-term (15 minutes) pretreatment of cells with either 5 mM Diprotin A or PBS before injecting cells into recipient animals. After 6 weeks, human cell engraftment was assessed by measuring human CD45 positive cells in recipient marrow. The engraftment of human cells in these transplanted NOD/SCID animals was easily detected using anti-human CD45 antibodies by FACS analysis without significant background staining from non-transplanted mice (Figure 2). Figures 3 and 4 show results of two independent transplant experiments using highly purified (>90%) CD34<sup>+</sup> cells from hUCB. Pretreatment of the cells with Diprotin A significantly enhanced engraftment of human cells in both experiments. The effect of Diprotin A was most pronounced when control cell engraftment was relatively low (<15%). When control cell engraftment was relatively high (80,000 cell group in Figure 4), Diprotin A did not significantly enhance engraftment. Diprotin A did not negatively affect engraftment, even when control cell engraftment was relatively high (Figure 4). Figure 5 shows another independent transplant experiment using CD34<sup>+</sup> cells that were less pure (<40% CD34<sup>+</sup>). Under these conditions, Diprotin A pretreatment again significantly enhanced engraftment of human CD45<sup>+</sup> cells. This condition of a relatively less pure CD34<sup>+</sup> cell sample may better reflect clinical CB transplantation, since not all clinical transplants are performed with pure HSC/HPC populations.

### Non-Transplanted NOD/SCID mouse



### Transplanted NOD/SCID mouse



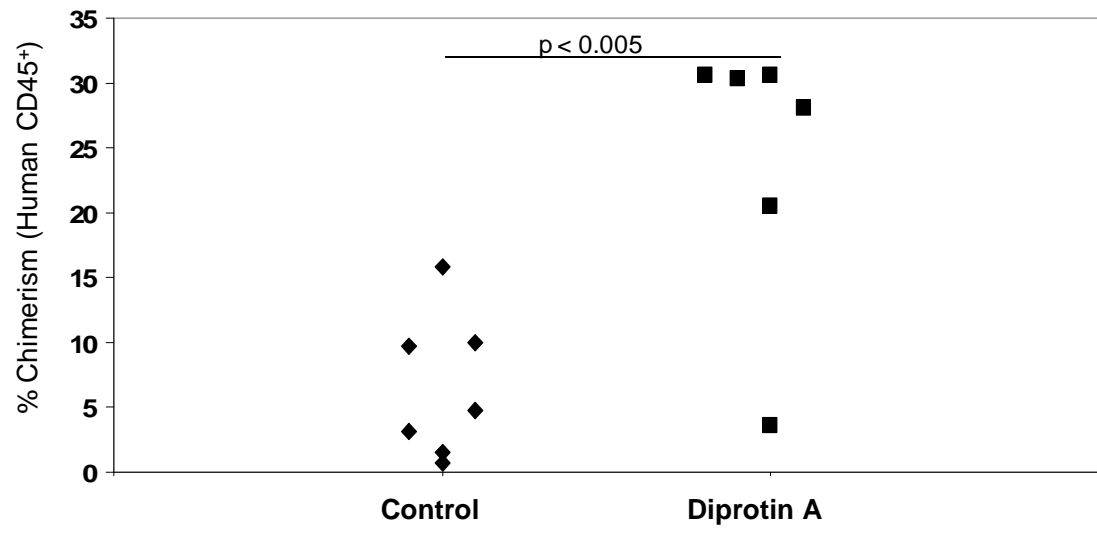
**Figure 2: Analysis of NOD/SCID mice for human hematopoietic cell engraftment.**

Shown are representative FACS plots illustrating no non-specific staining of NOD/SCID recipient mouse BM cells using the anti-human CD45 antibody (top), no non-specific staining of transplanted NOD/SCID mice using the isotype control antibody (middle), and specific detection of human cells in transplanted NOD/SCID mice using the anti-human CD45 antibody (bottom). Note: Fewer events are shown for the SSC-H versus FSC-H plots on the left side in order to accurately gate out sub cellular debris from the analysis.

Mean  $\pm$  SD

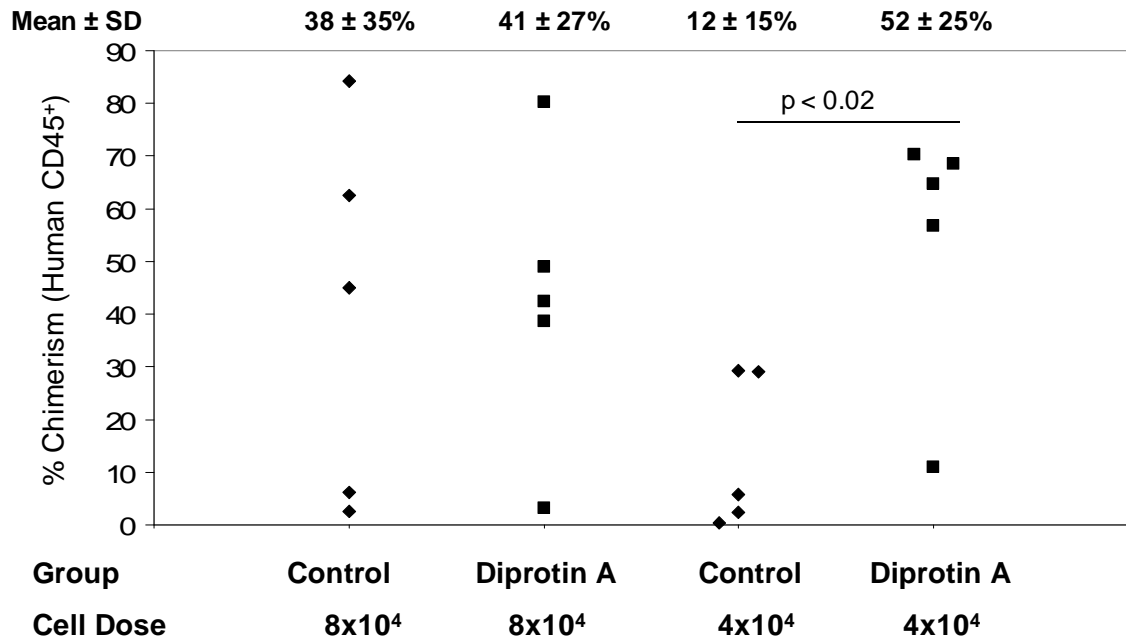
6.53  $\pm$  5.52%

23.95  $\pm$  10.70%

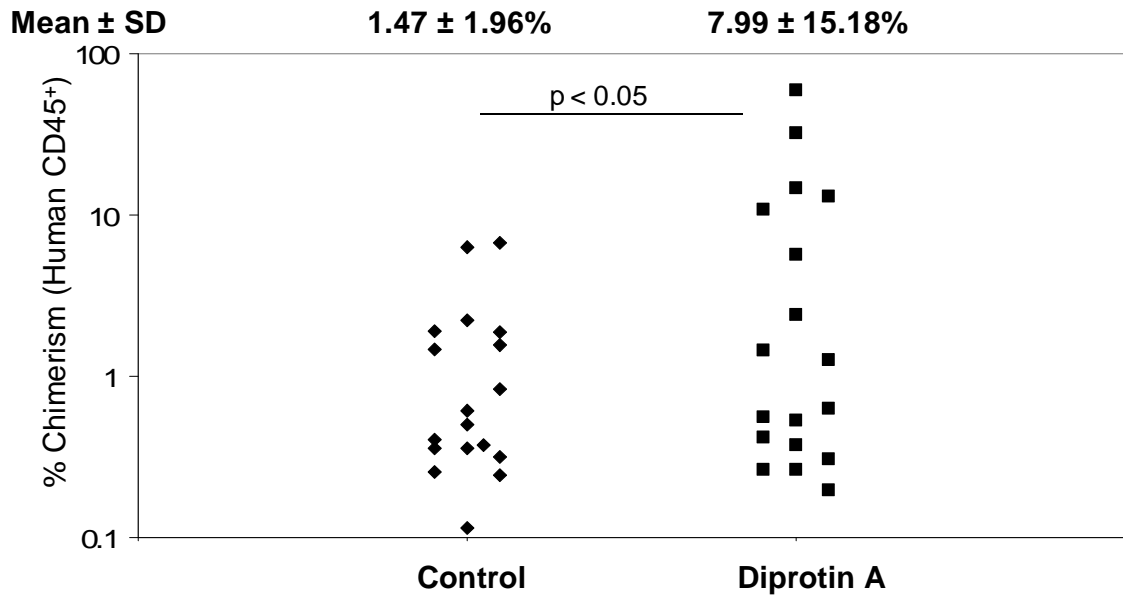




**Figure 3: CD26 inhibition enhanced NOD/SCID engraftment.** Purified CD34<sup>+</sup> cells were treated with either PBS (control) or 5 mM Diprotin A for 15 minutes at 37°C before injecting by tail vein into sublethally irradiated NOD/SCID mice (8 x 10<sup>4</sup> cells per animal). Bone marrow was isolated from 7 control and 6 Diprotin A animals 6 weeks after transplantation.



**Figure 4: CD26 inhibition enhanced NOD/SCID engraftment.** Purified CD34<sup>+</sup> cells were treated with either PBS (control) or 5 mM Diprotin A for 15 minutes at 37°C before injecting by tail vein into sub lethally irradiated NOD/SCID mice at either 4 x 10<sup>4</sup> or 8 x 10<sup>4</sup> cells per animal. Bone marrow was isolated from five animals in all four groups about 8 weeks after transplantation.

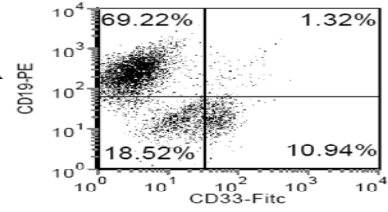
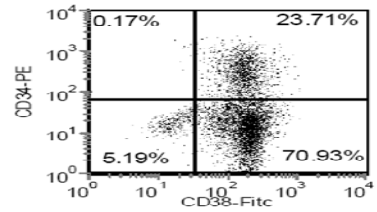
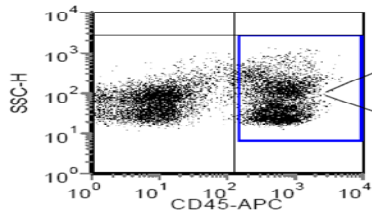


**Figure 5: CD26 inhibition enhanced NOD/SCID engraftment of <40% pure CD34<sup>+</sup> cells.** NOD/SCID animals were transplanted with various numbers of CD34<sup>+</sup> cells ( $2 \times 10^4$ ,  $4 \times 10^4$ , or  $8 \times 10^4$ ) which had been pre-treated with either PBS or 5 mM Diprotin A for 15 minutes at 37°C. Bone marrow was isolated from 18 animals in each group about 9 weeks after transplantation. Results of animals were pooled into two groups (Control vs. Diprotin A) because there was no dose response in engraftment due to number of cells transplanted.

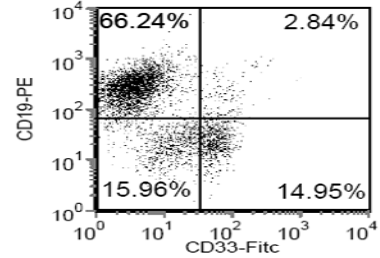
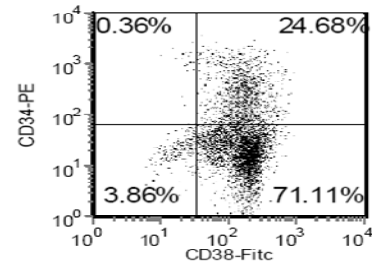
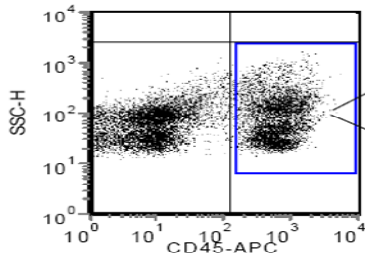
*CD26 pre-treatment did not affect multilineage differentiation of transplanted CD34<sup>+</sup>  
hUCB cells*

Any treatment or strategy which affects the engraftment of hematopoietic cells has to be analyzed for its role in affecting differentiation of the transplanted cells. For any treatment to be clinically effective, the transplanted cells must be able to differentiate into all lineages of the blood normally, otherwise the recipient could have serious complications and morbidity. To determine if CD26 inhibition impaired or skewed differentiation of transplanted human CD34<sup>+</sup> cells in the NOD/SCID mouse recipients, we analyzed the expression of various surface markers associated with primarily the myeloid and B lymphoid lineages, as well as markers for immature HSC populations. Transplanted mouse bone marrow cells were stained and analyzed by FACS for their expression of human CD34, CD38, CD19 and CD33, markers routinely used to determine multi-lineage potential of engrafted human cells in NOD/SCID mice (Bhatia et al. 1997b; Ishikawa et al. 2003). CD34 is a marker for human HSCs and HPCs (Janeway 2005). CD38 is used in conjunction with CD34 to delineate mature versus immature stem/progenitor populations (Ishikawa et al. 2003; Janeway 2005). CD19 is a B-cell marker (Janeway 2005) and CD33 is a myeloid cell marker (Janeway 2005). Figure 6 shows representative FACS plots from animals transplanted with control and Diprotin A treated cells demonstrating multi-lineage staining of the human cells. When mice exhibiting human chimerism greater than 20% were analyzed and the data pooled, no significant differences were noted in percentages of the various populations assayed (Table 1), suggesting that CD26 inhibition did not significantly affect lineage differentiation of the transplanted CD34<sup>+</sup> cells.

Representative control animal



Representative Diprotin A animal



**Figure 6: Effect of CD26 inhibition on multilineage differentiation of CD34<sup>+</sup> transplanted cells.** These FACS plots show representative NOD/SCID animals from the experiment in Figure 4. Gated human (CD45<sup>+</sup>) cells are shown expressing CD34, CD38, CD19 and CD33 surface markers.



	% CD38 <sup>+</sup> CD34 <sup>-</sup>	% CD38 <sup>+</sup> CD34 <sup>+</sup>	% CD34 <sup>+</sup> CD38 <sup>-</sup>
Control	68.4 ± 2.4	20.7 ± 3.8	0.25 ± 0.27
Diprotin A	68.6 ± 10.9	21.8 ± 10.0	0.17 ± 0.18

	% CD33 <sup>+</sup>	% CD19 <sup>+</sup>
Control	11.5 ± 8.5	64.5 ± 13.6
Diprotin A	5.7 ± 3.3	73.5 ± 7.5

**Table 2: Phenotype of engrafted human cells in NOD/SCID mice.** CD26 inhibition did not impair multilineage differentiation of transplanted CD34<sup>+</sup> cells. Table 2 shows data pooled from animals which had chimerism of greater than 20% (N=5 control and N=8 Diprotin A). The percentages shown are within the CD45<sup>+</sup> human population (Mean  $\pm$  SD). There were no significant differences in any of the populations.

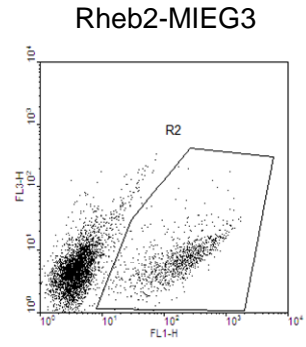
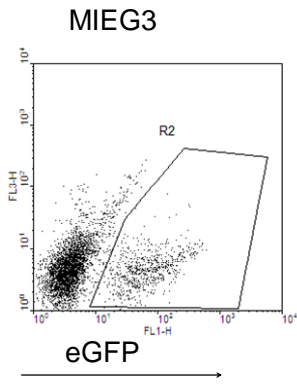
Part II: Overexpression of the novel Ras molecule, Rheb2, enhanced HPC and HSC growth and impaired HSC *in vivo* repopulating activity.

Understanding which signaling molecules and pathways affect hematopoietic cell proliferation, self-renewal, differentiation and engraftment is important for clinical uses of these cells, including *ex vivo* expansion, viral transduction to correct genetic deficiencies and immune cell manipulation to control GVHD.

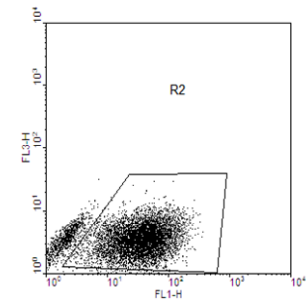
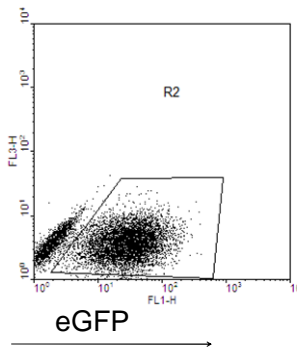
We were interested in the role of the novel ras molecule, Rheb2, in mouse hematopoietic cell function for two reasons: its expression pattern in mouse HSCs suggests it may be important for their function, and it activates the mTOR pathway, known to be involved in regulating hematopoietic cell growth, survival and other functions. We studied the role of Rheb2 in mouse hematopoietic cells by overexpressing it using the retroviral vector, Mieg3. The Mieg3 vector contains an enhanced GFP marker protein which allows for easy detection of transduced cells in various cell types. FACS analysis of transduced cells, including 3T3 and mouse primary BM cells, shown in Figure 7 illustrates that positively transduced cells can be separated easily from the non-transduced population.

We confirmed that the Rheb2-MIEG3 vector produced full-length recombinant Rheb2 protein by Western blotting cellular lysates with both anti-Flag and anti-Rheb2 antibodies in various cell lines, as shown in Figure 8. To further confirm the specificity of the band seen in the Western blots, we used an shRNA vector containing a sequence specific against Rheb2 cDNA and, using transient transfection, were able to knock-down the expression of the recombinant Rheb2 in cells overexpressing it as shown in Figure 9. These results confirmed that full-length Rheb2 was produced by the retroviral vector Rheb2-Mieg3.

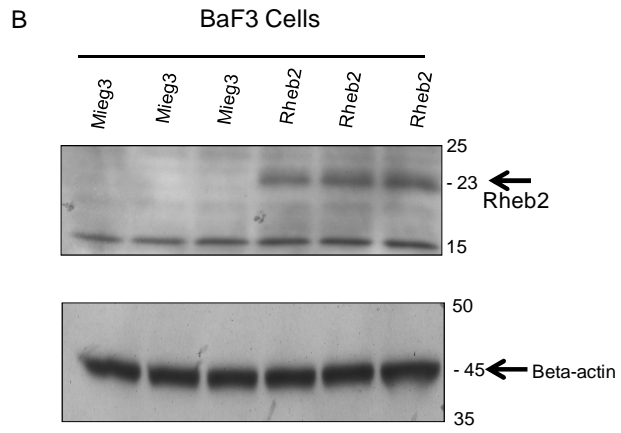
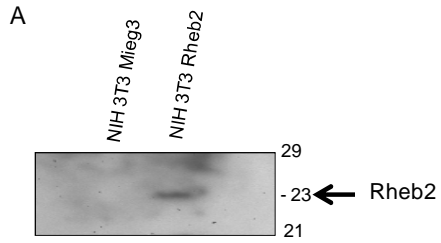
Mouse Bone Marrow  
Mononuclear Cells



NIH 3T3  
Cells



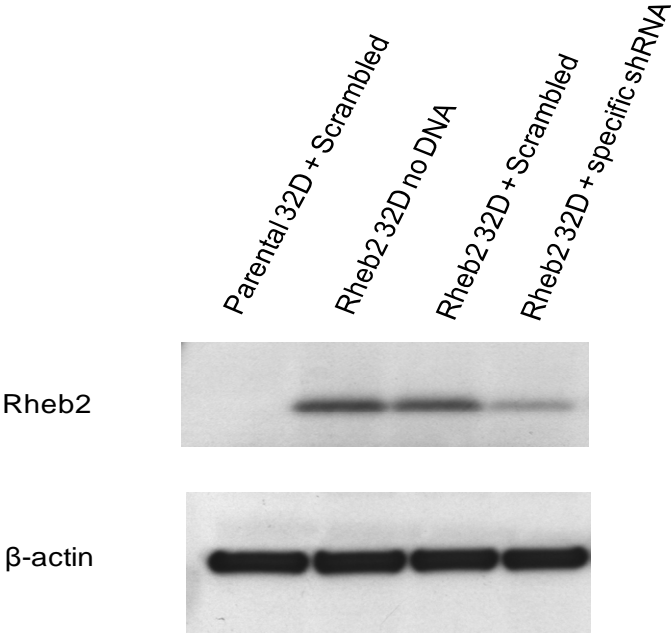
**Figure 7: Detection of GFP<sup>+</sup> cells after transduction with Mieg3 and Rheb2-Mieg3 retroviral vectors.** Mieg3 or Rheb2-Mieg3 retroviral supernatants were used to transduce primary mouse MNCs or 3T3 cells and GFP<sup>+</sup> cells were determined by FACS. Shown are representative FACS plots illustrating that both retrovirus transduce cells equally well leading to GFP expression which can be easily differentiated using FACS analysis.



**Figure 8: Production of Rheb2 protein by transduction with the Rheb2-Mieg3 retrovirus.** Mieg3 and Rheb2-Mieg3 retroviral supernatants were used to transduce NIH-3T3 (A) and BaF3 (B) cells; lysates were collected and production of full-length (about 20kD) Rheb2 protein was confirmed in cells transduced with the Rheb2-Mieg3 virus by blotting for Flag tag in A and anti-Rheb2 (Abnova) in B. Part B shows 3 samples per transduced BaF3 cell lines.

Mouse Rheb2 cDNA BC016521

5'atgccgctgggtgcgctacaggaaggtggccatcctagggt  
accgctCcgtagggaagacatccttggcacatcaattgtg  
gaaggcgagttCctggaaggctacgatcctacagtggaga  
atactta ..... 3'





**Figure 9: Effect of shRNA knock-down in cells overexpressing Rheb2.** Transduced 32D cells overexpressing Rheb2 protein (labeled as Rheb2 32D) were transfected with plasmid DNA vectors encoding scrambled or specific Rheb2 shRNA constructs, and protein lysates were analyzed for Rheb2 protein by Western Blot. The sequence of Rheb2 used to design the shRNA vectors is shown in the gray box above. The scrambled vector had no ability to knock-down Rheb2 protein compared to a negative control transfected without DNA. The specific anti-Rheb2 vector was able to partially silence the expression of Rheb2 in these cells. Transfection efficiency in these experiments varied between 40 and 60%. Representative of more than 3 independent experiments.

*Overexpression of Rheb2 enhanced proliferation and mTOR signaling in two mouse hematopoietic progenitor cell lines*

To test the ability of overexpressed Rheb2 protein to activate the mTOR pathway, we created stable, transduced mouse BaF3 and 32D cell lines by transduction with Mieg3 and Rheb2-Mieg3 viruses and FACS sorting followed by expansion. BaF3 is a mouse pro B-cell line, and 32D is a mouse myeloid progenitor cell line. Both of these are dependent on the growth factor IL-3 for growth and survival. In BaF3 cells, signaling through the mTOR pathway is important for mediating IL-3-induced growth (Cruz et al. 2005). Therefore, we tested the ability of Rheb2 to enhance proliferation and mTOR signaling in response to delayed addition of IL-3.

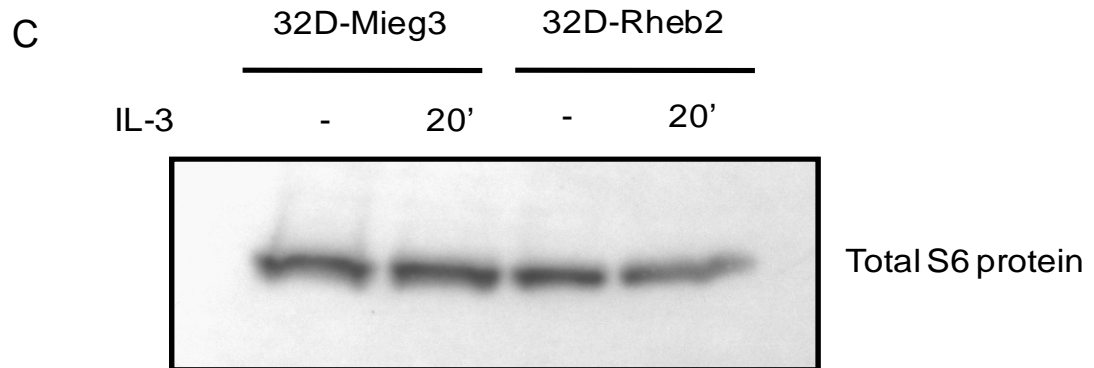
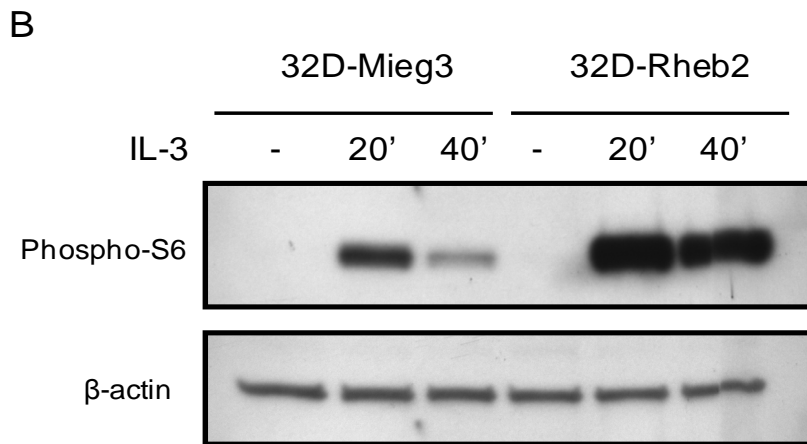
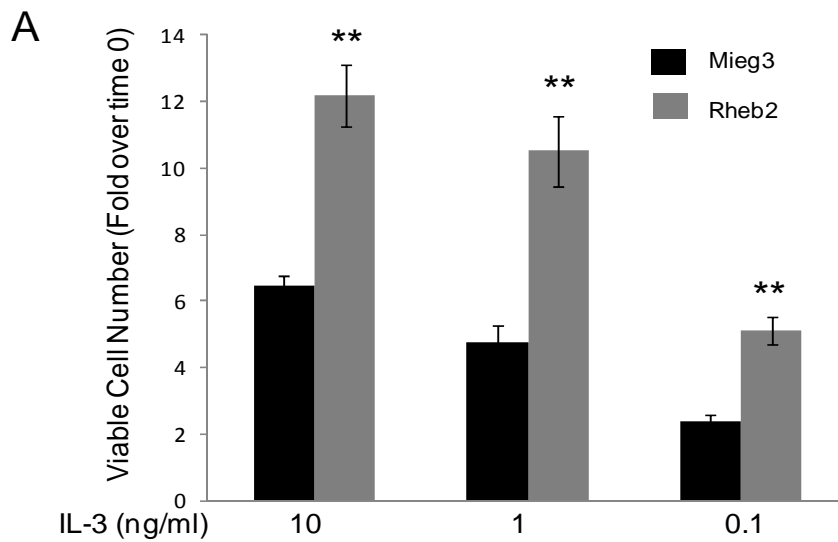
32D cells were plated in the absence of IL-3 and incubated for 8 hours before IL-3 was added back to the cultures. Illustrated in Figure 10A, 32D cells overexpressing Rheb2 proliferated significantly greater than empty vector transduced cells in response to delayed IL-3 addition. This enhancement of proliferation was significant at three different concentrations of IL-3 added back to the cultures. In addition to the proliferation of 32D cells, signaling through the mTOR pathway was measured after delayed IL-3 addition by Western blotting for phosphorylated S6 ribosomal protein, a known downstream effector of mTOR commonly used to measure its activity and inhibited by rapamycin treatment (Raslova et al. 2006). As shown in Figure 10B, 32D cells overexpressing Rheb2 had hyperactive mTOR activity in response to IL-3 addition compared to empty vector cells. This hyperactive mTOR signaling was evident at 20 and 40 minutes after IL-3 addition.

BaF3 cells were transduced and analyzed in a similar fashion as 32D cells. In response to 12 hour IL-3 starvation followed by IL-3 addition, Rheb2-BaF3 cells proliferated significantly greater than Mieg3-32D cells as shown in Figure 11A. When measuring

mTOR activity after starvation and IL-3 addition, Rheb2-BaF3 had increased signaling through this pathway as seen in Figure 11B.

These results demonstrate that Rheb2 overexpression increased the proliferation in response to delayed IL-3 addition of two hematopoietic progenitor cell lines, 32D and BaF3. Correlated with the increased proliferation, Rheb2 overexpression increased signaling through the mTOR pathway, confirming that Rheb2 can activate this pathway.

### 32D Cells 8 hour IL-3 starvation

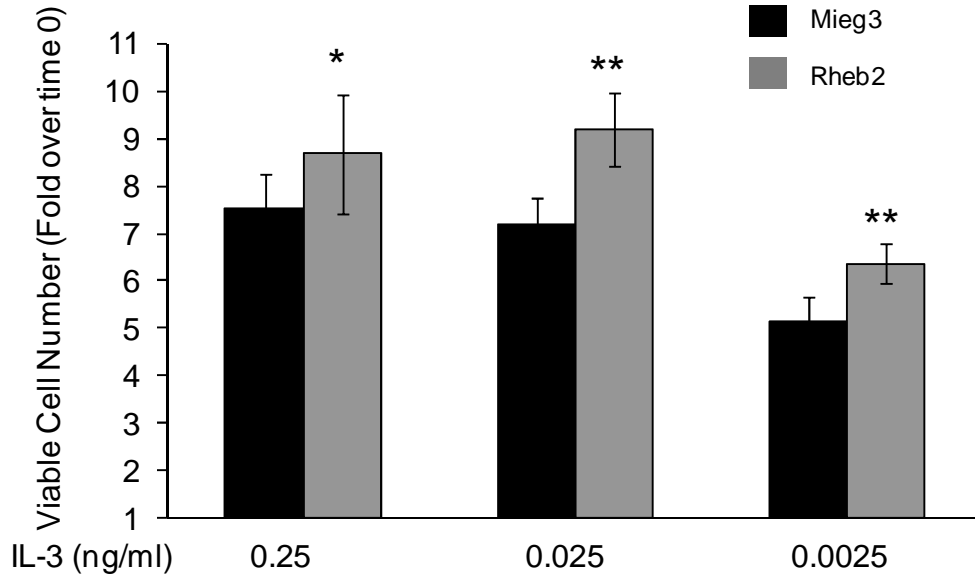


**Figure 10: Effect of Rheb2 overexpression on 32D proliferation and mTOR**

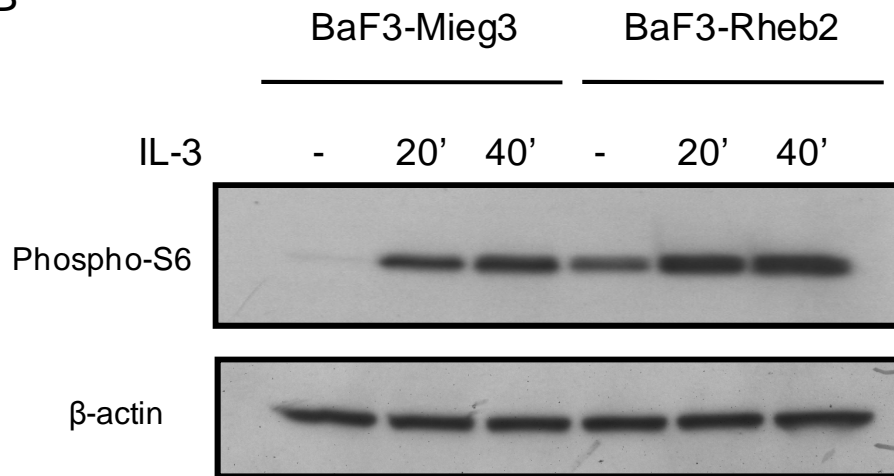
**signaling.** 32D cells were transduced with Mieg3 or Rheb2-Mieg3 retroviral supernatants, GFP<sup>+</sup> cells FACS sorted and expanded to create stably expressing cell lines. In part A, cells were plated in triplicate at  $1 \times 10^5$  cells per well without IL-3 and starved for 8 hours. After 8 hours, IL-3 (10 ng/ml, 0.1 ng/ml or 0.01 ng/ml) was added back to the cultures and viable cell counts were taken after 48 hours in culture by trypan blue exclusion counting. Combined data from 2 independent experiments (mean  $\pm$  SD). \*\*  $p < 0.01$ . For part B, 32D cells were plated at  $1 \times 10^6$  cells per well, starved without IL-3 for 8 hours. After 8 hours, IL-3 (10ng/ml) was added to the wells for 20 or 40 minutes. Negative controls had PBS added for 40 minutes. Cellular lysates were taken and Western blots were performed for phosphorylated ribosomal S6 protein levels. Representative of two independent experiments. Part C shows levels of total ribosomal S6 protein in representative 32D samples indicating that total S6 was not increased in the Rheb2 cell line.

# BaF3 Cells 12 hour IL-3 starvation

A



B



**Figure 11: Effect of Rheb2 overexpression on BaF3 cell proliferation and mTOR signaling.** BaF3 cells were transduced with Mieg3 or Rheb2-Mieg3 retroviral supernatants, GFP<sup>+</sup> cells FACS sorted and expanded to create stably expressing cell lines. In part A, cells were plated in triplicate at  $1 \times 10^5$  cells per well without IL-3 and starved for 12 hours. After 12 hours, IL-3 (0.25 ng/ml, 0.025 ng/ml or 0.0025 ng/ml) was added back to the cultures and viable cell counts were taken after at least 33 hours in culture by trypan blue exclusion counting. Combined data from 3 independent experiments (mean  $\pm$  SD). \*  $p < 0.05$ . For part B, BaF3 cells were plated at  $1 \times 10^6$  cells per well, starved without IL-3 for 12 hours. After 12 hours, IL-3 (10ng/ml) was added to the wells for 20 or 40 minutes. Negative controls had PBS added for 40 minutes. Cellular lysates were taken and Western blots were performed for phosphorylated ribosomal S6 protein levels. In addition to 12 hour starvation, hyperactivation of mTOR pathway was observed after 8 hour IL-3 starvation.

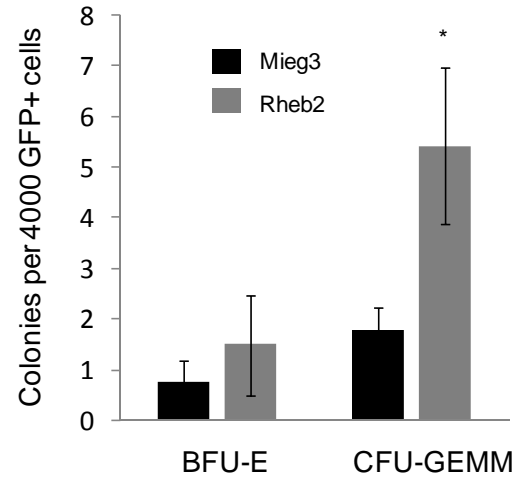
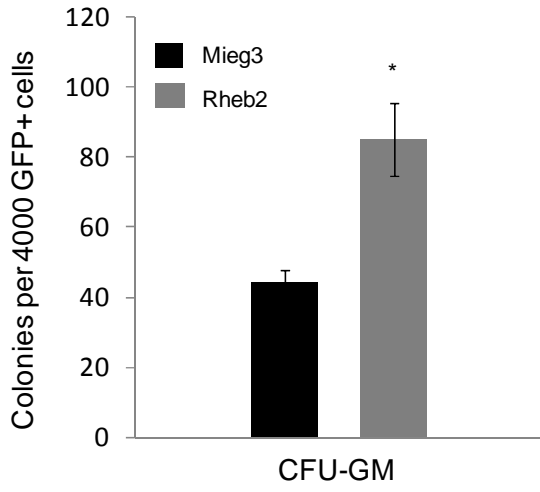
*Overexpression of Rheb2 enhanced colony-forming ability of primary mouse hematopoietic progenitor cells*

To determine the effect of overexpressing Rheb2 on the colony-forming ability of hematopoietic cells, primary mouse BM cells were transduced with Mieg3 and Rheb2-Mieg3 vectors, GFP<sup>+</sup> cells sorted by FACS and plated in semi-solid methylcellulose colony assays containing growth factors conducive for progenitor cell growth including PWMSCM, hemin, FBS and SCF (see Materials and Methods for further explanation). CFU-GM, BFU-E and CFU-GEMM colonies were counted after 6-8 days incubation. Overexpression of Rheb2 in these cells significantly increased the colony-forming ability compared to vector control cells as illustrated in Figure 12. Average numbers of CFU-GM colonies per 4000 cells plated significantly increased from  $44 \pm 3.6$  SEM in Mieg3 plates to  $85 \pm 10.6$  SEM in Rheb2 plates. The average number of CFU-GEMM increased from  $1.8 \pm 0.5$  SEM in Mieg3 plates to  $5.4 \pm 1.5$  SEM in Rheb2 plates. In addition to these two colony types, the average number of Bfu-E colonies per 4000 cells increased due to Rheb2 overexpression; however, the numbers were very low and did not attain statistical significance.

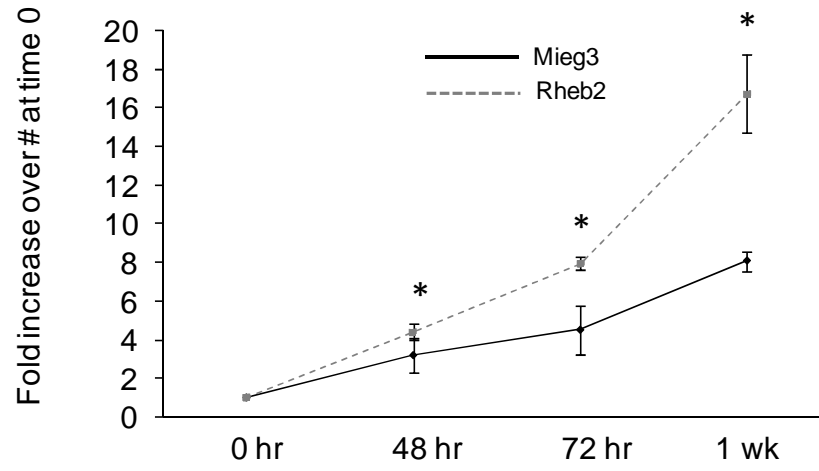
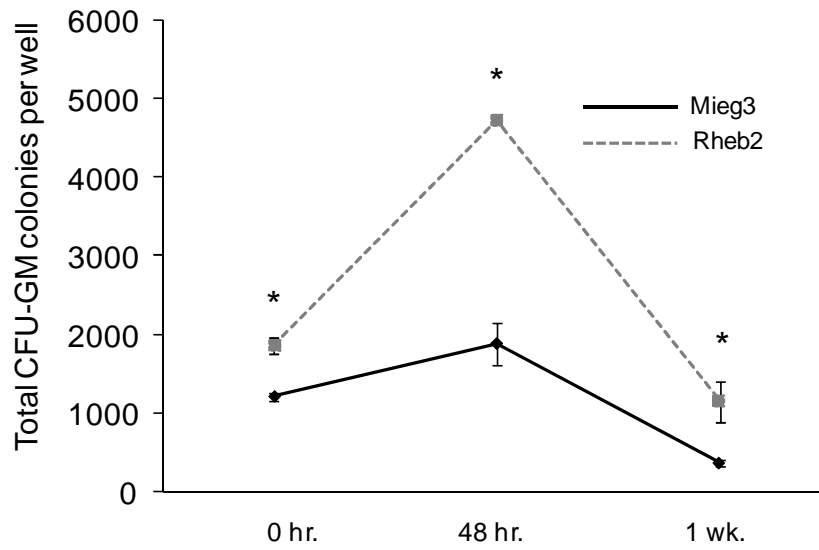
In addition to plating GFP<sup>+</sup> cells directly in colony assays after FACS sorting, we cultured these cells up to one week after sorting in liquid culture containing SFT (50 ng/ml each) growth factors. Cells were removed from the liquid cultures at 48 hours and 1 week after seeding and plated in methylcellulose colony assays. Figure 13 shows in part A the expansion of the total number of GFP<sup>+</sup> cells determined by trypan blue exclusion counting and in part B the expansion of CFU-GM progenitors as determined by methylcellulose colony assays. The numbers of total GFP<sup>+</sup> cells were significantly increased up to two-fold over Mieg3 cells by Rheb2 overexpression as shown in Figure 13A. Consistent with an expansion of total cells, Rheb2 overexpression expanded



colony-forming CFU-GM progenitors greater than 2-fold over Mieg3 progenitor numbers at 48 hours and 1 week after plating in liquid culture. These results demonstrate that overexpression of Rheb2 can expand total cell numbers as well as progenitor cell numbers in liquid cultures.



**Figure 12: Effect of Rheb2 overexpression on primary mBM MNC colony formation.** Mouse BM MNCs were transduced with either Mieg3 or Rheb2-Mieg3 retroviral supernatants. GFP<sup>+</sup> cells were FACS sorted and plated in triplicate in methylcellulose colony assays containing PWMSCM, FBS, hemin and SCF (50 ng/ml) and colony numbers scored after 6-7 days incubation at low oxygen tension (O<sub>2</sub> = 5%). Results are combined for three independent experiments (mean ± SEM). \* p<0.05

**A****B**

**Figure 13: Effect of Rheb2 overexpression on colony-forming cell expansion *in vitro*.** GFP<sup>+</sup> cells ( $5 \times 10^4$  per well) were cultured in triplicate in medium containing SFT (50 ng/ml each) for the indicated times. At each time point, total viable cell numbers were measured by trypan blue exclusion counting (part A), and cells were removed from the liquid cultures and plated in triplicate in methylcellulose colony assays. Numbers of CFU-GM progenitors per well were calculated by numbers of progenitors per input cells in methylcellulose assays (part B). Mean  $\pm$  SD. \*  $p < 0.05$ .

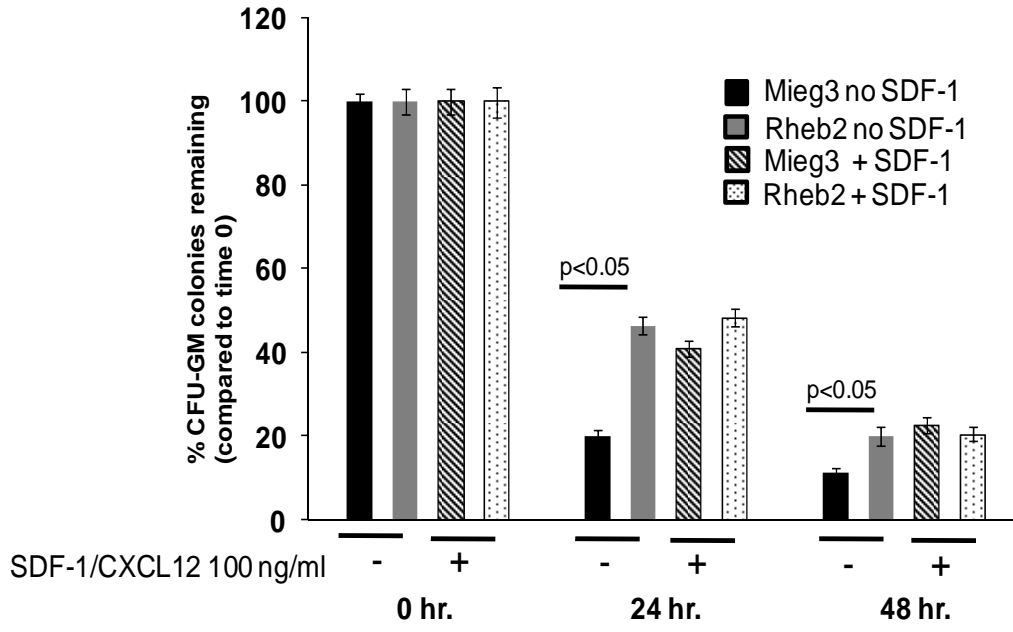
*Rheb2 overexpression enhanced the survival of primary hematopoietic progenitors in delayed growth factor addition colony assays*

The mTOR pathway, downstream of Rheb2, is a positive regulator of cell survival of various cell types under disparate stimuli. Therefore, we tested the ability of overexpressed Rheb2 to enhance the survival of primary hematopoietic progenitor cells using a delayed growth factor colony assay system. Primary mouse MNCs were transduced and FACS sorted. The GFP<sup>+</sup> cells were plated in methylcellulose colony assays in the absence of colony-stimulating growth factors as described in Materials and Methods. At 0, 24 and 48 hours after plating, growth factors were added to individual plates. Colonies were scored after 6-8 days of incubation. In addition, we tested the ability of CXCL12/SDF-1, a known pro-survival chemokine, to enhance the colony formation of these transduced cells by adding the chemokine or PBS to the plates at time 0.

The results from these delayed growth factor addition assays are shown in Figure 14. In the absence of CXCL12/SDF-1, overexpression of Rheb2 significantly enhanced, compared to empty vector, progenitor survival when standardized to time 0 CFU-GM colony numbers (Figure 14). In the presence of CXCL12/SDF-1, the survival of Mieg3 progenitor cells was enhanced as expected; however, the survival of Rheb2 overexpressing cells was not enhanced as shown in Figure 14. These results suggest that Rheb2 enhances the survival of progenitor cells, and that this effect is not increased by CXCL12/SDF-1 stimulation.

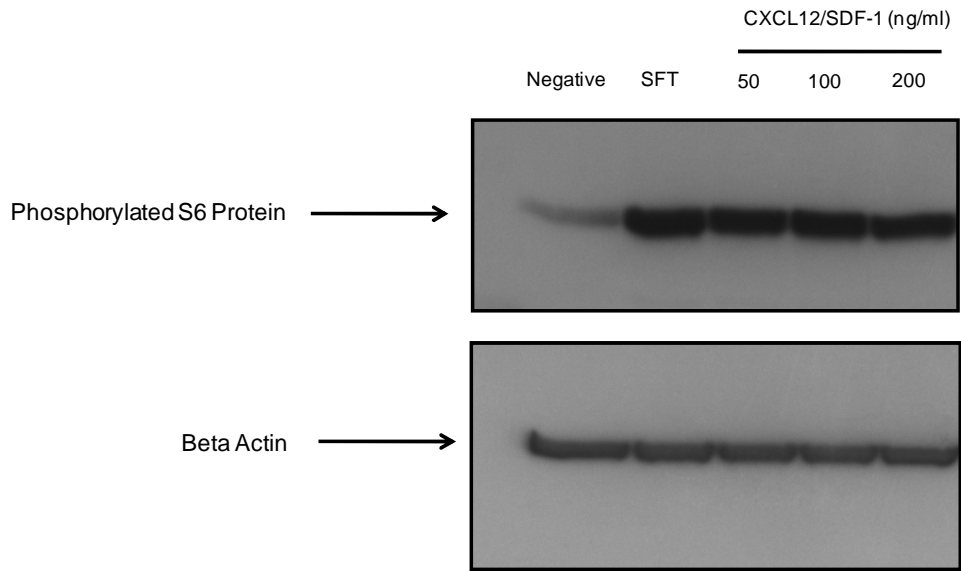
There are many possible explanations for the lack of enhancement of Rheb2 transduced cell survival by SDF-1. The survival of these cells may already be maximally stimulated by Rheb2 overexpression and introducing additional survival factors may be masked by

its effect. In addition, Rheb2 transduced cells may have downregulated the SDF-1 receptor CXCR4, thus preventing the cells from responding to SDF-1. An interesting possibility is that both SDF-1 and Rheb2 stimulate common intracellular pathways leading to enhanced survival. Specifically, both SDF-1 and Rheb2 may stimulate the mTOR pathway. We tested this hypothesis by stimulating cultured mouse MNCs after a period of starvation with various concentrations of CXCL12/SDF-1, isolating cellular lysates and Western blotting for mTOR activity using anti-phospho S6 ribosomal protein, a known downstream effector of mTOR commonly used to measure mTOR activity and inhibited by rapamycin treatment (Raslova et al. 2006). We found that indeed SDF-1 activated the mTOR activity when compared to unstimulated cells (Figure 15). This suggests that Rheb2 and SDF-1 may share downstream effectors leading to enhanced cell survival.





**Figure 14: Rheb2 overexpression enhanced progenitor survival.** Mouse BM MNCs were transduced and FACS sorted for GFP<sup>+</sup> cells. GFP<sup>+</sup> cells were plated in methylcellulose colony assays absent of the growth factors hemin, PWMSCM and SCF. At the indicated time points, these GFs were added to the methylcellulose plates. Colony assays were incubated a total of 6-7 days and colonies counted. The percent remaining at each time point was calculated by standardizing to the number of colonies at time 0 in both Mieg3 and Rheb2-Mieg3 assays. Representative of two independent experiments (mean  $\pm$  SD).



**Figure 15: Effect of SDF-1/CXCL12 stimulation on mTOR activity in mBM MNCs.**

MNCs were cultured in medium containing SFT (50 ng/ml each) for 48 hours. Cells were starved of SFT for 8 hours and stimulated with either PBS (negative control), SFT (50 ng/ml each, positive control) or various concentrations of SDF-1/CXCL12 as indicated for 40 minutes. Cellular lysates were harvested and Western blots were performed to measure the level of phosphorylated ribosomal S6 protein. Beta actin was used as a loading control. Representative of two independent experiments.

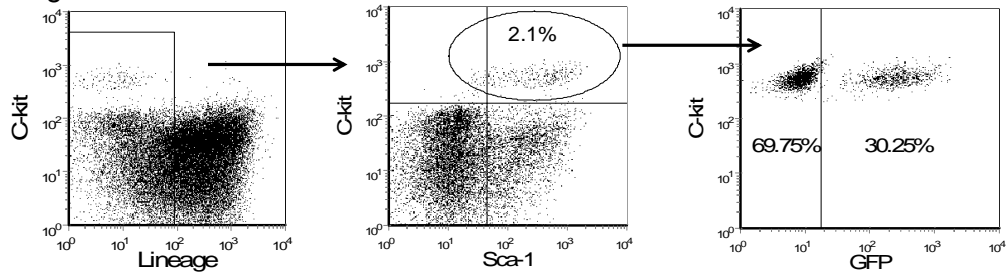
*Overexpression of Rheb2 expanded phenotypic HSCs in vitro – a process partially dependent upon mTOR signaling*

We have shown thus far that overexpression of Rheb2 had positive effects on growth and survival of transduced mouse hematopoietic progenitor cells. To further define the role of Rheb2 on immature HSC populations, we transduced mouse MNCs and cultured them *in vitro* in the combination of SFT (50 ng/ml each) for 4 days after transduction. After 4 days, we determined the percentage of phenotypic hematopoietic stem cells, defined as c-kit<sup>+</sup>, sca-1<sup>+</sup> and lineage<sup>-</sup> (KSL) in the cultures. In addition, we cultured the cells in the absence or presence of rapamycin (50 nM) to determine if mTOR inhibition could help to reverse the expansion of the HSCs. The results of these experiments are shown in Figure 16. In 16A, representative FACS plots of cultured cells clearly show an increased percentage of Rheb2 GFP<sup>+</sup> (transduced) cells within the total KSL population compared to Mieg3 GFP<sup>+</sup> cells. Importantly the percent of total GFP<sup>+</sup> cells (25% for Mieg3 and 23% for Rheb2), as well as total cell numbers were similar in Mieg3 and Rheb2 cultures, suggesting a specific effect of Rheb2 overexpression on immature KSL cells. The graph in Figure 16B shows tabulated data on expansion of KSL cells. Overexpression of Rheb2 significantly expanded the percent of GFP<sup>+</sup>KSL cells four-fold over empty vector transduced cells. Importantly, the percent of GFP<sup>-</sup>KSL cells was not significantly affected by Rheb2 overexpression, illustrating that these results are not due to general KSL expansion but are specific for the GFP<sup>+</sup> transduced fraction. In addition, the percent of GFP<sup>+</sup>KSL cells in both Mieg3 and Rheb2 cultures shortly after transduction (less than 1 day) were not dramatically different (0.08% in Mieg3 and 0.12% in Rheb2 cultures), suggesting that the Rheb2 retrovirus was not simply targeting immature cells preferentially during the transduction period.

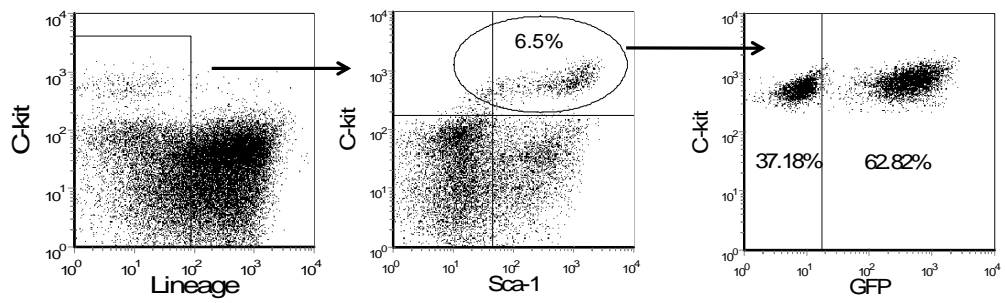
The addition of rapamycin to these cultures decreased the percentage of KSL cells in both the GFP<sup>-</sup> and GFP<sup>+</sup> fractions in both Mieg3 and Rheb2 cultures. This decrease was significant for Rheb2 GFP<sup>+</sup> cells but did not reach significance for Mieg3 GFP<sup>+</sup> cells. Rapamycin did not completely reverse the expansion of KSL cells due to Rheb2, and there was still a significant increase in GFP<sup>+</sup>KSL percentage in Rheb2 versus Mieg3 cultures. This suggests that either the concentration of rapamycin was not high enough at 50 nM or that the mechanism controlling this expansion is mediated by mTOR as well as other cellular pathways. Additionally, the use of rapamycin in the context of constitutive Rheb2 activity (overexpression) may release autoinhibitory feedback loops known to be downstream of mTOR acting on Akt signaling (explained further in Discussion). Overall, these results show that Rheb2 can expand KSL cells *in vitro* which can be partially inhibited by blocking mTOR activity.

A

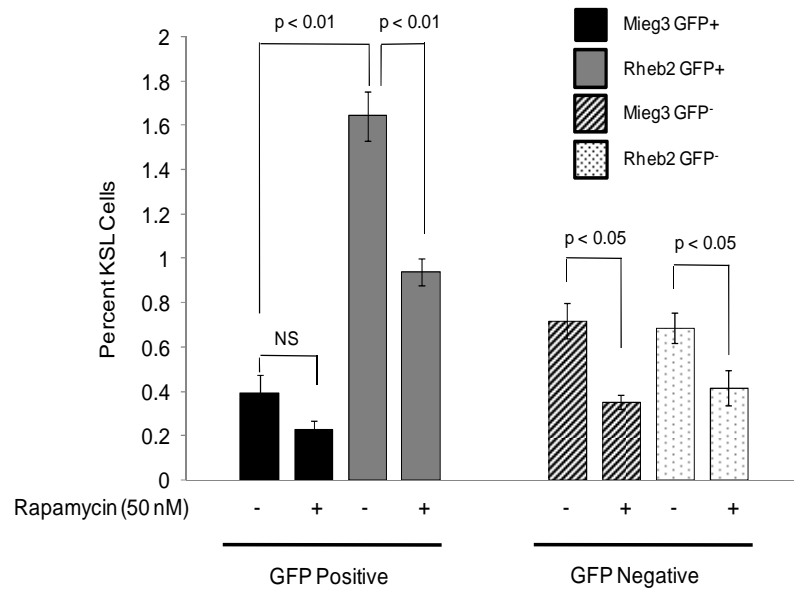
Mieg3



Rheb2



B



**Figure 16: Rheb2 overexpression expanded immature hematopoietic cells *in vitro*.**

Mouse BM MNCs were transduced and cultured in medium containing SFT (50 ng/ml) with either rapamycin (50 nM) or vehicle control (methanol) in duplicate for each condition for 4 days after transduction (total of 8 days after isolation from mice). The percentage of KSL cells out of the total population was determined in GFP<sup>+</sup> (transduced) and GFP<sup>-</sup> (non-transduced) populations. Representative FACS plots shown in part A, note the percentage in the middle plots refer to the percentage of KSL cells out of the lineage negative population. Combined results from two independent experiments (mean  $\pm$  SEM) are shown in part B. p values are shown on the graph.

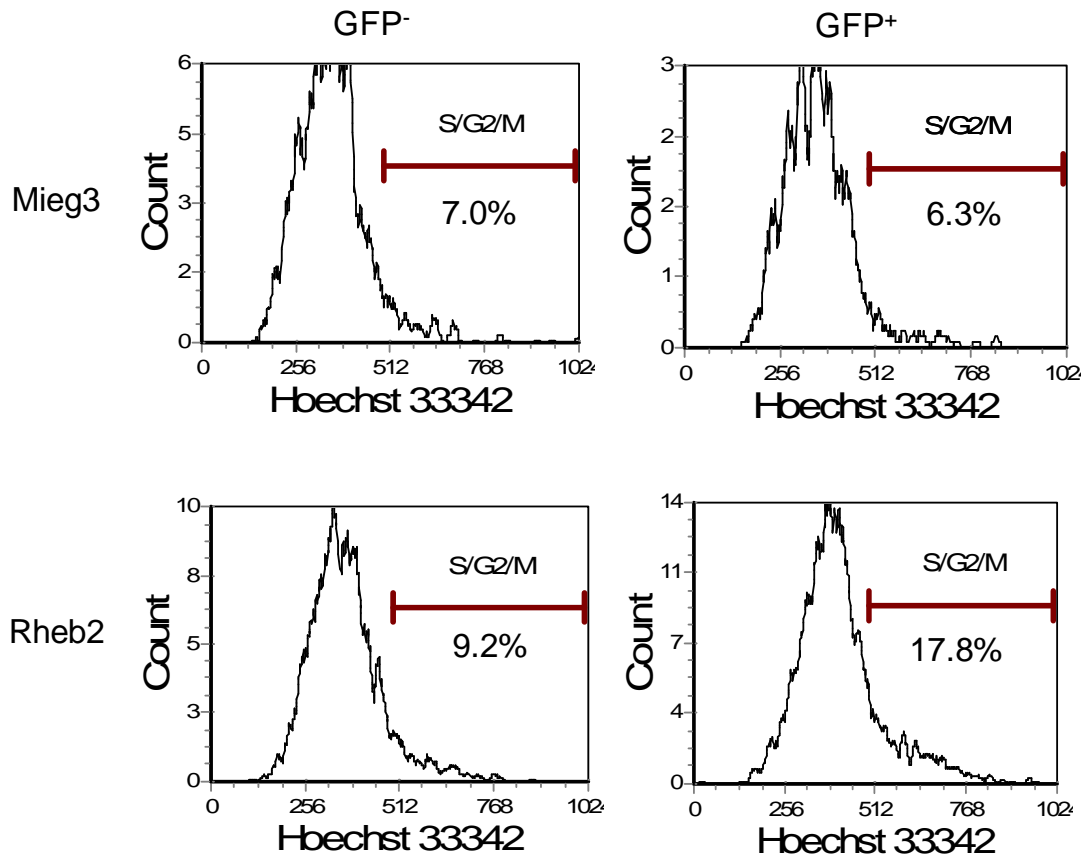
*Overexpression of Rheb2 enhanced the cell cycling status of mouse KSL cells expanded in liquid culture*

To test whether the expansion of KSL cells due to Rheb2 overexpression accompanied an increase in the cycling of the KSL cells, we used Hoescht 33342 DNA staining to determine the percent of KSL cells in G1 versus S/G2/M *in vitro*.

In *in vitro* expanded cultures, Rheb2 transduced GFP<sup>+</sup> KSL cells had a significantly greater percentage of cells in S/G2/M compared to Mieg3 GFP<sup>+</sup>KSL cells (Figures 17 and 18). In Figure 18, two independent experiments analyzing the percentage of cycling S/G2/M cells in Mieg3 and Rheb2 GFP<sup>-</sup> and GFP<sup>+</sup> fractions cultured in the absence or presence of 50 nM rapamycin are shown. In the first experiment, cells were cultured for 3 or 4 days after transduction and analyzed by FACS. We found a significant increase in the percent of S/G2/M cells in the Rheb2 GFP<sup>+</sup> fraction compared to Mieg3 GFP<sup>+</sup> cells at day 3 but not day 4 cultures, suggesting that this difference was detectable at early but not late culture time points. This significant increase was also found in day 3 cultures containing rapamycin, confirming increased cycling due to Rheb2 overexpression. The percent of Rheb2 GFP<sup>+</sup> S/G2/M cells was decreased due to rapamycin treatment; however, this difference was not significant. Similar to the results of KSL expansion discussed earlier (Figure 16), this suggests that either mTOR is partially responsible for this increased cycling or that the intricacies of the mTOR signaling pathway confound this analysis. It is also possible that rapamycin inhibits the movement of cells out of S/G2/M by a checkpoint block; however, this is not likely given evidence that rapamycin blocks the transition from G1 to S in the cell cycle (Dutcher 2004). In the second experiment, Hoechst staining was performed at 4 and 5 days culture after transduction. The percent of cycling S/G2/M Rheb2 GFP<sup>+</sup> cells was increased at both time points compared to Mieg3 GFP<sup>+</sup> cells, although these were not significant. Interestingly, when comparing the GFP<sup>+</sup> transduced population to the GFP<sup>-</sup> population within Rheb2

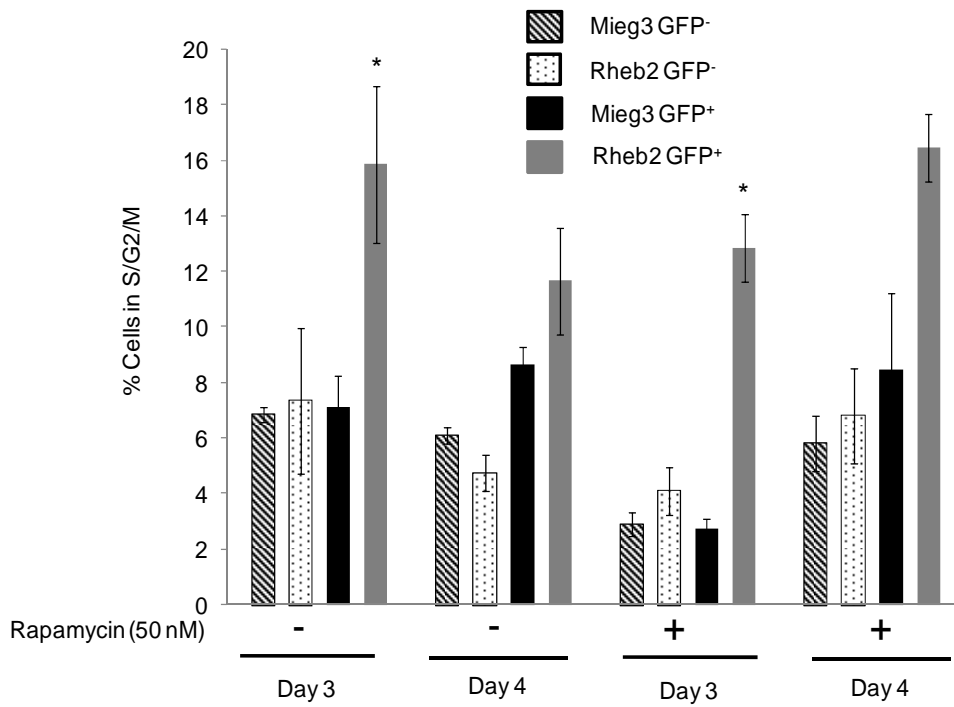


cultures, the percent of cycling S/G2/M GFP<sup>+</sup> cells was significantly greater. This difference was found in cultures without and with rapamycin at both day 4 and day 5 (Figure 18). Again, these results suggest that Rheb2 can increase the entrance of KSL cells into S/G2/M causing expansion of this population.

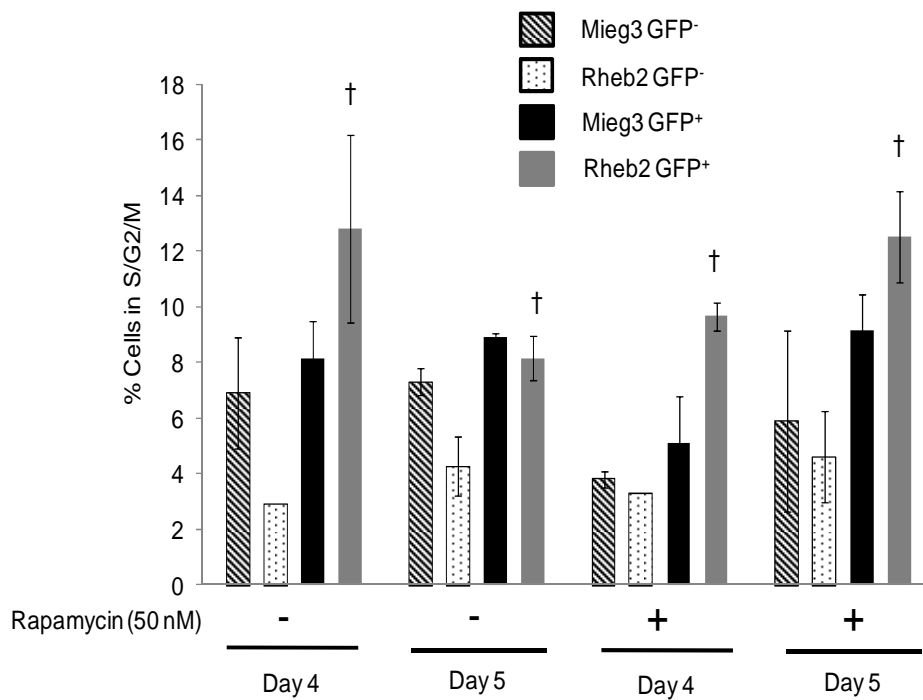


**Figure 17: Effect of Rheb2 on KSL cycling *in vitro*.** Transduced mouse BM MNCs were culture for 3 or 4 days (experiment 1) or 4 or 5 days (experiment 2) and stained for KSL markers as well as Hoechst DNA staining. Above are representative FACS plots from Day 3 cultures without rapamycin in experiment 1, illustrating that a higher percentage of GFP<sup>+</sup> KSL cells in the Rheb2 GFP<sup>+</sup> cultures fall within the S/G2/M region of Hoechst staining (right side of figure). The KSL gating parameters used were the same as those used in Figure 16. The Hoechst profile in GFP<sup>-</sup> populations was similar between Mieg3 and Rheb2 cultures (left side of figure). Tabulated data from both experiments is shown in Figure 18.

### Experiment 1



### Experiment 2



**Figure 18: Effect of Rheb2 on KSL cycling *in vitro*.** The percent of cycling KSL cells was measured by Hoechst staining. Two independent experiments are shown (mean  $\pm$  SD). In experiment 1, cells were stained after 3 or 4 days in culture. In experiment 2, cells were stained after 4 or 5 days in culture. \* indicates  $p < 0.05$  compared to Mieg3 GFP<sup>+</sup> cells, while † indicates  $p < 0.05$  compared to the Rheb2 GFP<sup>-</sup> fraction within the same sample.

*Overexpression of Rheb2 impairs in vivo competitive repopulation of total transduced cells, while expanding immature hematopoietic cells in a short-term model*

To determine the effect of Rheb2 overexpression on mouse HSC functions such as differentiation and self-renewal, we performed *in vivo* competitive repopulation assays. As described in Materials and Methods, we transduced donor MNCs from C57/Bl6 mice (CD45.2<sup>+</sup>). These cells were mixed at a 1:1 ratio with competitor BM cells from BoyJ mice (CD45.1<sup>+</sup>) and were transplanted into heterozygous C57/Bl6-BoyJ CD45.1<sup>+</sup>/CD45.2<sup>+</sup> recipient mice conditioned with 950 Rad of total body irradiation. To determine chimerism of donor cells, we isolated both PB and BM at 1, 2 and 4 weeks after transplantation and stained the cells with anti-CD45.1 and anti-CD45.2 antibodies. In addition, we stained the BM samples with c-kit, sca-1 and lineage antibodies to determine GFP<sup>+</sup> cell numbers within immature populations of hematopoietic cells.

Representative FACS plots are shown in Figure 19, demonstrating that donor (CD45.2<sup>+</sup>45.1<sup>-</sup>), competitor (CD45.1<sup>+</sup>45.2<sup>-</sup>) and recipient (CD45.1<sup>+</sup>45.2<sup>+</sup>) cells, as well as GFP<sup>+</sup> and GFP<sup>-</sup> cells within the donor population can easily be detected in transplanted animal PB samples using FACS. Figure 20A shows the tabulated PB chimerism of donor transduced cells at 1, 2 and 4 weeks after transplantation. At 2 and 4 weeks, Rheb2 transduced GFP<sup>+</sup> cells had significantly impaired PB chimerism compared to Mieg3 GFP<sup>+</sup> cells (Figure 20A). At four weeks, the percent of CD45.2<sup>+</sup>GFP<sup>+</sup> PB cells in animals transplanted with Rheb2 transduced cells was decreased 2-fold compared to the same population in animals transplanted with Mieg3 transduced cells. Importantly, the engraftment of CD45.2<sup>+</sup>GFP<sup>-</sup> cells was not significantly different between animals transplanted with Mieg3 or Rheb2 cells, suggesting a specific effect on the GFP<sup>+</sup> fraction and confirming that transplanted cell numbers were equal between these groups.

In addition to PB, we determined the effect of Rheb2 overexpression on BM cell engraftment at 1, 2 and 4 weeks after transplantation. The engraftment of total donor CD45.2<sup>+</sup> GFP<sup>+</sup> cells is shown in Figure 20B, where we observed a similar trend as in PB samples. Animals transplanted with Rheb2 cells had significantly lower donor GFP<sup>+</sup> cell engraftment compared to animals transplanted with Mieg3 cells at 2 and 4 weeks after transplantation. In the BM the impairment of engraftment due to Rheb2 was more dramatic than PB, with a greater than 5-fold reduction in total CD45.2<sup>+</sup>GFP<sup>+</sup> in animals transplanted with Rheb2 cells compared to Mieg3 cells at four weeks.

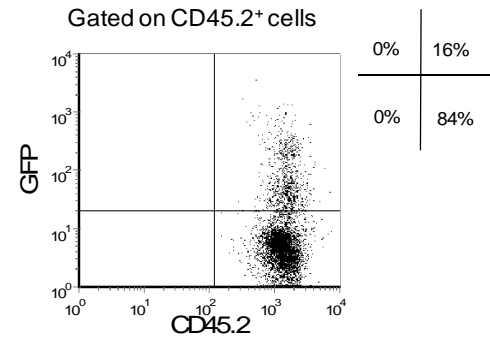
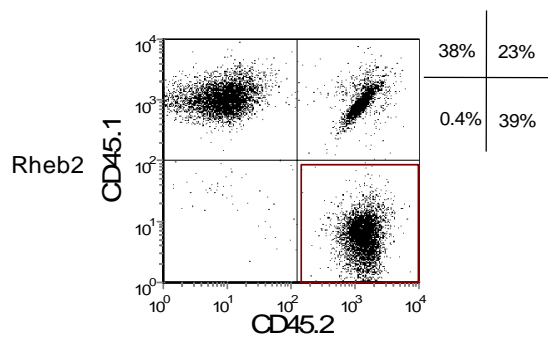
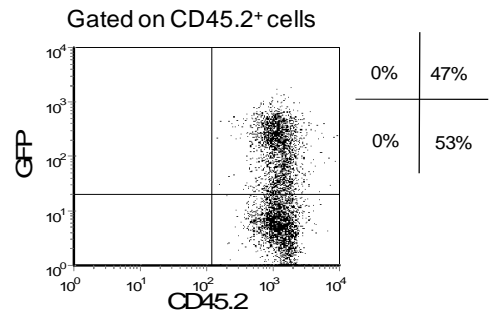
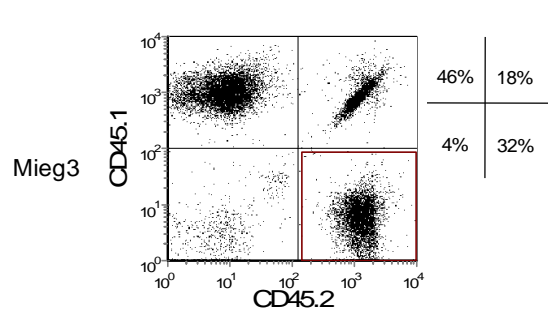
These results suggest that Rheb2 overexpression significantly impairs the ability of transduced cells to engraft recipient mice in an *in vivo* competitive repopulation model. This effect occurred shortly after transplantation, detected at two weeks after injecting the cells.

To further elucidate the effect of Rheb2 overexpression *in vivo*, we determined absolute numbers of GFP<sup>+</sup>sca-1<sup>+</sup>lineage<sup>-</sup> (GFP<sup>+</sup>LS) cells in transplanted animals at 1, 2 and 4 weeks after transplantation. The LS population was analyzed instead of KSL for two reasons. First, cell numbers in these animals were very low, especially at 1 week after transplantation, and second, the expression of c-kit was dim at 1 week after transplantation. The LS phenotype still defines an immature population of hematopoietic stem and progenitor cells. Figure 21A shows representative FACS plots of animals transplanted with Mieg3 or Rheb2 cells at 2 weeks after transplantation. Similar to our *in vitro* KSL expansion data, these plots show an increased percentage of GFP<sup>+</sup>LS cells in animals transplanted with Rheb2 cells. We determined the average absolute numbers of GFP<sup>+</sup>LS cells per mouse and show these results in Figure 21B. At 2 weeks after transplantation, animals transplanted with Rheb2 cells had significantly greater numbers of GFP<sup>+</sup>LS cells (19,457 ± 2250 GFP<sup>+</sup>LS cells in animals transplanted with Mieg3 cells

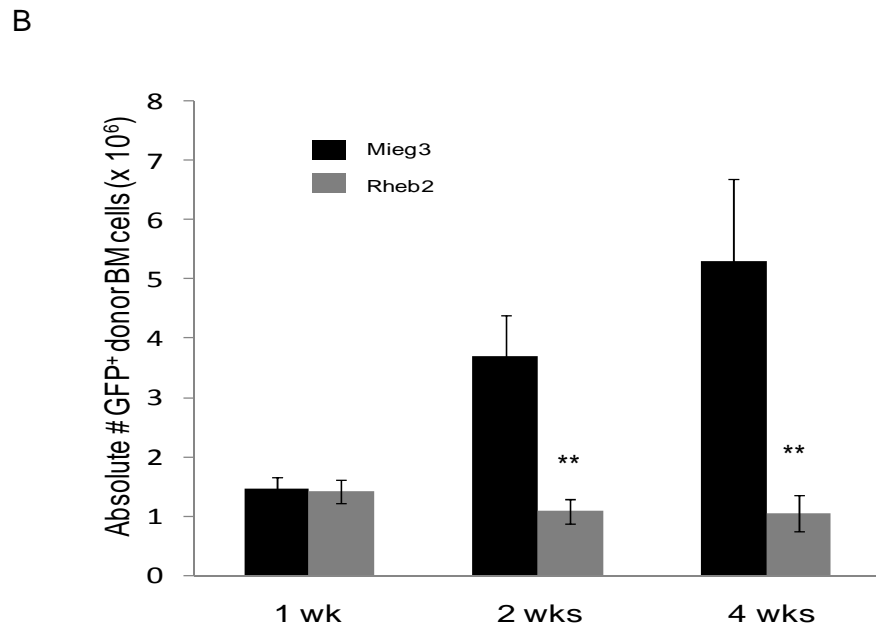
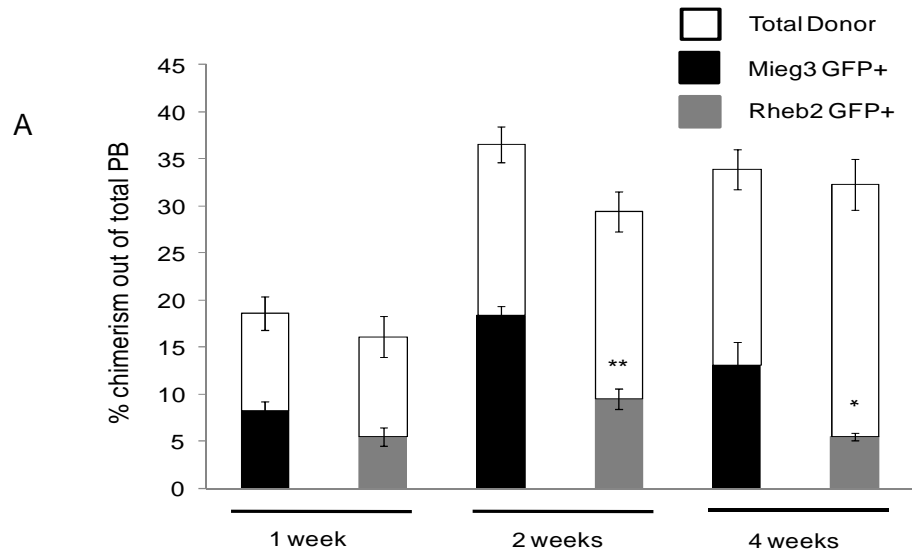
versus  $31,132 \pm 4340$  GFP<sup>+</sup>LS cells in animals transplanted with Rheb2 cells, Mean  $\pm$  SEM). This was a dramatic increase considering the impairment of overall donor GFP<sup>+</sup> cells found in these animals at this time point. At 1 week post transplantation, numbers of Mieg3 and Rheb2 GFP<sup>+</sup> LS cells were similar in recipient animals, suggesting that the increase observed at 2 weeks after transplantation was not due to early differences in homing of these cells. At 4 weeks post-transplantation, the numbers of GFP<sup>+</sup>LS cells in animals transplanted with Rheb2 cells were lower compared to Mieg3 cells, suggesting a transient effect of Rheb2 on the LS population.

The data from these experiments suggest that Rheb2 expands immature hematopoietic cells *in vivo*, transiently increasing the absolute number of these cells, but causing a significant impairment in total cell engraftment, suggesting an ability of Rheb2 overexpression to interfere with the normal repopulating ability or differentiation of these cells.



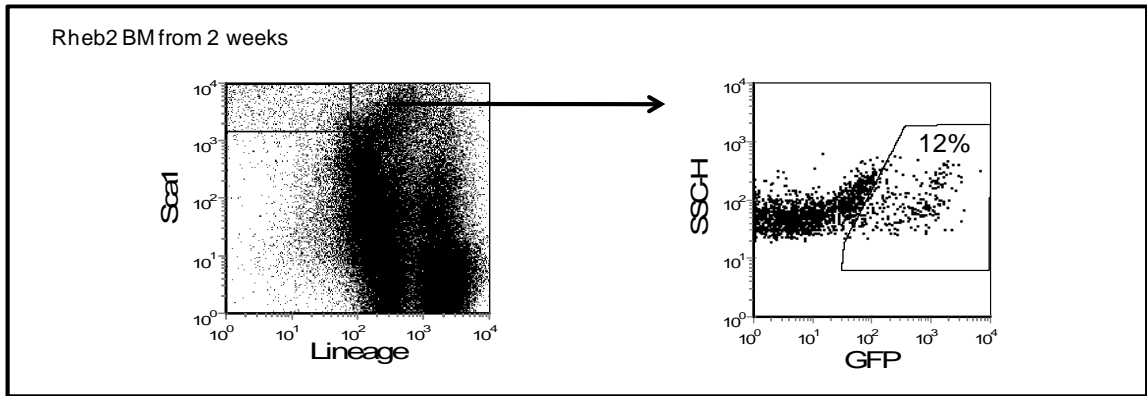
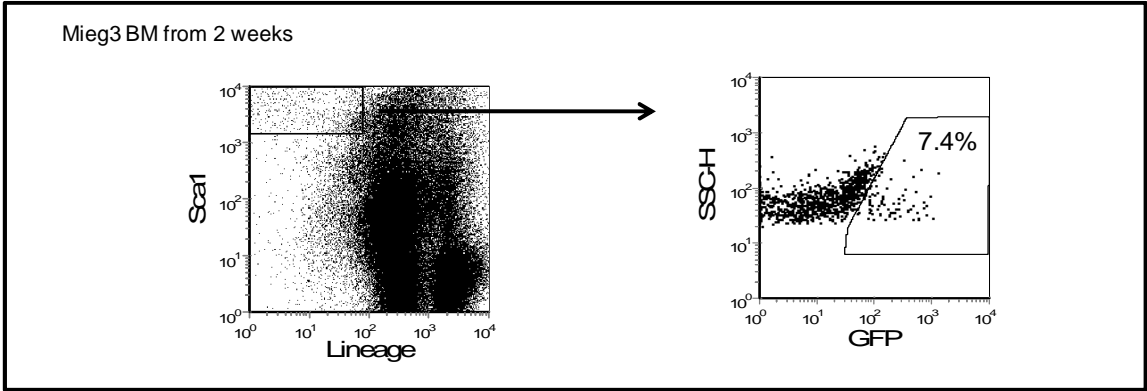


**Figure 19: Donor, competitor and recipient cells are easily distinguished in competitive repopulation recipients.** Representative FACS plots are shown for a recipient C57/BoyJ mouse transplanted with either Mieg3 or Rheb2-Mieg3 transduced cells. This illustrates that donor (CD45.2<sup>+</sup>45.1<sup>-</sup>), competitor (CD45.1<sup>+</sup>45.2<sup>-</sup>) and resident recipient (CD45.2<sup>+</sup>45.1<sup>+</sup>) cells were discriminated (left side of the figure). In addition, GFP<sup>+</sup> cells within the donor population were identified in these recipients (right side of figure). Percentages are shown next to the FACS plots. The fluorochromes used for these analyses were CD45.1-PE and CD45.2-APC.

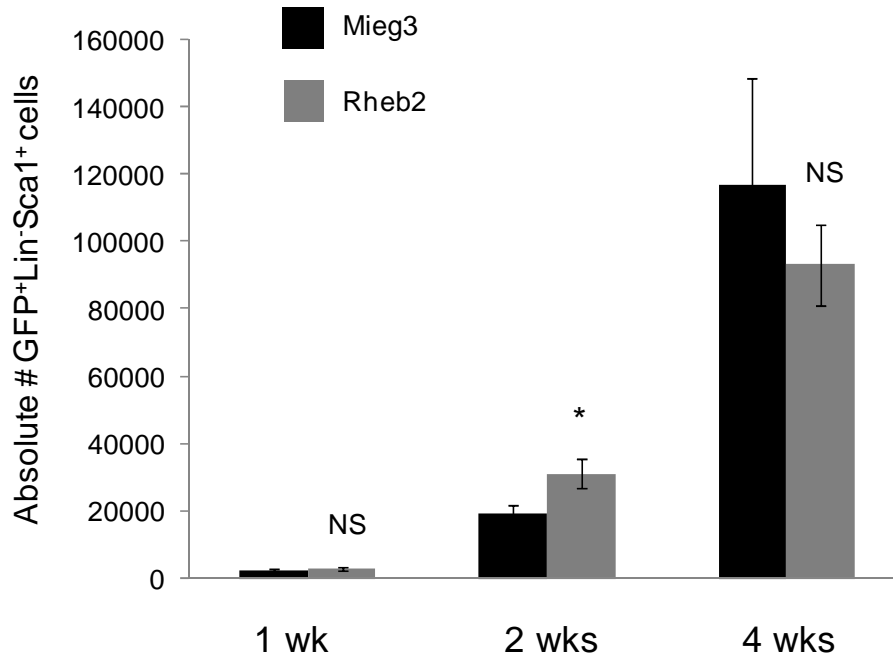


**Figure 20: Effect of Rheb2 overexpression on *in vivo* short-term competitive repopulation.** Transduced, unsorted mBM MNCs from C57/Bl6 animals were mixed with competitor BM cells from BoyJ animals and transplanted into irradiated C57/Bl6:BoyJ recipients. PB and BM were harvested at 1,2 and 4 weeks after transplantation. (A) The chimerism of transduced cells in the PB of recipient animals. (B) The chimerism of absolute numbers of transduced GFP<sup>+</sup> donor cells in the BM of recipients. At least 5 in each group at each time point (mean  $\pm$  SEM).  
\* p<0.05; \*\* p<0.01.

A



B

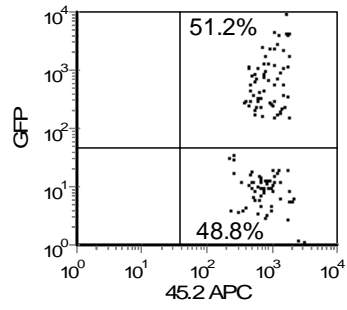


**Figure 21: Effect of Rheb2 overexpression on engraftment of immature LS cells *in vivo*.** (A) Representative FACS plots showing the percentage of GFP<sup>+</sup> cells within the immature LS population in recipient animals at two weeks after transplantation. (B) Graph shows the absolute numbers of GFP<sup>+</sup>LS cells in the BM of recipients (at least 5 recipients in each group at each time point, mean  $\pm$  SEM). \*  $p < 0.05$ . NS = Not-significant.

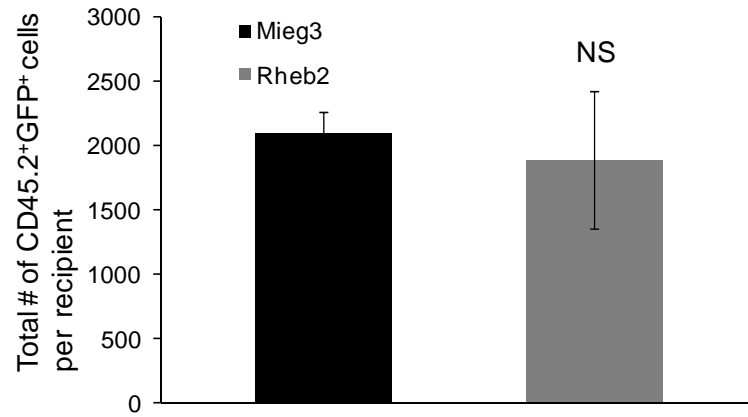
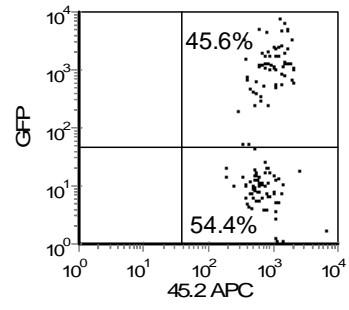
*Rheb2 overexpression did not impair short-term 24 hour homing of transplanted cells*

Engraftment is a complex process involving many physiological processes. The ability of transplanted cells to move from the PB to the BM shortly after transplantation is called homing and is crucial for establishing stable engraftment of donor cells. To determine if the impaired engraftment of Rheb2 overexpressing cells was due to decreased homing of the cells, we transplanted transduced donor C57/Bl6 CD45.2 cells into irradiated recipient BoyJ CD45.1 mice without competitor cells and analyzed the BM of the recipient animals 24 hours after transplantation by FACS for chimerism of donor cells. Figure 22 shows representative FACS plots from recipient animals in A and tabulated total numbers of donor CD45.2<sup>+</sup>GFP<sup>+</sup> cells in the graph in B. These results show that Rheb2 overexpression did not significantly impair the ability of the total transduced cell population to home to the BM, suggesting that the impaired engraftment is likely due to some other mechanism than homing. It should be noted that we did not determine the homing of immature cell populations such as lin<sup>-</sup>sca-1<sup>+</sup> in this experiment, and these rarer populations could have been affected by Rheb2 overexpression without affecting the homing of the total GFP<sup>+</sup> population.

Mieg3 Animal (Gated on CD45.2<sup>+</sup>)



Rheb2 Animal (Gated on CD45.2<sup>+</sup>)





**Figure 22: Effect of Rheb2 overexpression on short-term homing *in vivo*.**  $1 \times 10^6$  transduced, unsorted C57 BM MNCs were transplanted into irradiated BoyJ recipients. (A) Representative FACS plots gated on the donor (CD45.2<sup>+</sup>) population showing the percentage of GFP<sup>+</sup> cells. (B) Graph shows total number of GFP<sup>+</sup> donor cells per recipient (3 animals in each group). NS = not significant.

*Overexpression of Rheb2 impaired long-term competitive repopulation of transduced cells*

We found that overexpression of Rheb2 expanded immature cells *in vitro* and *in vivo* while impairing the engraftment of total transduced cells, suggesting Rheb2 could impair the long-term engraftment ability of HSCs. To examine this, we performed additional competitive repopulation assays and followed donor cell engraftment for longer periods of time. These long-term engraftment experiments test the ability of HSCs to maintain repopulation of recipient animals, and are considered gold-standard assays for HSC self-renewal. We transduced donor cells from C57/Bl6 CD45.2<sup>+</sup> mice and mixed with competitor cells from BoyJ CD45.1<sup>+</sup> mice at two ratios, 1:1 and 0.4:1 donor to competitor, and transplanted the mixed cells into irradiated BoyJ CD45.1 recipient animals. PB chimerism of donor GFP<sup>+</sup> cells was followed periodically by FACS staining for CD45.1 and CD45.2 markers. In addition, we determined the multilineage differentiation of Mieg3 and Rheb2 GFP<sup>+</sup> cells by staining PB samples at 1 month post-transplantation for lymphoid and myeloid cell markers.

Figure 23 shows the results of PB chimerism from competitive repopulation assays using a 1:1 ratio of donor to competitor cells. Beginning at 1 month after transplantation and continuing through 5 months after transplantation, the percent of Rheb2 CD45.2<sup>+</sup>GFP<sup>+</sup> cells out of total PB was significantly, at least 2-fold, lower than Mieg3 CD45.2<sup>+</sup>GFP<sup>+</sup> cells. The percent of CD45.2<sup>+</sup>GFP<sup>-</sup> cells was similar between both groups of recipients, suggesting a specific effect on the transduced fraction and indicating that similar numbers of cells were transplanted into both groups of recipients. Although numbers of Rheb2 CD45.2<sup>+</sup>GFP<sup>+</sup> cells were lower at all time points, the percent was stable from month to month and did not decrease further as the experiment continued, indicating that long-term repopulating cells were indeed present in the Rheb2 GFP<sup>+</sup> fraction, but at

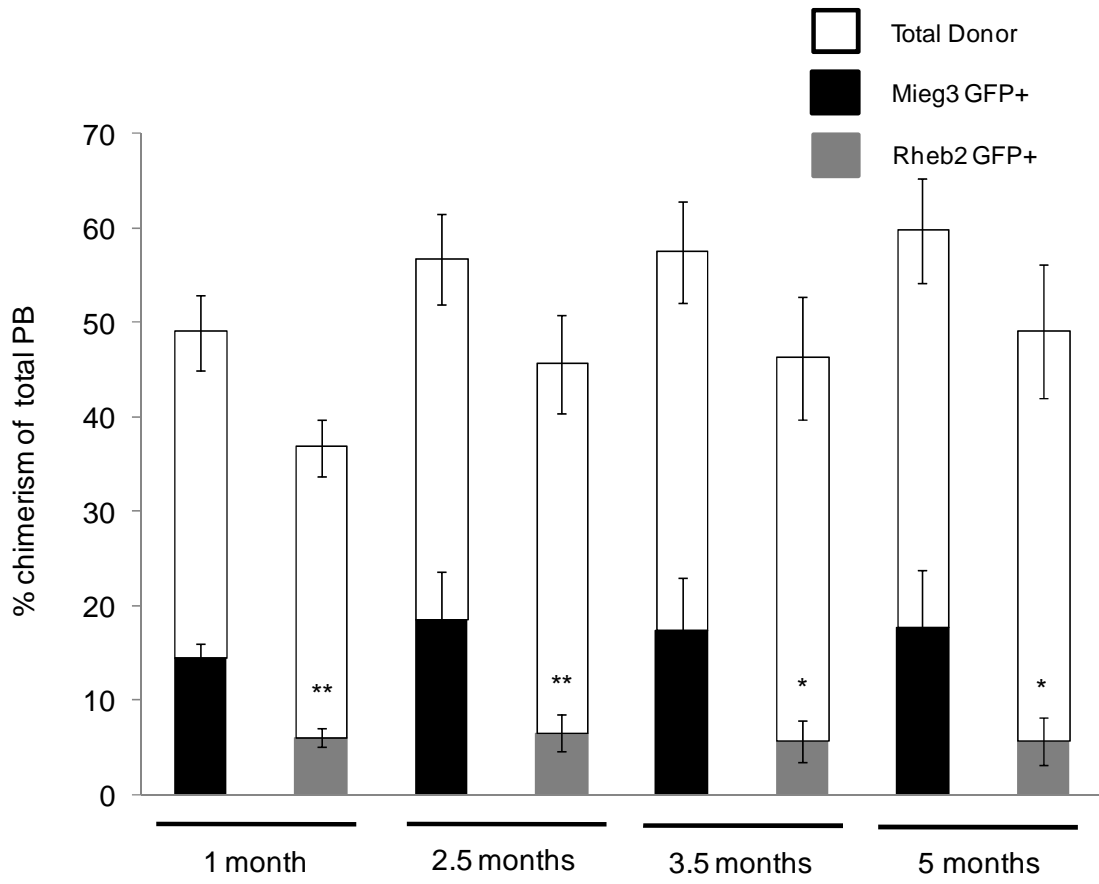
significantly lower numbers. Furthermore, it suggests that changes occurring shortly after transplantation were sufficient to cause impairment of the engraftment of long-term HSCs, a point discussed later in this thesis.

Figure 24 shows PB chimerism from recipient animals transplanted with 0.4:1 donor to competitor cells. The same trend found in the 1:1 group was observed in these experiments with significantly lower numbers of Rheb2 CD45.2<sup>+</sup>GFP<sup>+</sup> cells compared to Mieg3 CD45.2<sup>+</sup>GFP<sup>+</sup> cells found in recipient animals at all time points after transplantation. Again, the engraftment of these cells was stable during the course of the experiment, suggesting that long-term engrafting HSCs were present in Rheb2 cells, albeit at lower numbers than Mieg3 cells.

*Rheb2 overexpression impaired secondary repopulation of transduced hematopoietic cells*

We have shown that animals transplanted with Rheb2 transduced cells had fewer numbers of GFP<sup>+</sup> cells from one month through 5 months after transplantation. These data suggested that the numbers of long-term HSCs were decreased due to Rheb2. To determine the effect of Rheb2 overexpression on the self-renewal capacity of transduced HSCs in these primary recipients, we performed secondary transplantation. We isolated BM from primary transplant recipients 5 months after transplantation and transplanted these cells into irradiated BoyJ CD45.1<sup>+</sup> recipients. We followed the chimerism in the PB of the animals periodically. Figure 25 shows BM chimerism in primary recipients at 5 months after transplantation (Figure 25A) as well as the PB chimerism of secondary transplanted animals up to 2 months after transplantation (Figure 25B). Similar to primary transplant recipients, secondary recipients transplanted with Rheb2 transduced cells had a significantly lower percent of GFP<sup>+</sup> cells in the PB compared to Mieg3

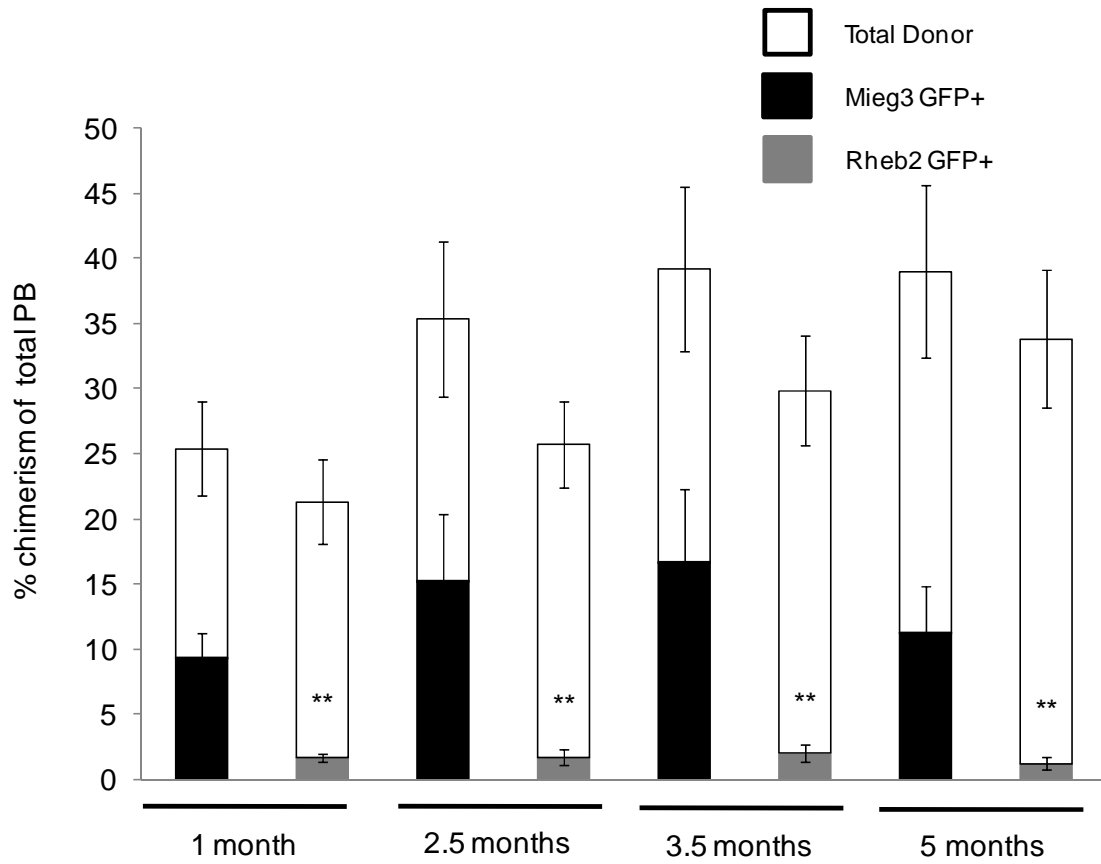
transduced cells. It is important to note that in the secondary transplant recipients, but not in primary recipients, the percentage of donor cells which are GFP<sup>-</sup> was slightly increased in animals transplanted with Rheb2 compared to Mieg3 transduced cells (28.0 ± 5.4% in animals transplanted with Mieg3 cells versus 36.5 ± 5.1% in animals transplanted with Rheb2 cells, Mean ± SEM). Importantly, this increase is not significant. A possible explanation for these results is that GFP expression is preferentially silenced in cells overexpressing Rheb2. This is not supported by our data from primary transplant recipients showing that the percentage of donor GFP<sup>-</sup> cells was similar in animals transplanted with either Mieg3 or Rheb2 transduced cells (see Figures 23 and 24). Future experiments using real-time PCR detection of proviral DNA in GFP<sup>-</sup> and GFP<sup>+</sup> cell populations will rule-out a GFP silencing effect. Overall, our data support the conclusion that Rheb2 overexpression decreases numbers of long-term HSCs capable of repopulating secondary recipients.



**Figure 23: Effect of Rheb2 overexpression on long-term engraftment *in vivo*.**

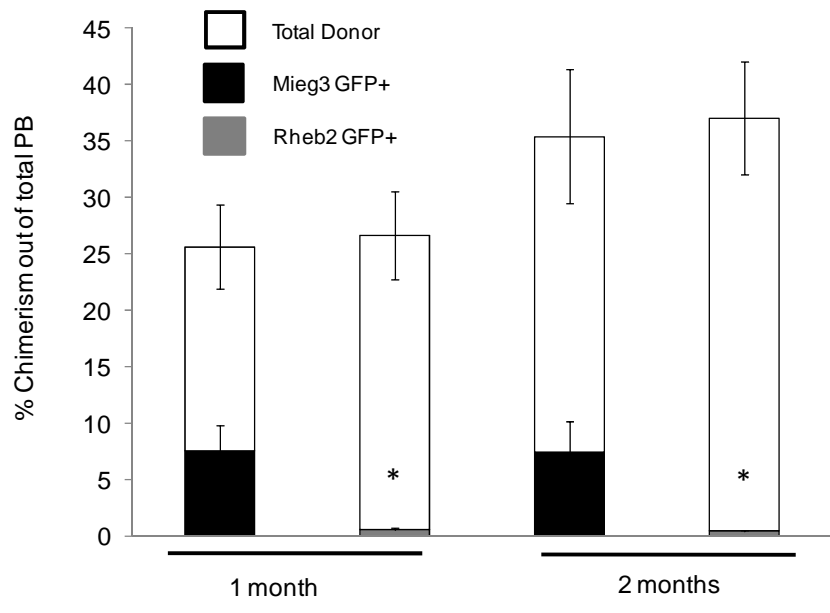
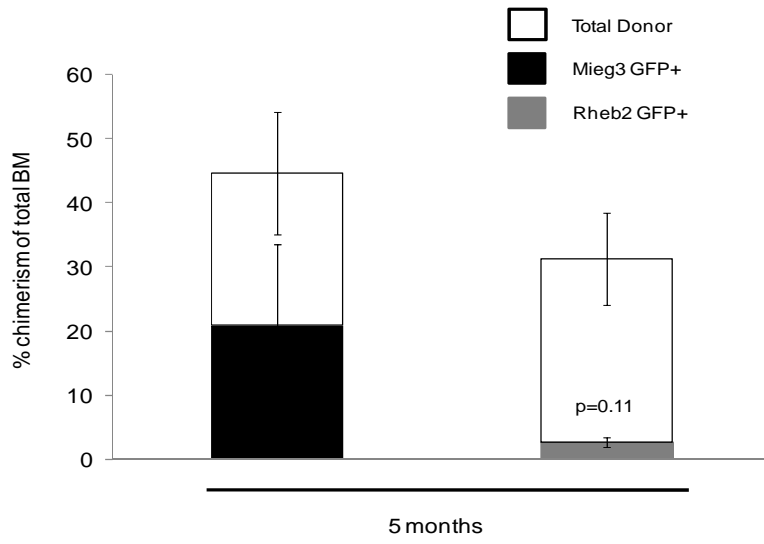
Transduced, unsorted mBM MNCs were mixed with BM cells from BoyJ animals at a 1:1 donor to competitor cell ratio and transplanted into irradiated BoyJ recipients. PB was monitored in these animals at 1, 2.5, 3.5 and 5 months after transplantation. Two experiments combined, at least 11 animals in each group (Mean  $\pm$  SEM).

\*  $p < 0.05$ ; \*\*  $p < 0.02$ .



**Figure 24: Effect of Rheb2 overexpression on long-term engraftment of transduced cells *in vivo*.** Transduced, unsorted mBM MNCs were mixed with BM cells from BoyJ animals at a 0.4:1 donor to competitor cell ratio and transplanted into irradiated BoyJ recipients. PB was monitored in these animals at 1, 2.5, 3.5 and 5 months after transplantation. Two experiments combined, at least 11 animals in each group (Mean  $\pm$  SEM). \*\*  $p < 0.02$



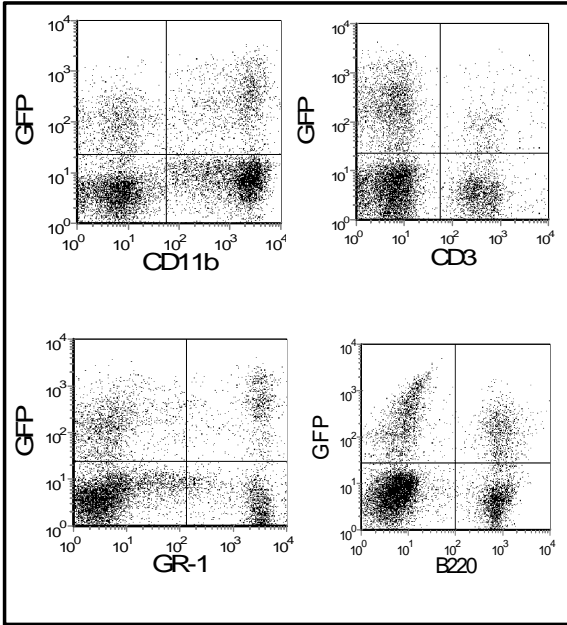


**Figure 25: Effect of Rheb2 overexpression on engraftment of secondary transplant recipients *in vivo*.** Part A shows primary recipient BM chimerism in 1:1 group 5 months after transplantation (6 Mieg3 and 5 Rheb2 primary animals, Mean  $\pm$  SEM). Part B shows PB chimerism in secondary animals transplanted with BM cells ( $2 \times 10^6$  each) from part A (total of 20 animals per group, Mean  $\pm$  SEM). Note: 4 secondary animals transplanted from one Mieg3 primary animal died prematurely and were excluded from analysis of secondary transplant recipients. \*  $p < 0.05$ .

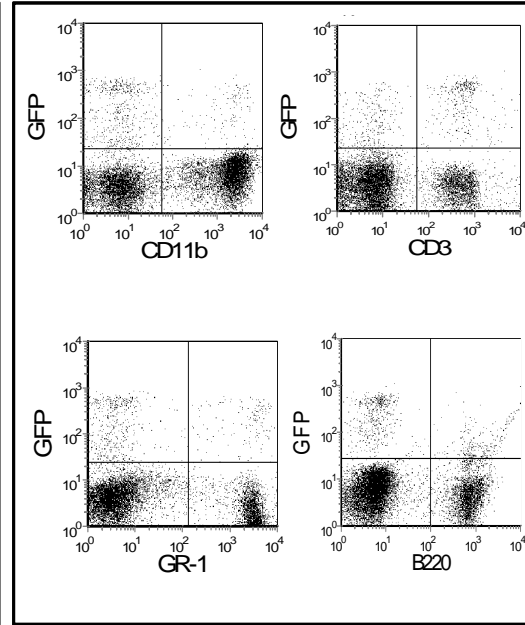
*Rheb2 overexpression did not affect multilineage differentiation as measured by phenotypic surface markers*

To determine if both Mieg3 and Rheb2 transduced cells, once transplanted into recipient mice, exhibited multilineage differentiation, we analyzed the PB of 1:1 competitive transplant recipients (from Figure 23) by FACS at 1 month after transplantation for the surface molecules CD11b, CD3, Gr1 (L6-G) and B220. CD11b and Gr1 (L6-G) are myeloid markers, specifically monocyte and neutrophil markers. CD3 is a pan T-cell marker, and B220 is a B-cell marker. Therefore, this combination of surface markers determined if multilineage differentiation occurred in recipient mice. In Figure 26, we present FACS plots from representative Mieg3 and Rheb2 recipient animals showing that both the GFP<sup>-</sup> and GFP<sup>+</sup> cell fractions exhibited multilineage surface molecule staining. Table 3 shows combined data from Mieg3 and Rheb2 animals showing the percentages within GFP<sup>-</sup> and GFP<sup>+</sup> fractions of CD11b<sup>+</sup>, CD3<sup>+</sup>, GR1 (L6-G)<sup>+</sup> and B220<sup>+</sup> cells. Importantly, there were no significant differences in these percentages in Rheb2 GFP<sup>+</sup> cells compared to Rheb2 GFP<sup>-</sup> cells nor compared to Mieg3 GFP<sup>+</sup> cells, indicating that overexpression of Rheb2 did not dramatically skew the transduced cells towards one lineage at the expense of another or block differentiation towards certain lineages.

Representative Mieg3 animal



Representative Rheb2 animal



**Figure 26: Rheb2 transduced cells exhibited multilineage differentiation *in vivo*.**

Figure shows FACS plots of PB cells from representative animals transplanted with Mieg3 and Rheb2 transduced cells from the 1:1 competitive repopulation group shown in Figure 21 at 1 month after transplantation. Both Mieg3 and Rheb2 GFP<sup>+</sup> populations expressed myeloid (CD11b and GR-1) and lymphoid (B220 and CD3) surface antigens, indicating multilineage differentiation. Tabulated data from 3 recipients in each group is shown in Table 3.

<b>Mean ± S.D.</b>	<b>% CD11b</b>	<b>% CD3</b>	<b>% GR1</b>	<b>% B220</b>
<b>Mieg3 GFP<sup>-</sup></b>	50.9 ± 2.6	25.1 ± 0.7	32 ± 3.6	24.7 ± 4.4
<b>Mieg3 GFP<sup>+</sup></b>	47.8 ± 8.8	29 ± 6	30.6 ± 2.9	36.1 ± 1.4
<b>Rheb2 GFP<sup>-</sup></b>	60.9 ± 15.9	19.9 ± 5.6	43 ± 13.7	18.7 ± 10.2
<b>Rheb2 GFP<sup>+</sup></b>	49 ± 31	30.5 ± 26	40.6 ± 23.4	27.9 ± 10.9

**Table 3: Rheb2 overexpression did not affect multilineage differentiation of transduced cells *in vivo*.** Table shows combined data from 3 Mieg3 and 3 Rheb2-Mieg3 transplant recipients. No significant differences were found between Mieg3 and Rheb2 GFP<sup>+</sup> cells.

## DISCUSSION

### Inhibition of CD26 peptidase activity: a strategy to increase hematopoietic cell engraftment

The CD34<sup>+</sup> fraction of hUCB contains HSCs capable of engrafting NOD/SCID mice *in vivo*. Our results demonstrated that inhibition of CD26 peptidase activity by pretreatment of donor CD34<sup>+</sup> cells with 5 mM Diprotin A increased their *in vivo* engraftment in NOD/SCID mice, without affecting differentiation of the cells. In our experience, this enhancement was apparent when engraftment of control CD34<sup>+</sup> cells was low. Additionally, the engrafting capability of NOD/SCID mice transplanted with CD34<sup>+</sup> cells varied greatly between experiments. In one experiment, not shown, the engraftment of both control and Diprotin A-treated cells was greater than 55% and not significantly different when  $1.8 \times 10^5$  CD34<sup>+</sup> cells were injected. In another experiment we noted no detectable engraftment with injection of a similar number ( $1.8 \times 10^5$ ) of CD34<sup>+</sup> cells, with or without Diprotin A pretreatment. As shown in Figure 5, we did not detect a dose-response in engraftment of varying cell numbers, but the Diprotin A pretreated group was enhanced in engraftment at each of these different cell infusion numbers, and this enhancement was significant after pooling the animals into two groups. Thus, enhancement of engraftment with Diprotin A related more to levels of control cell engraftment, so long as these levels were detectable, than to the numbers of cells transplanted into the recipients.

Our present study, in light of previous work (Tian et al. 2006; Peranteau et al. 2006; Christopherson et al. 2004) on mouse to mouse transplantation, strongly suggests that CD26 activity plays an important role in hematopoietic cell engraftment. A practical strength of these studies is the ability to enhance transplantation by a simple 15 minute



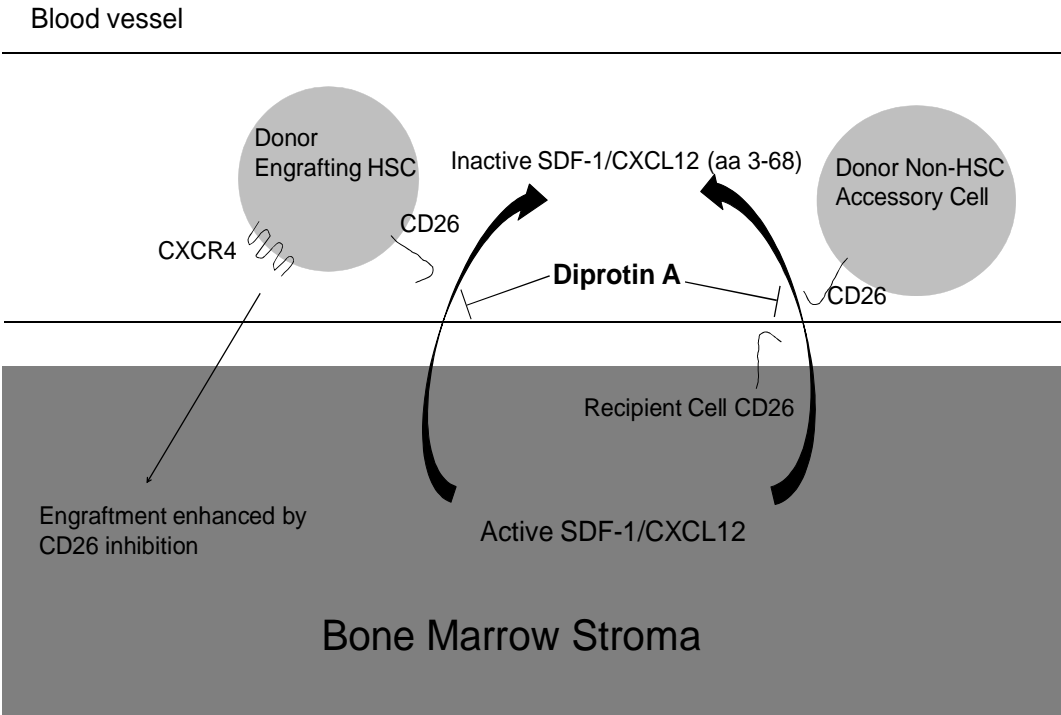
pretreatment of cells to diminish CD26 activity (Christopherson et al. 2004). This treatment requires very little *ex vivo* manipulation of donor cells. While this study demonstrates the positive effects of treating just the donor cells, it is possible that treatment of both donor cells and recipients may have greater effects on enhancing transplantation, especially given the fact that CD26 is expressed in many different tissue beds within the body (Pro and Dang 2004; Demuth et al. 2005). Our group has shown that *in vivo* treatment of recipient mice with Diprotin A enhanced the primary competitive and secondary non-competitive repopulating capacity of untreated congenic mouse bone marrow donor HSCs (Broxmeyer et al. 2007). However, an advantage of treating donor cells alone is that it would avoid possible side effects of potential total body CD26-inhibition of recipients, even though CD26 inhibitors are reversible.

It was important to determine whether CD26 was expressed on phenotypically defined subsets of human HSCs. Since we found preferential expression of CD26 on more immature cells within the CD34<sup>+</sup> population, this suggests that strategies to eliminate CD26 activity by depletion of CD26<sup>+</sup> cells could potentially have a negative impact on transplantation outcomes, since this might eliminate HSCs. Thus, strategies to inhibit CD26 peptidase activity, rather than remove CD26-expressing cells, may be more appropriate for clinical use.

The exact mechanism explaining the *in vivo* effects we and other laboratories have observed due to inhibition of CD26 has not been fully elucidated. There is ample evidence suggesting that CD26 is acting through its ability to cleave SDF-1/CXCL12 to an inactive form, thus negatively affecting the homing and engraftment of HSCs *in vivo*. First, we know that CD26 acts *in vitro* to cleave SDF-1/CXCL12 and that this form negatively affects the chemotaxis. Second, it is likely that the peptidase activity of CD26 is responsible for these observations, since Diprotin A acts by competitively inhibiting

this property of the enzyme (Rahfeld et al. 1991). Third, the increased short-term homing observed by our laboratory in mouse congenic transplants using Diprotin A pre-treated or CD26<sup>-/-</sup> cells was reversed by treatment with AMD3100, a selective CXCR4 antagonist, suggesting that the effects are mediated through the SDF-1: CXCR4 axis (Christopherson et al. 2004). Figure 27 shows a diagram of the proposed mechanism of action of CD26 *in vivo* based on evidence to this point and emphasizing its effect on SDF-1/CXCL12. It is likely that multiple mechanisms account for the effect of CD26 inhibition. Multiple cell populations express CD26, including HSCs, accessory donor cells and recipient stromal cells. Under conditions where CD26 peptidase is active, SDF-1 is cleaved to an inactive, antagonist form capable of inhibiting migration of HSCs into the BM stroma. After Diprotin A treatment of any of the CD26<sup>+</sup> cell populations, SDF-1 is preserved in its full-length active form and stimulates migration of HSCs into the BM stroma (Figure 27). However, there are likely additional processes occurring which account for some of the mechanism. First, we have summarized here the wide effect CD26 has on many different chemokines. It is possible that *in vivo* CD26 could be cleaving other chemokines, in addition to SDF-1/CXCL12, that affect the homing of HSCs and/or accessory cells leading to effects on establishment of the hematopoietic niche. Furthermore, in addition to its well-established peptidase activity, CD26 has been shown to bind to components of the extracellular matrix (ECM) as well as interact with cell-surface signaling proteins, including fibronectin, collagen, adenosine deaminase (ADA) and CD45 (Pro and Dang 2004; De Meester et al. 1999). The physiological relevance of these interactions is not well known, but they illustrate the point that CD26 is more than a simple dipeptidase enzyme. There is some signaling associated with CD26 as well, as studies using cross-linking anti-CD26 antibodies have shown multiple tyrosine phosphorylation events occur downstream (De Meester et al. 1999). This is probably not a direct effect of CD26, since the cytoplasmic region is very short, and is

likely mediated by a CD26 binding partner. Inhibiting the peptidase activity of CD26, although unlikely, could conceivably have an effect on any or all of these non-enzymatic functions and could be part of the CD26 mechanism of action *in vivo*. Finally, there is evidence that CD26 can functionally bind to CXCR4, and this CD26: CXCR4 complex can be internalized by SDF-1/CXCL12 stimulation of lymphocytes (Herrera et al. 2001). This internalization, if occurring in a fraction of the CXCR4 pool, could have an amplifying effect on CD26 inhibition by decreasing CD26 expression on the cell surface. Whether this interaction is modulated by cleaved or full-length SDF-1 and whether this interaction is affected by inhibition of CD26 peptidase activity is an interesting avenue of further study.



**Figure 27: Proposed mechanism of CD26/DPPIV action *in vivo*.** Transplanted HSCs migrate into the recipient BM due to various mechanisms, one of which is SDF-1/CXCL12 signaling. CD26 molecules, expressed on donor HSCs, donor accessory cells (non-HSCs) and recipient BM cells are proposed to cleave SDF-1/CXCL12 *in vivo* to an inactive, truncated form which negatively affects the engraftment of the donor HSCs. In the presence of CD26 inhibitors such as Diprotin A, this cleavage of SDF-1 is blocked and the HSCs are enhanced in migration into and retention within the BM.

Studies by two independent laboratories published at the same time as our own confirmed the effect of inhibition of CD26 on engraftment of human HSCs (Christopherson II et al. 2007; Kawai et al. 2007). The first, published by Christopherson *et al* (Christopherson II et al. 2007) showed that pre-treatment of either CD34<sup>+</sup>-purified or lineage-depleted human CB cells with Diprotin A resulted in enhanced engraftment of NOD/SCID $\beta$ 2m<sup>null</sup> mice. The NOD/SCID $\beta$ 2m<sup>null</sup> model is an improvement of the NOD/SCID strain by deletion of the  $\beta$ <sub>2</sub>-microglobulin gene, leading to fewer natural killer (NK) cells and better engraftment of the human cells (Christianson et al. 1997; Kollet et al. 2000). The effect of Diprotin A in this study was more dramatic on the lineage-negative CB population, which had 7.5 fold increased engraftment compared to a 4 fold increase in highly purified CD34<sup>+</sup> cell treated with Diprotin A. The percent of CD26<sup>+</sup> cells was higher in the lineage-negative population compared to the CD34<sup>+</sup> population as was the CD26 activity, while only about 30% of the lineage-negative population was CD34<sup>+</sup>. This suggests that CD34<sup>-</sup>CD26<sup>+</sup> accessory cells negatively affect engraftment of the repopulating HSCs and that inhibiting these cells as well as the CD34<sup>+</sup>CD26<sup>+</sup> cells leads to dramatic increases in cell engraftment. The second study on human HSCs and CD26 was published by Kawai *et al* (Kawai et al. 2007) and showed that *in vivo* treatment of NOD/SCID animals with Diprotin A significantly enhanced the engraftment of G-CSF mobilized peripheral blood (MPB) CD34<sup>+</sup> cells. In addition, this treatment enhanced the engraftment of retrovirally transduced human CD34<sup>+</sup> MPB cells. This enhancement of engraftment was found only when Diprotin A was injected into the animals at the same time as injection of CD34<sup>+</sup> cells, suggesting that CD26-inhibition of the recipient mouse cells may be more important than pre-treatment of the donor cells. This lack of pre-treatment effect of Diprotin A was found despite the fact that the cells expressed CD26 and exhibited CD26 activity after an *ex vivo* culture period. The discrepancy in this study could reflect a difference between the HSCs derived from G-CSF MPB and those from

CB used in ours (Campbell et al. 2007a) and Christopherson's (Christopherson II et al. 2007) studies. Additionally, it illustrates the probable role of the recipient BM stroma in affecting engraftment of donor cells through CD26 mechanisms.

The potential clinical use of this strategy will undoubtedly require more work with animal models, such as canine and primate, using CB as well as other transplantable sources such as MPB and BM. As stated above, it will be important to determine whether treating the donor cells or recipient is more effective at enhancing engraftment. For MPB donor cells, treatment of the cells alone was not effective at increasing engraftment, but for CB donor cells this pre-treatment did enhance engraftment. It may be that a combination of treating the cells and the recipients is best given the fact that CD26 is expressed in many tissue beds and found in a soluble form in the plasma (Pro and Dang 2004).

CD26 plays a role in other biological processes. Much work has been done on the relationship between CD26, glucose metabolism and Type 2 diabetes. CD26 inhibitors are currently in clinical use for the treatment of diabetes. CD26 can cleave hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP), both of which can stimulate insulin release in the post-prandial response to foods (Deacon and Holst 2006; Demuth et al. 2005). CD26 cleavage of these hormones has a negative effect on glucose metabolism, and inactivation of CD26 activity is thought to extend their half-life leading to better blood glucose responses in diabetics. The fact that CD26 plays an important role in glucose metabolism led various companies to develop multiple CD26 inhibitors, with a least one, Sitagliptin (Merck) having FDA approval (Nathan 2007). These compounds vary in their structures, mechanism of action (competitive vs. non-competitive), and duration (long-lasting vs. short-lasting). The ability of these newer, clinical grade CD26 inhibitors to affect HSC biology should be tested, first in animal models, to determine if they can work in these contexts. The results of

clinical trials on diabetes to date have shown minimal toxicity due to these compounds (Deacon and Holst 2006). However, it is important to note that long-term follow up has not taken place and firm conclusions about the safety of these inhibitors cannot be made at this time. In addition, there are safety factors to consider in transplant patients who are immunosuppressed and have hematologic pathologies; the fact that these drugs are safe in one population does not guarantee that they will be safe in another. There is also a concern that inhibiting CD26 in a human transplant recipient could lead to HSCs seeding other tissue beds, besides BM, in the body that produce SDF-1/CXCL12 gradients (e.g. lung and lymphoid organs), a phenomenon known to occur with BM cells (Dooner et al. 2004). Whether the HSCs in these non-BM environments could thrive, proliferate and possibly harm the patient may be unlikely but is still a concern.

It is our goal that the ability to increase engraftment due to inhibition of CD26 activity will be able, alone or combined with other strategies such as *ex vivo* expansion and immunotherapy, to extend the potential therapeutic uses of limiting hematopoietic cell populations, such as those from human CB. This therapy may provide more patients with the best chance of a successful outcome after hematopoietic cell transplantation.



The novel ras molecule Rheb2: diverse roles in expansion and repopulation of hematopoietic cells

The second part of our study involved the role of Rheb2 in affecting hematopoietic cell functions, especially *in vivo* engraftment. We studied Rheb2 mainly because of its role in mTOR signaling. Studying the role of intracellular signaling proteins, including members of the ras family, is important for determining how HSC functions such as self-renewal and differentiation are regulated. Understanding these processes is important for clinical expansion of HSCs, transduction and other therapies.

We report here that overexpression of Rheb2 leads to increased mTOR signaling in two mouse hematopoietic cell lines, 32D and BaF3. Although Rheb2-mTOR signaling has been demonstrated in other cell types, no researchers have studied the signaling of this molecule in hematopoietic cells. In addition to activating mTOR signaling, we found that Rheb2 overexpression enhanced proliferation of 32D and BaF3 cells in response to delayed IL-3 addition, suggesting that Rheb2 plays a role in IL-3 mediated survival and/or proliferation. In fact, the importance of mTOR in mediating IL-3 effects has been previously reported (Cruz et al. 2005).

We found that overexpression of Rheb2 enhanced the *in vitro* growth and survival of primary mouse hematopoietic cells, including the progenitor cell subsets. Total proliferation of transduced FACS sorted GFP<sup>+</sup> mouse BM MNCs was enhanced by Rheb2. In addition, the colony-forming ability of mouse hematopoietic progenitors and their expansion in liquid culture was enhanced by Rheb2 overexpression. These positive effects can be explained by a variety of mechanisms including enhancement of proliferation, differentiation or survival. Increased proliferation is supported by our data showing expansion of progenitors over time in liquid culture due to Rheb2

overexpression (Figure 13). In addition to proliferation, enhanced survival played an important role in Rheb2 effects, as demonstrated by increased colony-formation in delayed growth factor addition colony assays. Interestingly, we found that the pro-survival chemokine SDF-1/CXCL12 could not further enhance the survival of Rheb2 transduced cells. This suggests that SDF-1 and Rheb2 may stimulate the same signaling pathways leading to enhanced cell survival, or alternatively, that there is an upper limit to enhanced survival and Rheb2 overexpression may reach that limit. We showed that SDF-1 stimulation of mouse BM MNCs enhanced signaling through the mTOR pathway, suggesting that this pathway may be activated by both SDF-1 and Rheb2 leading to cell survival. To our knowledge, this is the first report of mTOR activation by SDF-1 stimulation. Further experiments need to be performed to fully elucidate the relationship between Rheb2, SDF-1 and mTOR, but our preliminary data suggest this could be an interesting avenue of study.

Our final piece of *in vitro* data showed that Rheb2 overexpression expanded the percent of KSL cells in liquid cultures. Rheb2 had a substantial effect on this expansion, with a greater than 4-fold increase in KSL percentage 4 days after transduction. This expansion was specific for the GFP<sup>+</sup> transduced cell fraction, and percent KSL cells was similar in the non-transduced GFP<sup>-</sup> fraction in both Mieg3 and Rheb2 cultures. These data suggest, along with the colony assays, that Rheb2 overexpression expands immature hematopoietic cells. It is important to point out that the KSL phenotype does not identify a pure population of mouse HSCs, and there exists the possibility that an immature progenitor cell population was expanded in the Rheb2 liquid cultures, as opposed to true multipotent HSCs. In the future, more combinations of surface markers, such as CD34 and thy1.1 can be used to further delineate this expanded population. Alternatively, this population can be FACS sorted and transplanted *in vivo* to assess multipotentiality.

Treatment of these liquid cultured cells with 50 nM of rapamycin, a specific mTOR inhibitor, significantly decreased the percent of GFP<sup>+</sup> KSL cells in Rheb2 cultures. The fact that rapamycin only partially reduced the percentage of Rheb2 GFP<sup>+</sup> KSL cells, and these percentages were still enhanced compared to Mieg3 cultures, suggests that other signaling pathways may be involved in this expansion. Alternatively, this may suggest that our concentration of rapamycin was too low to completely reverse the Rheb2 overexpression. In fact, this 50 nM concentration is lower than studies on megakaryocyte differentiation which used 100 nM rapamycin without significant cell death (Raslova et al. 2006). Additionally, rapamycin may need to be periodically replenished in these cultures in order to maintain stable levels of the compound, a change we will test in the future. Finally, mTOR signaling is complicated with various negative and positive feedback mechanisms regulating the signaling upstream of mTOR through the Akt pathway. Under conditions of Rheb2 overexpression, the interplay between these positive and negative feedback loops is likely altered, partly explaining why rapamycin treatment only partially reduced the expansion of KSL cells. Also, only one of the two mTOR complexes (mTORC1) is inhibited by rapamycin. The possible activity of Rheb2 on mTORC2, which is unaffected by rapamycin, could also explain the lack of reversal of KSL expansion due to rapamycin, especially given the fact that this complex can activate signaling through the Akt pathway (Wullschleger et al. 2006). As previously mentioned, Rheb1 does not have appreciable activity on mTORC2; however, no information is available on the activity of Rheb2 on this complex.

Overall our *in vitro* data showed that Rheb2 overexpression positively regulates hematopoietic cell growth and survival. Furthermore, we provide evidence that phenotypic HSCs, identified as KSL cells, are expanded by Rheb2, a process at least partially mediated by mTORC1. The next question we addressed, using a variety of *in*

*vivo* assays, was whether this expansion of immature cells *in vitro* could translate into enhanced engraftment *in vivo*, a goal of any *ex vivo* expansion protocol.

In contrast to the *in vitro* expansion data, Rheb2 overexpression significantly impaired the engraftment of transduced cells in both short-term and long-term competitive repopulation assays. In short-term studies, the engraftment of total GFP<sup>+</sup> transduced cells was impaired at 2 and 4 weeks post-transplantation in both the PB and BM of recipient animals. However, similar to the *in vitro* expansion data, immature LS cells were transiently expanded at 2 weeks post-transplantation in recipient animals due to Rheb2 overexpression, suggesting that Rheb2 positively affected the proliferation of these cells after transplantation. This effect was short-lived and not found at 1 month post-transplantation. From 1 month on in long-term competitive transplant recipients, Rheb2 overexpression resulted in significantly decreased percentages of GFP<sup>+</sup> donor transduced cells in the PB. This trend was found in animals transplanted with a 1:1 or 0.4:1 ratio of donor to competitor cells. Additionally, the engraftment of donor Rheb2 GFP<sup>+</sup> cells was significantly lower in secondary transplant recipients, measuring the self-renewal of long-term HSCs. Overall, the data on *in vivo* donor cell engraftment suggest that Rheb2 expands immature cells after transplantation resulting in depletion of their short and long-term repopulating ability.

Interestingly, the level of donor GFP<sup>+</sup> cells was stable in animals transplanted with Rheb2 cells from 1 month on in competitive assays, suggesting that long-term repopulating HSCs were indeed present in these animals, but at significantly lower numbers than animals transplanted with control Mieg3 cells. This suggests that Rheb2 effects occur shortly after transduction and transplantation to deplete the numbers of HSCs. The cells that remain engrafted in these animals have likely either adapted to these adverse effects or have downmodulated the expression of Rheb2 in order to

maintain themselves. These are hypotheses we can test in future studies using real-time PCR or Western blotting to follow the expression of Rheb2 *in vivo* over time and the phosphorylation status of mTOR pathway effectors.

At first glance, the *in vivo* data presented seem to conflict with our *in vitro* data. On the one hand, Rheb2 expanded phenotypic immature cells *in vitro* and *in vivo*. On the other hand, Rheb2 depleted the engraftment of functional repopulating HSCs *in vivo*. This begs the question of what mechanism explains the Rheb2 effect on repopulating ability (i.e. self-renewal) of mouse HSCs. More generally, it suggests a negative relationship between proliferation of immature hematopoietic cells and maintenance of their repopulating activity. Previous work (Cheng et al. 2000; Yilmaz et al. 2006) suggests that, in fact, this is often times the case and factors which expand HSCs by pushing them into a proliferative state have negative consequences on engraftment and repopulating ability.

*The Relationship between proliferation and maintenance of repopulating activity in HSC expansion*

For any *ex vivo* expansion approach to be clinically effective at treating patients, the expanded HSC population must be similar to fresh HSCs in their ability to maintain long-term repopulation. A general problem with *ex vivo* HSC expansion is the impact of proliferation on the ability of HSCs to maintain long-term engraftment. The majority of long-term repopulating HSCs are found in a quiescent state *in vivo* under steady-state conditions (Bradford et al. 1997; Cheshier et al. 1999; Passegue et al. 2005; Orschell-Traycoff et al. 2000), and the balance between quiescence and proliferation *in vivo* during hematopoietic repopulation is thought to be important for maintaining stable repopulation in transplant recipients. A significantly higher percentage of phenotypic LT-

HSC in the G<sub>0</sub> (quiescent) phase of the cell cycle are able to stably engraft recipient animals compared to cells in G<sub>1</sub> or S-G<sub>2</sub>/M phases (Passegue et al. 2005), suggesting that specific molecular changes occurring as cells move through the cell cycle impact their homing and engrafting ability. This concept has been observed repeatedly in the past few years as researchers analyze genes responsible for controlling proliferation and/or cell cycle in HSCs.

A well-known example of how proliferation impacts repopulating ability comes from a study of p21 knockout mice (Cheng et al. 2000). p21<sup>cip1/waf1</sup> is a member of the family of cyclin dependent kinase inhibitors (CDKI) and specifically regulate the movement of cells out of G<sub>1</sub> through the rest of the cell cycle (Cheng et al. 2000). Researchers discovered after knocking out p21 in mice that fewer BM cells were found in a quiescent G<sub>0</sub> cell-cycle state, as measured by pyronin RNA staining. This increase in cycling of the BM cells correlated with an increased number of immature colony-forming cells under basal conditions using a cobblestone area forming assay, a surrogate *in vitro* assay for quantifying stem cells. However, HSC numbers were dramatically decreased in response to BM conditioning by 5-FU, which kills cycling BM cells and causes the stem cells to undergo stress in replenishing them. Furthermore, in transplant studies, p21 knockout bone marrow cells were significantly impaired in their ability to maintain engraftment and survival in serially transplanted recipients, suggesting that p21 loss negatively affects the ability of HSCs to maintain self-renewal capacity and engraft recipient animals (Cheng et al. 2000).

Further information on proliferation and repopulation has been found in studies on many different molecules, including the transcription factor Gfi1, the GTPase Cdc42, Wnt extracellular signaling molecules, and the phosphatase molecules Shp-2 and PTEN

(Zeng et al. 2004; Yang et al. 2007; Fleming et al. 2008; Chan et al. 2006; Yilmaz et al. 2006; Zhang et al. 2006).

Gfi is a nuclear transcription factor important in T cell proliferation and maturation and in interacting with signal transducing transcription factors such as STAT proteins (Rodel et al. 2000). Studies of the Gfi<sup>-/-</sup> knockout mouse revealed a role for this protein in restraining mouse HSC proliferation and maintenance of long-term repopulating activity (Zeng et al. 2004). Gfi<sup>-/-</sup> mice had reduced numbers of HSCs and HPCs *in vivo* under steady-state conditions, which were significantly impaired in their ability to repopulate transplanted recipient mice, were highly proliferative and not quiescent, suggesting that loss of this transcription factor puts HSCs into cell cycle and out of a quiescent state and leads to their exhaustion (Zeng et al. 2004). This mechanism of loss of repopulating ability may involve a p21 mediated mechanism as this molecule was downregulated in cells lacking Gfi (Zeng et al. 2004). A similar phenotype was discovered by researchers studying the knockout mouse model of the Rho GTPase protein Cdc42, a protein involved in cytoskeletal dynamics and adhesion (Yang et al. 2007; Etienne-Manneville and Hall 2002). Loss of Cdc42 led to HSC proliferation, less quiescent HSCs, expansion *in vivo* and impaired repopulation in transplant recipients (Yang et al. 2007). Contrary to the p21 and Gfi studies, the loss of Cdc42 dramatically impaired the ability of HSCs to home to and lodge within the BM of recipients, adhere and migrate in *in vitro* assays, and interact with the BM niche/stroma *in vivo* (Yang et al. 2007). Again, the loss of Cdc42 results in a decrease in p21 expression, suggesting a role for this protein in the mechanism (Yang et al. 2007).

Stimulation of Frizzled receptors by Wnt proteins can lead to expansion of HSCs *in vitro*. The mechanism of this expansion, and the effect of Wnt proteins on HSC repopulating activity, had not been studied thoroughly until recently. A group using a transgenic

mouse expressing an inhibitor of Wnt signaling, Dickkopf1, restricted to osteoblast expression, found that decreased Wnt signaling in HSCs within *in vivo* BM niches decreased the percentage of quiescent HSCs and impaired their long-term repopulation in serially transplanted recipient mice (Fleming et al. 2008). This study suggests a role for Wnt signaling in maintaining HSC quiescence *in vivo*, preventing exhaustion of the repopulating activity.

The phosphatases Shp-2 and PTEN play important roles in cell growth, differentiation and survival of hematopoietic and other tissues. Shp-2 is involved in modulating signaling via various growth factor receptor classes (Qu 2000), impacting the signaling from cytokines such as SCF, IL-3, M-CSF, GM-CSF and EPO (Chan et al. 2006).

Decreased function of Shp-2 in haploinsufficient BM cells caused a decrease in the percentage of quiescent cells and dramatically decreased the long-term repopulating ability of the cells (Chan et al. 2006). Importantly, Shp2<sup>+/-</sup> cells were able to home to recipient BM normally compared to wild-type cells, suggesting that the engraftment defects were not due to a defective ability of the cells to reach the BM after transplantation (Chan et al. 2006).

Phosphatase and tensin homologue (PTEN) is an important phosphatase involved in modulating the PI3K pathway by removing phosphate groups from inositol tri-phosphate, a second mediator in this pathway. PTEN mutations are found in many cancers, including leukemia (Cheong et al. 2003; Roman-Gomez et al. 2004; Aggerholm et al. 2000; Dahia et al. 1999). PTEN knockout is embryonic lethal in the mouse, but recently a conditional knockout mouse was developed and analyzed for changes in the HSC compartment (Yilmaz et al. 2006; Zhang et al. 2006). Two independent groups found that loss of PTEN dramatically affected HSC growth and behavior (Yilmaz et al. 2006; Zhang et al. 2006). Loss of PTEN reduced the percentage of quiescent HSCs, which



was correlated with a transient, short-lived increase in the number of HSCs *in vivo*, followed by a decrease in these numbers, and a concomitant increase in these numbers in peripheral tissues, indicating an increased mobilization tendency in cells lacking PTEN (Zhang et al. 2006). *In vivo* repopulating ability of PTEN knockout BM cells was significantly impaired compared to wild-type mice (Yilmaz et al. 2006; Zhang et al. 2006). Short-term homing of PTEN deficient cells was unaffected (Zhang et al. 2006), suggesting that the HSCs are exhausted by the loss of PTEN, due to increased cycling and an inability to maintain quiescence. Both groups found that the loss of PTEN led to leukemia; however, the depletion/exhaustion of HSCs occurred in animals in which leukemia never developed, suggesting that this depletion is not secondary to BM crowding or other changes due to leukemic development (Yilmaz et al. 2006). Additionally, one of the two groups studying this PTEN conditional knockout found that inhibiting the mTOR pathway by treating the mice *in vivo* with rapamycin led to the resolution of leukemia and, importantly, the restoration of normal numbers of HSCs by preventing increased cycling and exhaustion. This study suggests that activation of mTOR, a downstream effector of PI3K signaling, is responsible for some of the effects seen due to loss of PTEN (Yilmaz et al. 2006).

It is clear from the above discussion that various molecules have been studied regarding their ability to affect proliferation and maintenance of repopulating ability of HSCs. The goal of *ex vivo* expansion of HSCs is made more difficult by these studies showing a trade-off between proliferation and maintaining repopulating activity in HSCs.

In the current study, we found similar data suggesting that Rheb2 pushes HSCs into cycle at the expense of their repopulating ability (Figures 17 and 18). We found that a higher percentage of Rheb2 GFP<sup>+</sup> cells were in cycling S/G2/M status compared to Mieg3 and the GFP<sup>-</sup> fractions. This is one explanation for the loss of repopulating ability

when Rheb2 is overexpressed in immature hematopoietic cells. Under these circumstances, similar to the PTEN or p21 knockout models, the transduced HSCs may lose their ability to maintain the balance between proliferation and self-renewal/repopulating ability, leading to their exhaustion *in vivo*.

Other mechanisms could be involved in the phenotype observed due to Rheb2 overexpression, including possible effects on differentiation and survival. No defect in differentiation was observed at 1 month after competitive transplantation. However, earlier time points after transplantation were not measured and it is possible that a transient effect on differentiation, similar to the effect on LS expansion, could be present in these recipient mice. It is less likely that Rheb2 is preventing differentiation of progenitor cells, since we found increased colony numbers in methylcellulose colony assays. It is possible that Rheb2 can enhance differentiation, resulting in more colonies in the methylcellulose assays and depletion of HSCs *in vivo*. There also exists the possibility that Rheb2 overexpression negatively affects survival of HSCs. However, in progenitor cells, Rheb2 enhanced survival in delayed growth factor methylcellulose assays. We did not determine levels of apoptosis in more immature HSC populations.

Hematopoietic cell transplantation (HCT) is a complex process which has progressed dramatically in the past 20 years but still has limitations. In this study, two different approaches hypothesized as important in hematopoietic cell engraftment have been evaluated: modulation of engraftment by factors affecting homing and modulation of engraftment by signaling molecules involved in hematopoietic cell proliferation. First, expanding on studies using mouse to mouse transplantation, we found that inhibition of the peptidase CD26/DPPIV enhances engraftment of hUCB CD34<sup>+</sup> cells in a xenograph model. This provides a rationale for continuing study and determination of whether this strategy will have a positive effect in human hematopoietic transplantation, especially

using human umbilical cord blood as a donor source. In the study on Rheb2 overexpression, we found that Rheb2 could indeed expand hematopoietic progenitor and stem cells, but impaired the repopulating ability of the HSCs. Elucidating the downstream effectors responsible for these observations could help determine which molecular targets are responsible for maintaining the delicate balance between proliferation and repopulating activity and how manipulation of these pathways could be used clinically to enhance HCT.

## FUTURE OBJECTIVES

### Testing FDA-approved CD26 inhibitors in animal models

As stated in the Discussion, FDA CD26/DPPIV inhibitors are available for the treatment of type II diabetes. Our goal is to test these inhibitors in animal models of hematopoietic engraftment to determine if they can enhance this engraftment similar to Diprotin A. Our plans are to test these on the donor cells as well as recipient cells to determine which treatment or combination of treatments will be more effective at enhancing engraftment.

### CD26 expression on human HSCs and mechanism

We found that a higher percentage of immature CD38<sup>-</sup>CD34<sup>+</sup> cells expressed CD26 compared to more mature CD38<sup>+</sup>CD34<sup>+</sup> cells. This suggests a relationship between purity of HSCs and CD26/DPPIV expression, indicating part of the mechanism responsible for Diprotin A effects may be inhibition of CD26 on immature HSCs, enhancing their homing and engraftment. To test this hypothesis, we can FACS sort CD26<sup>+</sup>CD34<sup>+</sup> and CD26<sup>-</sup>CD34<sup>+</sup> and pretreat with Diprotin A before transplantation into NOD/SCID mice to test whether the CD26<sup>+</sup> population contains a higher proportion of *in vivo* engrafting HSCs.

To determine if the SDF-1-CXCR4 axis accounts for the effects due to CD26 expression, we can perform *in vivo* homing experiments using either CD34<sup>+</sup> or Lineage-negative hUCB cells in the presence or absence of AMD3100 treatment. We would expect this treatment to block the Diprotin A enhanced engraftment.

### Rheb2 in vivo mechanisms

We have provided evidence that Rheb2 enhances the cycling of immature hematopoietic populations *in vitro*, correlated with a transient increase in these numbers *in vivo*

followed by their exhaustion. We propose that the *in vivo* mechanism responsible for this exhaustion is similar to the *in vitro* increased cycling observed. Therefore, to test this, we will transplant recipient mice with Rheb2 transduced cells and follow the numbers of immature cells (as measured by Lin<sup>-</sup>Sca-1<sup>+</sup>) at least weekly after transplantation. At the same time, we will determine the cycling status of these cells *in vivo* using Hoechst 33342 staining, and we will incorporate Pyronin Y staining to measure the percentage of cells in G0 versus G1 stages. Our hypothesis is that Rheb2 overexpression will increase the cycling of cells *in vivo* corresponding to their increased numbers, followed by a decrease in cycling and a decrease in the total numbers.

#### Silencing of Rheb2 and effects on hematopoiesis

As shown in Figure 9, we have developed an shRNA retroviral vector capable of knocking down the expression of Rheb2. Our future goals will be to silence Rheb2 in primary hematopoietic cells and use these in functional assays such as *in vitro* colony-forming assays and *in vivo* competitive transplantations. We expect that Rheb2 knock-down will decrease the colony-forming ability of the transduced cells *in vitro*. *In vivo*, we expect knock-down of Rheb2 may prevent exhaustion of HSCs leading to increased long-term numbers. However, if Rheb2 knock-down negatively affects survival, as may be expected, we may find a loss of HSCs *in vivo* due to cell death. In addition to knock-down of Rheb2, we plan to study the level of expression of Rheb1 in hematopoietic cells and compare this to Rheb2 expression to understand better which protein is involved in mouse hematopoietic cell functions.

#### Rheb2 effects on mTORC2

Our *in vitro* experiments suggested that Rheb2 enhanced mTORC1 signaling in hematopoietic cell lines; however, rapamycin only partially decreased BM KSL

expansion in liquid cultures. First, we will test higher doses of rapamycin and replenish it frequently in these liquid cultures to determine if mTORC1 is involved to a greater extent than our current studies indicate. It is interesting to note that the role of Rheb2 on mTORC2 has not been published and may help to explain some of the effects we have observed. To test the relationship between Rheb2 and mTORC2, we will use Rheb2 overexpressing and Rheb2 silenced cell lines to determine if downstream mTORC2 targets are affected by Rheb2 modulation. One target of mTORC2 is AKT which is phosphorylated at Serine 473 by mTORC2 (Corradetti and Guan 2006). By Western blotting for these and other effectors of mTORC2, we can determine the role of Rheb2 in its modulation – a potentially novel finding.

## REFERENCES

- Aggerholm, A., K. Gronbaek, P. Guldberg and P. Hokland (2000). "Mutational analysis of the tumour suppressor gene MMAC1/PTEN in malignant myeloid disorders." Eur J Haematol **65**(2): 109-13.
- Aiuti, A., I. J. Webb, C. Bleul, T. Springer and J. C. Gutierrez-Ramos (1997). "The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood." J Exp Med **185**(1): 111-20.
- Akashi, K., D. Traver, T. Miyamoto and I. L. Weissman (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**(6774): 193-7.
- Antonchuk, J., G. Sauvageau and R. K. Humphries (2002). "HOXB4-induced expansion of adult hematopoietic stem cells ex vivo." Cell **109**(1): 39-45.
- Astrinidis, A. and E. P. Henske (2005). "Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease." Oncogene **24**(50): 7475-81.
- Austin, T. W., G. P. Solar, F. C. Ziegler, L. Liem and W. Matthews (1997). "A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells." Blood **89**(10): 3624-35.
- Baggiolini, M. (1998). "Chemokines and leukocyte traffic." Nature **392**(6676): 565-8.
- Basso, A. D., A. Mirza, G. Liu, B. J. Long, W. R. Bishop and P. Kirschmeier (2005). "The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity." J Biol Chem **280**(35): 31101-8.

- Bellantuono, I. (2004). "Haemopoietic stem cells." Int J Biochem Cell Biol **36**(4): 607-20.
- Bertrand, F. E., J. D. Spengeman, J. G. Shelton and J. A. McCubrey (2005). "Inhibition of PI3K, mTOR and MEK signaling pathways promotes rapid apoptosis in B-lineage ALL in the presence of stromal cell support." Leukemia **19**(1): 98-102.
- Bhatia, M., D. Bonnet, U. Kapp, J. C. Wang, B. Murdoch and J. E. Dick (1997a). "Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture." J Exp Med **186**(4): 619-24.
- Bhatia, M., J. C. Wang, U. Kapp, D. Bonnet and J. E. Dick (1997b). "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice." Proc Natl Acad Sci U S A **94**(10): 5320-5.
- Bodine, D. M., S. Karlsson and A. W. Nienhuis (1989). "Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into hematopoietic stem cells." Proc Natl Acad Sci U S A **86**(22): 8897-901.
- Bodine, D. M., D. Orlic, N. C. Birkett, N. E. Seidel and K. M. Zsebo (1992). "Stem cell factor increases colony-forming unit-spleen number in vitro in synergy with interleukin-6, and in vivo in SI/Sld mice as a single factor." Blood **79**(4): 913-9.
- Bollag, G., D. W. Clapp, S. Shih, F. Adler, Y. Y. Zhang, P. Thompson, B. J. Lange, M. H. Freedman, F. McCormick, T. Jacks and K. Shannon (1996). "Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells." Nat Genet **12**(2): 144-8.
- Bradford, G. B., B. Williams, R. Rossi and I. Bertoncello (1997). "Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment." Exp Hematol **25**(5): 445-53.



- Braun, B. S., D. A. Tuveson, N. Kong, D. T. Le, S. C. Kogan, J. Rozmus, M. M. Le Beau, T. E. Jacks and K. M. Shannon (2004). "Somatic activation of oncogenic Kras in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder." Proc Natl Acad Sci U S A **101**(2): 597-602.
- Brenner, S., N. Whiting-Theobald, T. Kawai, G. F. Linton, A. G. Rudikoff, U. Choi, M. F. Ryser, P. M. Murphy, J. M. Sechler and H. L. Malech (2004). "CXCR4-transgene expression significantly improves marrow engraftment of cultured hematopoietic stem cells." Stem Cells **22**(7): 1128-33.
- Broxmeyer, H. E. (2001). "Regulation of hematopoiesis by chemokine family members." Int J Hematol **74**(1): 9-17.
- Broxmeyer, H. E. (2006). Umbilical Cord Blood Stem Cells: Collection, Processing, and Transplantation. Blood Banking and Transfusion Medicine: Basic Principles and Practice. C. D. Hillyer, Silberstein, L.E., Ness, P.M., Anderson, K.C., and Roback, J., Churchill Livingstone, an imprint of Elsevier, Inc.: 823-832.
- Broxmeyer, H. E. (2008). "Chemokines in hematopoiesis." Curr Opin Hematol **15**(1): 49-58.
- Broxmeyer, H. E., S. Cooper, L. Kohli, G. Hangoc, Y. Lee, C. Mantel, D. W. Clapp and C. H. Kim (2003a). "Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/antiapoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo." J Immunol **170**(1): 421-9.
- Broxmeyer, H. E., G. Hangoc, S. Cooper, T. Campbell, S. Ito and C. Mantel (2007). "AMD3100 and CD26 modulate mobilization, engraftment, and survival of hematopoietic stem and progenitor cells mediated by the SDF-1/CXCL12-CXCR4 axis." Ann N Y Acad Sci **1106**: 1-19.

- Broxmeyer, H. E., L. Kohli, C. H. Kim, Y. Lee, C. Mantel, S. Cooper, G. Hangoc, M. Shaheen, X. Li and D. W. Clapp (2003b). "Stromal cell-derived factor-1/CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells." J Leukoc Biol **73**(5): 630-8.
- Broxmeyer, H. E., C. M. Orschell, D. W. Clapp, G. Hangoc, S. Cooper, P. A. Plett, W. C. Liles, X. Li, B. Graham-Evans, T. B. Campbell, G. Calandra, G. Bridger, D. C. Dale and E. F. Srour (2005). "Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist." J Exp Med **201**(8): 1307-18.
- Brun, A. C., X. Fan, J. M. Bjornsson, R. K. Humphries and S. Karlsson (2003). "Enforced adenoviral vector-mediated expression of HOXB4 in human umbilical cord blood CD34+ cells promotes myeloid differentiation but not proliferation." Mol Ther **8**(4): 618-28.
- Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, L. A. Milner, H. M. Kronenberg and D. T. Scadden (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature **425**(6960): 841-6.
- Campbell, T. B., G. Hangoc, Y. Liu, K. Pollok and H. E. Broxmeyer (2007a). "Inhibition of CD26 in Human Cord Blood CD34(+) Cells Enhances Their Engraftment of Nonobese Diabetic/Severe Combined Immunodeficiency Mice." Stem Cells Dev **16**(3): 347-54.
- Campbell, T. B., G. Hangoc, Y. Liu, K. Pollok and H. E. Broxmeyer (2007b). "Inhibition of CD26 in human cord blood CD34+ cells enhances their engraftment of nonobese diabetic/severe combined immunodeficiency mice." Stem Cells Dev **16**(3): 347-54.

- Cancelas, J. A., M. Jansen and D. A. Williams (2006). "The role of chemokine activation of Rac GTPases in hematopoietic stem cell marrow homing, retention, and peripheral mobilization." Exp Hematol **34**(8): 976-85.
- Cashen, A. F., H. M. Lazarus and S. M. Devine (2007). "Mobilizing stem cells from normal donors: is it possible to improve upon G-CSF?" Bone Marrow Transplant **39**(10): 577-88.
- Chae, H. D., K. E. Lee, D. A. Williams and Y. Gu (2008). "Cross-talk between RhoH and Rac1 in regulation of actin cytoskeleton and chemotaxis of hematopoietic progenitor cells." Blood **111**(5): 2597-605.
- Chan, R. J., Y. Li, M. N. Hass, A. Walter, C. S. Voorhorst, W. C. Shelley, Z. Yang, C. M. Orschell and M. C. Yoder (2006). "Shp-2 heterozygous hematopoietic stem cells have deficient repopulating ability due to diminished self-renewal." Exp Hematol **34**(9): 1230-9.
- Chan, S. M., A. P. Weng, R. Tibshirani, J. C. Aster and P. J. Utz (2007). "Notch signals positively regulate activity of the mTOR pathway in T-cell acute lymphoblastic leukemia." Blood **110**(1): 278-86.
- Chen, L., Z. Gao, J. Zhu and G. P. Rodgers (2007). "Identification of CD13+CD36+ cells as a common progenitor for erythroid and myeloid lineages in human bone marrow." Exp Hematol **35**(7): 1047-55.
- Cheng, T., N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes and D. T. Scadden (2000). "Hematopoietic stem cell quiescence maintained by p21cip1/waf1." Science **287**(5459): 1804-8.
- Cheong, J. W., J. I. Eom, H. Y. Maeng, S. T. Lee, J. S. Hahn, Y. W. Ko and Y. H. Min (2003). "Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome." Br J Haematol **122**(3): 454-6.

- Cheshier, S. H., S. J. Morrison, X. Liao and I. L. Weissman (1999). "In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells." Proc Natl Acad Sci U S A **96**(6): 3120-5.
- Choo, A. Y., P. P. Roux and J. Blenis (2006). "Mind the GAP: Wnt steps onto the mTORC1 train." Cell **126**(5): 834-6.
- Christensen, J. L. and I. L. Weissman (2001). "Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells." Proc Natl Acad Sci U S A **98**(25): 14541-6.
- Christianson, S. W., D. L. Greiner, R. A. Hesselton, J. H. Leif, E. J. Wagar, I. B. Schweitzer, T. V. Rajan, B. Gott, D. C. Roopenian and L. D. Shultz (1997). "Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice." J Immunol **158**(8): 3578-86.
- Christopherson II, K. W., L. A. Paganessi, S. Napier and N. K. Porecha (2007). "CD26 Inhibition on CD34(+) or Lineage(-) Human Umbilical Cord Blood Donor Hematopoietic Stem Cells/Hematopoietic Progenitor Cells Improves Long-Term Engraftment into NOD/SCID/Beta2(null) Immunodeficient Mice." Stem Cells Dev **16**(3): 355-60.
- Christopherson, K. W., 2nd, S. Cooper and H. E. Broxmeyer (2003). "Cell surface peptidase CD26/DPPIV mediates G-CSF mobilization of mouse progenitor cells." Blood **101**(12): 4680-6.
- Christopherson, K. W., 2nd, G. Hangoc and H. E. Broxmeyer (2002). "Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells." J Immunol **169**(12): 7000-8.

- Christopherson, K. W., 2nd, G. Hangoc, C. R. Mantel and H. E. Broxmeyer (2004). "Modulation of hematopoietic stem cell homing and engraftment by CD26." Science **305**(5686): 1000-3.
- Christopherson, K. W., 2nd, S. E. Uralil, N. K. Porecha, R. C. Zabriskie, S. M. Kidd and S. M. Ramin (2006). "G-CSF- and GM-CSF-induced upregulation of CD26 peptidase downregulates the functional chemotactic response of CD34+CD38- human cord blood hematopoietic cells." Exp Hematol **34**(8): 1060-8.
- Chute, J. P. (2006). "Stem cell homing." Curr Opin Hematol **13**(6): 399-406.
- Clark-Lewis, I., K. S. Kim, K. Rajarathnam, J. H. Gong, B. Dewald, B. Moser, M. Baggiolini and B. D. Sykes (1995). "Structure-activity relationships of chemokines." J Leukoc Biol **57**(5): 703-11.
- Corradetti, M. N. and K. L. Guan (2006). "Upstream of the mammalian target of rapamycin: do all roads pass through mTOR?" Oncogene **25**(48): 6347-60.
- Cottler-Fox, M. H., T. Lapidot, I. Petit, O. Kollet, J. F. DiPersio, D. Link and S. Devine (2003). "Stem cell mobilization." Hematology Am Soc Hematol Educ Program: 419-37.
- Crump, M. P., J. H. Gong, P. Loetscher, K. Rajarathnam, A. Amara, F. Arenzana-Seisdedos, J. L. Virelizier, M. Baggiolini, B. D. Sykes and I. Clark-Lewis (1997). "Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1." Embo J **16**(23): 6996-7007.
- Cruz, R., L. Hedden, D. Boyer, M. G. Kharas, D. A. Fruman and K. K. Lee-Fruman (2005). "S6 kinase 2 potentiates interleukin-3-driven cell proliferation." J Leukoc Biol **78**(6): 1378-85.

- Dahia, P. L., R. C. Aguiar, J. Alberta, J. B. Kum, S. Caron, H. Sill, D. J. Marsh, J. Ritz, A. Freedman, C. Stiles and C. Eng (1999). "PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies." Hum Mol Genet **8**(2): 185-93.
- De Meester, I., S. Korom, J. Van Damme and S. Scharpe (1999). "CD26, let it cut or cut it down." Immunol Today **20**(8): 367-75.
- Deacon, C. F. and J. J. Holst (2006). "Dipeptidyl peptidase IV inhibitors: a promising new therapeutic approach for the management of type 2 diabetes." Int J Biochem Cell Biol **38**(5-6): 831-44.
- Deeg, H. J., K. M. Sullivan, C. D. Buckner, R. Storb, F. R. Appelbaum, R. A. Clift, K. Doney, J. E. Sanders, R. P. Witherspoon and E. D. Thomas (1986). "Marrow transplantation for acute nonlymphoblastic leukemia in first remission: toxicity and long-term follow-up of patients conditioned with single dose or fractionated total body irradiation." Bone Marrow Transplant **1**(2): 151-7.
- Demuth, H. U., C. H. McIntosh and R. A. Pederson (2005). "Type 2 diabetes--therapy with dipeptidyl peptidase IV inhibitors." Biochim Biophys Acta **1751**(1): 33-44.
- Desponts, C., A. L. Hazen, K. H. Paraiso and W. G. Kerr (2006). "SHIP deficiency enhances HSC proliferation and survival but compromises homing and repopulation." Blood **107**(11): 4338-45.
- Diaconescu, R. and R. Storb (2005). "Allogeneic hematopoietic cell transplantation: from experimental biology to clinical care." J Cancer Res Clin Oncol **131**(1): 1-13.
- Dooner, M., J. Cerny, G. Colvin, D. Demers, J. Pimentel, D. Greer, M. Abedi, C. McAuliffe and P. Quesenberry (2004). "Homing and conversion of murine hematopoietic stem cells to lung." Blood Cells Mol Dis **32**(1): 47-51.
- Dutcher, J. P. (2004). "Mammalian target of rapamycin inhibition." Clin Cancer Res **10**(18 Pt 2): 6382S-7S.

- Etienne-Manneville, S. and A. Hall (2002). "Rho GTPases in cell biology." Nature **420**(6916): 629-35.
- Fingar, D. C. and J. Blenis (2004). "Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression." Oncogene **23**(18): 3151-71.
- Fleming, H. E., V. Janzen, C. Lo Celso, J. Guo, K. M. Leahy, H. M. Kronenberg and D. T. Scadden (2008). "Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo." Cell Stem Cell **2**(3): 274-83.
- Follo, M. Y., S. Mongiorgi, C. Bosi, A. Cappellini, C. Finelli, F. Chiarini, V. Papa, M. Libra, G. Martinelli, L. Cocco and A. M. Martelli (2007). "The Akt/mammalian target of rapamycin signal transduction pathway is activated in high-risk myelodysplastic syndromes and influences cell survival and proliferation." Cancer Res **67**(9): 4287-94.
- Fukuda, S., H. Bian, A. G. King and L. M. Pelus (2007). "The chemokine GRO{beta} mobilizes early hematopoietic stem cells characterized by enhanced homing and engraftment." Blood.
- Gammaitoni, L., S. Bruno, F. Sanavio, M. Gunetti, O. Kollet, G. Cavalloni, M. Falda, F. Fagioli, T. Lapidot, M. Aglietta and W. Piacibello (2003). "Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution." Exp Hematol **31**(3): 261-70.
- Gluckman, E., H. A. Broxmeyer, A. D. Auerbach, H. S. Friedman, G. W. Douglas, A. Devergie, H. Esperou, D. Thierry, G. Socie, P. Lehn and et al. (1989). "Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling." N Engl J Med **321**(17): 1174-8.

- Gu, Y., M. D. Filippi, J. A. Cancelas, J. E. Sieftring, E. P. Williams, A. C. Jasti, C. E. Harris, A. W. Lee, R. Prabhakar, S. J. Atkinson, D. J. Kwiatkowski and D. A. Williams (2003). "Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases." Science **302**(5644): 445-9.
- Guan, E., J. Wang and M. A. Norcross (2004). "Amino-terminal processing of MIP-1beta/CCL4 by CD26/dipeptidyl-peptidase IV." J Cell Biochem **92**(1): 53-64.
- Guan, E., J. Wang, G. Roderiquez and M. A. Norcross (2002). "Natural truncation of the chemokine MIP-1 beta /CCL4 affects receptor specificity but not anti-HIV-1 activity." J Biol Chem **277**(35): 32348-52.
- Hammerman, P. S., C. J. Fox, M. J. Birnbaum and C. B. Thompson (2005). "Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival." Blood **105**(11): 4477-83.
- Hansen, J. A., T. A. Gooley, P. J. Martin, F. Appelbaum, T. R. Chauncey, R. A. Clift, E. W. Petersdorf, J. Radich, J. E. Sanders, R. F. Storb, K. M. Sullivan and C. Anasetti (1998). "Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia." N Engl J Med **338**(14): 962-8.
- Hao, Q. L., A. J. Shah, F. T. Thiemann, E. M. Smogorzewska and G. M. Crooks (1995). "A functional comparison of CD34 + CD38- cells in cord blood and bone marrow." Blood **86**(10): 3745-53.
- Hart, C., D. Drewel, G. Mueller, J. Grassinger, M. Zaiss, L. A. Kunz-Schughart, R. Andreesen, A. Reichle, E. Holler and B. Hennemann (2004). "Expression and function of homing-essential molecules and enhanced in vivo homing ability of human peripheral blood-derived hematopoietic progenitor cells after stimulation with stem cell factor." Stem Cells **22**(4): 580-9.
- Heike, T. and T. Nakahata (2002). "Ex vivo expansion of hematopoietic stem cells by cytokines." Biochim Biophys Acta **1592**(3): 313-21.



- Herrera, C., C. Morimoto, J. Blanco, J. Mallol, F. Arenzana, C. Lluís and R. Franco (2001). "Comodulation of CXCR4 and CD26 in human lymphocytes." J Biol Chem **276**(22): 19532-9.
- Hidalgo, A., L. A. Weiss and P. S. Frenette (2002). "Functional selectin ligands mediating human CD34(+) cell interactions with bone marrow endothelium are enhanced postnatally." J Clin Invest **110**(4): 559-69.
- Hoebeker, I., M. De Smedt, F. Stolz, K. Pike-Overzet, F. J. Staal, J. Plum and G. Leclercq (2007). "T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(-)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes." Leukemia **21**(2): 311-9.
- Hsu, Y. C., J. J. Chern, Y. Cai, M. Liu and K. W. Choi (2007). "Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase." Nature **445**(7129): 785-8.
- Huang, J., C. C. Dibble, M. Matsuzaki and B. D. Manning (2008). "The TSC1-TSC2 complex is required for proper activation of mTOR complex 2." Mol Cell Biol **28**(12): 4104-15.
- Hurley, C. K., M. Fernandez Vina and M. Setterholm (2003). "Maximizing optimal hematopoietic stem cell donor selection from registries of unrelated adult volunteers." Tissue Antigens **61**(6): 415-24.
- Im, E., F. C. von Lintig, J. Chen, S. Zhuang, W. Qui, S. Chowdhury, P. F. Worley, G. R. Boss and R. B. Pilz (2002). "Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells." Oncogene **21**(41): 6356-65.

- Inoki, K., H. Ouyang, T. Zhu, C. Lindvall, Y. Wang, X. Zhang, Q. Yang, C. Bennett, Y. Harada, K. Stankunas, C. Y. Wang, X. He, O. A. MacDougald, M. You, B. O. Williams and K. L. Guan (2006). "TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth." Cell **126**(5): 955-68.
- Ishikawa, F., A. G. Livingston, H. Minamiguchi, J. R. Wingard and M. Ogawa (2003). "Human cord blood long-term engrafting cells are CD34+ CD38." Leukemia **17**(5): 960-4.
- Ivanova, N. B., J. T. Dimos, C. Schaniel, J. A. Hackney, K. A. Moore and I. R. Lemischka (2002). "A stem cell molecular signature." Science **298**(5593): 601-4.
- Janeway, C. (2005). Immunobiology : the immune system in health and disease. New York, Garland Science.
- Kahn, J., T. Byk, L. Jansson-Sjostrand, I. Petit, S. Shivtiel, A. Nagler, I. Hardan, V. Deutsch, Z. Gazit, D. Gazit, S. Karlsson and T. Lapidot (2004). "Overexpression of CXCR4 on human CD34+ progenitors increases their proliferation, migration, and NOD/SCID repopulation." Blood **103**(8): 2942-9.
- Karanu, F. N., B. Murdoch, L. Gallacher, D. M. Wu, M. Koremoto, S. Sakano and M. Bhatia (2000). "The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells." J Exp Med **192**(9): 1365-72.
- Karbowiczek, M., G. P. Robertson and E. P. Henske (2006). "Rheb inhibits C-raf activity and B-raf/C-raf heterodimerization." J Biol Chem **281**(35): 25447-56.
- Kawai, T., U. Choi, P. C. Liu, N. L. Whiting-Theobald, G. F. Linton and H. L. Malech (2007). "Diprotin A Infusion into Nonobese Diabetic/Severe Combined Immunodeficiency Mice Markedly Enhances Engraftment of Human Mobilized CD34(+) Peripheral Blood Cells." Stem Cells Dev **16**(3): 361-370.

- Khalaf, W. F., H. White, M. J. Wenning, A. Orazi, R. Kapur and D. A. Ingram (2005). "K-Ras is essential for normal fetal liver erythropoiesis." Blood **105**(9): 3538-41.
- Kim, C. H. and H. E. Broxmeyer (1998). "In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment." Blood **91**(1): 100-10.
- Kollet, O., A. Peled, T. Byk, H. Ben-Hur, D. Greiner, L. Shultz and T. Lapidot (2000). "beta2 microglobulin-deficient (B2m(null)) NOD/SCID mice are excellent recipients for studying human stem cell function." Blood **95**(10): 3102-5.
- Kollet, O., A. Spiegel, A. Peled, I. Petit, T. Byk, R. Hershkovich, E. Guetta, G. Barkai, A. Nagler and T. Lapidot (2001). "Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice." Blood **97**(10): 3283-91.
- Kondo, M., I. L. Weissman and K. Akashi (1997). "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell **91**(5): 661-72.
- Krosil, J., P. Austin, N. Beslu, E. Kroon, R. K. Humphries and G. Sauvageau (2003). "In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein." Nat Med **9**(11): 1428-32.
- Laird, D. J., U. H. von Andrian and A. J. Wagers (2008). "Stem cell trafficking in tissue development, growth, and disease." Cell **132**(4): 612-30.
- Lapidot, T., A. Dar and O. Kollet (2005). "How do stem cells find their way home?" Blood **106**(6): 1901-10.
- Lapidot, T., Y. Fajerman and O. Kollet (1997). "Immune-deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis." J Mol Med **75**(9): 664-73.

- Lapidot, T. and I. Petit (2002). "Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells." Exp Hematol **30**(9): 973-81.
- Lessard, J., A. Faubert and G. Sauvageau (2004). "Genetic programs regulating HSC specification, maintenance and expansion." Oncogene **23**(43): 7199-209.
- Lewis, I. D. (2002). "Clinical and experimental uses of umbilical cord blood." Intern Med J **32**(12): 601-9.
- Li, Y., M. N. Corradetti, K. Inoki and K. L. Guan (2004a). "TSC2: filling the GAP in the mTOR signaling pathway." Trends Biochem Sci **29**(1): 32-8.
- Li, Y., K. Inoki and K. L. Guan (2004b). "Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity." Mol Cell Biol **24**(18): 7965-75.
- Link, H., L. Arseniev, O. Bahre, J. G. Kadar, H. Diedrich and H. Poliwoda (1996). "Transplantation of allogeneic CD34+ blood cells." Blood **87**(11): 4903-9.
- Linnekin, D. (1999). "Early signaling pathways activated by c-Kit in hematopoietic cells." Int J Biochem Cell Biol **31**(10): 1053-74.
- Long, X., Y. Lin, S. Ortiz-Vega, K. Yonezawa and J. Avruch (2005a). "Rheb binds and regulates the mTOR kinase." Curr Biol **15**(8): 702-13.
- Long, X., S. Ortiz-Vega, Y. Lin and J. Avruch (2005b). "Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency." J Biol Chem **280**(25): 23433-6.
- Lorenz, E., D. Uphoff, T. R. Reid and E. Shelton (1951). "Modification of irradiation injury in mice and guinea pigs by bone marrow injections." J Natl Cancer Inst **12**(1): 197-201.

- Ludwig, A., F. Schiemann, R. Mentlein, B. Lindner and E. Brandt (2002). "Dipeptidyl peptidase IV (CD26) on T cells cleaves the CXC chemokine CXCL11 (I-TAC) and abolishes the stimulating but not the desensitizing potential of the chemokine." J Leukoc Biol **72**(1): 183-91.
- MacKenzie, K. L., A. Dolnikov, M. Millington, Y. Shounan and G. Symonds (1999). "Mutant N-ras induces myeloproliferative disorders and apoptosis in bone marrow repopulated mice." Blood **93**(6): 2043-56.
- Morgensztern, D. and H. L. McLeod (2005). "PI3K/Akt/mTOR pathway as a target for cancer therapy." Anticancer Drugs **16**(8): 797-803.
- Morrison, S. J. and I. L. Weissman (1994). "The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype." Immunity **1**(8): 661-73.
- Mungamuri, S. K., X. Yang, A. D. Thor and K. Somasundaram (2006). "Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53." Cancer Res **66**(9): 4715-24.
- Murdoch, B., K. Chadwick, M. Martin, F. Shojaei, K. V. Shah, L. Gallacher, R. T. Moon and M. Bhatia (2003). "Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo." Proc Natl Acad Sci U S A **100**(6): 3422-7.
- Murphy, P. M. (1994). "The molecular biology of leukocyte chemoattractant receptors." Annu Rev Immunol **12**: 593-633.
- Nathan, D. M. (2007). "Finding new treatments for diabetes--how many, how fast... how good?" N Engl J Med **356**(5): 437-40.
- Nervi, B., D. C. Link and J. F. DiPersio (2006). "Cytokines and hematopoietic stem cell mobilization." J Cell Biochem **99**(3): 690-705.

- Ohishi, K., B. Varnum-Finney and I. D. Bernstein (2002). "Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells." J Clin Invest **110**(8): 1165-74.
- Ohtsuki, T., O. Hosono, H. Kobayashi, Y. Munakata, A. Souta, T. Shioda and C. Morimoto (1998). "Negative regulation of the anti-human immunodeficiency virus and chemotactic activity of human stromal cell-derived factor 1alpha by CD26/dipeptidyl peptidase IV." FEBS Lett **431**(2): 236-40.
- Oravec, T., M. Pall, G. Roderiguez, M. D. Gorrell, M. Ditto, N. Y. Nguyen, R. Boykins, E. Unsworth and M. A. Norcross (1997). "Regulation of the receptor specificity and function of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by dipeptidyl peptidase IV (CD26)-mediated cleavage." J Exp Med **186**(11): 1865-72.
- Orschell-Traycoff, C. M., K. Hiatt, R. N. Dagher, S. Rice, M. C. Yoder and E. F. Srour (2000). "Homing and engraftment potential of Sca-1(+)lin(-) cells fractionated on the basis of adhesion molecule expression and position in cell cycle." Blood **96**(4): 1380-7.
- Passegue, E., A. J. Wagers, S. Giuriato, W. C. Anderson and I. L. Weissman (2005). "Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates." J Exp Med **202**(11): 1599-611.
- Passegue, E. and I. L. Weissman (2005). "Leukemic stem cells: where do they come from?" Stem Cell Rev **1**(3): 181-8.
- Peled, A., I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, O. Lider, R. Alon, D. Zipori and T. Lapidot (1999). "Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4." Science **283**(5403): 845-8.

- Pelus, L. M. and S. Fukuda (2006). "Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties." Exp Hematol **34**(8): 1010-20.
- Pelus, L. M. and S. Fukuda (2008). "Chemokine-mobilized adult stem cells; defining a better hematopoietic graft." Leukemia **22**(3): 466-73.
- Peranteau, W. H., M. Endo, O. O. Adibe, A. Merchant, P. W. Zoltick and A. W. Flake (2006). "CD26 inhibition enhances allogeneic donor-cell homing and engraftment after in utero hematopoietic-cell transplantation." Blood **108**(13): 4268-74.
- Perkins, A. C. and S. Cory (1993). "Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene." Embo J **12**(10): 3835-46.
- Petzer, A. L., P. W. Zandstra, J. M. Piret and C. J. Eaves (1996). "Differential cytokine effects on primitive (CD34+CD38-) human hematopoietic cells: novel responses to Flt3-ligand and thrombopoietin." J Exp Med **183**(6): 2551-8.
- Pro, B. and N. H. Dang (2004). "CD26/dipeptidyl peptidase IV and its role in cancer." Histol Histopathol **19**(4): 1345-51.
- Proost, P., I. De Meester, D. Schols, S. Struyf, A. M. Lambeir, A. Wuyts, G. Opdenakker, E. De Clercq, S. Scharpe and J. Van Damme (1998a). "Amino-terminal truncation of chemokines by CD26/dipeptidyl-peptidase IV. Conversion of RANTES into a potent inhibitor of monocyte chemotaxis and HIV-1-infection." J Biol Chem **273**(13): 7222-7.
- Proost, P., P. Menten, S. Struyf, E. Schutyser, I. De Meester and J. Van Damme (2000). "Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78beta into a most efficient monocyte attractant and CCR1 agonist." Blood **96**(5): 1674-80.

- Proost, P., E. Schutyser, P. Menten, S. Struyf, A. Wuyts, G. Opdenakker, M. Detheux, M. Parmentier, C. Durinx, A. M. Lambeir, J. Neyts, S. Liekens, P. C. Maudgal, A. Billiau and J. Van Damme (2001). "Amino-terminal truncation of CXCR3 agonists impairs receptor signaling and lymphocyte chemotaxis, while preserving antiangiogenic properties." Blood **98**(13): 3554-61.
- Proost, P., S. Struyf, D. Schols, C. Durinx, A. Wuyts, J. P. Lenaerts, E. De Clercq, I. De Meester and J. Van Damme (1998b). "Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha." FEBS Lett **432**(1-2): 73-6.
- Proost, P., S. Struyf, D. Schols, G. Opdenakker, S. Sozzani, P. Allavena, A. Mantovani, K. Augustyns, G. Bal, A. Haemers, A. M. Lambeir, S. Scharpe, J. Van Damme and I. De Meester (1999). "Truncation of macrophage-derived chemokine by CD26/ dipeptidyl-peptidase IV beyond its predicted cleavage site affects chemotactic activity and CC chemokine receptor 4 interaction." J Biol Chem **274**(7): 3988-93.
- Qu, C. K. (2000). "The SHP-2 tyrosine phosphatase: signaling mechanisms and biological functions." Cell Res **10**(4): 279-88.
- Rahfeld, J., M. Schierhorn, B. Hartrodt, K. Neubert and J. Heins (1991). "Are diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase IV?" Biochim Biophys Acta **1076**(2): 314-6.
- Raslova, H., V. Baccini, L. Loussaief, B. Comba, J. Larghero, N. Debili and W. Vainchenker (2006). "Mammalian target of rapamycin (mTOR) regulates both proliferation of megakaryocyte progenitors and late stages of megakaryocyte differentiation." Blood **107**(6): 2303-10.



- Rodel, B., K. Tavassoli, H. Karsunky, T. Schmidt, M. Bachmann, F. Schaper, P. Heinrich, K. Shuai, H. P. Elsasser and T. Moroy (2000). "The zinc finger protein Gfi-1 can enhance STAT3 signaling by interacting with the STAT3 inhibitor PIAS3." Embo J **19**(21): 5845-55.
- Roman-Gomez, J., A. Jimenez-Velasco, J. A. Castillejo, X. Agirre, M. Barrios, G. Navarro, F. J. Molina, M. J. Calasanz, F. Prosper, A. Heiniger and A. Torres (2004). "Promoter hypermethylation of cancer-related genes: a strong independent prognostic factor in acute lymphoblastic leukemia." Blood **104**(8): 2492-8.
- Rosu-Myles, M., L. Gallacher, B. Murdoch, D. A. Hess, M. Keeney, D. Kelvin, L. Dale, S. S. Ferguson, D. Wu, F. Fellows and M. Bhatia (2000). "The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression." Proc Natl Acad Sci U S A **97**(26): 14626-31.
- Sallusto, F., A. Lanzavecchia and C. R. Mackay (1998). "Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses." Immunol Today **19**(12): 568-74.
- Santos, G. W., P. J. Tutschka, R. Brookmeyer, R. Saral, W. E. Beschorner, W. B. Bias, H. G. Braine, W. H. Burns, G. J. Eifenbein, H. Kaizer and et al. (1983). "Marrow transplantation for acute nonlymphocytic leukemia after treatment with busulfan and cyclophosphamide." N Engl J Med **309**(22): 1347-53.
- Sauvageau, G., U. Thorsteinsdottir, C. J. Eaves, H. J. Lawrence, C. Largman, P. M. Lansdorp and R. K. Humphries (1995). "Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo." Genes Dev **9**(14): 1753-65.

- Scheele, J. S., D. Ripple and M. Lubbert (2000). "The role of ras and other low molecular weight guanine nucleotide (GTP)-binding proteins during hematopoietic cell differentiation." Cell Mol Life Sci **57**(13-14): 1950-63.
- Schiedlmeier, B., H. Klump, E. Will, G. Arman-Kalcek, Z. Li, Z. Wang, A. Rimek, J. Friel, C. Baum and W. Ostertag (2003). "High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation." Blood **101**(5): 1759-68.
- Shaheen, M., & H.E. Broxmeyer (2005). The humoral regulation of hematopoiesis. . Hematology: Basic Principles and Practice. R. Hoffman, E. Benz, S. Shattil, B. Furie, H. Cohen, L. Silberstein & P. McGlave Elsevier Churchill Livingstone: pp. 233-265.
- Shen, S. W., A. Dolnikov, T. Passioura, M. Millington, S. Wotherspoon, A. Rice, K. L. MacKenzie and G. Symonds (2004). "Mutant N-ras preferentially drives human CD34+ hematopoietic progenitor cells into myeloid differentiation and proliferation both in vitro and in the NOD/SCID mouse." Exp Hematol **32**(9): 852-60.
- Shioda, T., H. Kato, Y. Ohnishi, K. Tashiro, M. Ikegawa, E. E. Nakayama, H. Hu, A. Kato, Y. Sakai, H. Liu, T. Honjo, A. Nomoto, A. Iwamoto, C. Morimoto and Y. Nagai (1998). "Anti-HIV-1 and chemotactic activities of human stromal cell-derived factor 1alpha (SDF-1alpha) and SDF-1beta are abolished by CD26/dipeptidyl peptidase IV-mediated cleavage." Proc Natl Acad Sci U S A **95**(11): 6331-6.
- Shizuru, J. A., R. S. Negrin and I. L. Weissman (2005). "Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system." Annu Rev Med **56**: 509-38.

- Sorrentino, B. P. (2004). "Clinical strategies for expansion of haematopoietic stem cells." Nat Rev Immunol **4**(11): 878-88.
- Steelman, L. S., S. L. Abrams, J. Whelan, F. E. Bertrand, D. E. Ludwig, J. Basecke, M. Libra, F. Stivala, M. Milella, A. Tafuri, P. Lunghi, A. Bonati, A. M. Martelli and J. A. McCubrey (2008). "Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia." Leukemia **22**(4): 686-707.
- Stier, S., T. Cheng, D. Dombkowski, N. Carlesso and D. T. Scadden (2002). "Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome." Blood **99**(7): 2369-78.
- Struyf, S., I. De Meester, S. Scharpe, J. P. Lenaerts, P. Menten, J. M. Wang, P. Proost and J. Van Damme (1998a). "Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor." Eur J Immunol **28**(4): 1262-71.
- Struyf, S., P. Menten, J. P. Lenaerts, W. Put, A. D'Haese, E. De Clercq, D. Schols, P. Proost and J. Van Damme (2001). "Diverging binding capacities of natural LD78beta isoforms of macrophage inflammatory protein-1alpha to the CC chemokine receptors 1, 3 and 5 affect their anti-HIV-1 activity and chemotactic potencies for neutrophils and eosinophils." Eur J Immunol **31**(7): 2170-8.
- Struyf, S., P. Proost, D. Schols, E. De Clercq, G. Opendakker, J. P. Lenaerts, M. Detheux, M. Parmentier, I. De Meester, S. Scharpe and J. Van Damme (1999). "CD26/dipeptidyl-peptidase IV down-regulates the eosinophil chemotactic potency, but not the anti-HIV activity of human eotaxin by affecting its interaction with CC chemokine receptor 3." J Immunol **162**(8): 4903-9.

- Struyf, S., P. Proost, S. Sozzani, A. Mantovani, A. Wuyts, E. De Clercq, D. Schols and J. Van Damme (1998b). "Enhanced anti-HIV-1 activity and altered chemotactic potency of NH<sub>2</sub>-terminally processed macrophage-derived chemokine (MDC) imply an additional MDC receptor." J Immunol **161**(6): 2672-5.
- Tao, W., G. Hangoc, S. Cooper and H. E. Broxmeyer (2004). "SDF-1alpha/CXCL12 enhances retroviral-mediated gene transfer into immature subsets of human and murine hematopoietic progenitor cells." Gene Ther **11**(1): 61-9.
- Tee, A. R., J. Blenis and C. G. Proud (2005). "Analysis of mTOR signaling by the small G-proteins, Rheb and RhebL1." FEBS Lett **579**(21): 4763-8.
- Thomas, E. D., R. A. Clift, J. Hersman, J. E. Sanders, P. Stewart, C. D. Buckner, A. Fefer, R. McGuffin, J. W. Smith and R. Storb (1982). "Marrow transplantation for acute nonlymphoblastic leukemia in first remission using fractionated or single-dose irradiation." Int J Radiat Oncol Biol Phys **8**(5): 817-21.
- Thorsteinsdottir, U., G. Sauvageau, M. R. Hough, W. Dragowska, P. M. Lansdorp, H. J. Lawrence, C. Largman and R. K. Humphries (1997). "Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia." Mol Cell Biol **17**(1): 495-505.
- Tian, C., J. Bagley, D. Forman and J. Iacomini (2006). "Inhibition of CD26 peptidase activity significantly improves engraftment of retrovirally transduced hematopoietic progenitors." Gene Ther **13**(7): 652-8.
- Tuveson, D. A., A. T. Shaw, N. A. Willis, D. P. Silver, E. L. Jackson, S. Chang, K. L. Mercer, R. Grochow, H. Hock, D. Crowley, S. R. Hingorani, T. Zaks, C. King, M. A. Jacobetz, L. Wang, R. T. Bronson, S. H. Orkin, R. A. DePinho and T. Jacks (2004). "Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects." Cancer Cell **5**(4): 375-87.

- Van Den Berg, D. J., A. K. Sharma, E. Bruno and R. Hoffman (1998). "Role of members of the Wnt gene family in human hematopoiesis." Blood **92**(9): 3189-202.
- van der Loo, J. C., X. Xiao, D. McMillin, K. Hashino, I. Kato and D. A. Williams (1998). "VLA-5 is expressed by mouse and human long-term repopulating hematopoietic cells and mediates adhesion to extracellular matrix protein fibronectin." J Clin Invest **102**(5): 1051-61.
- Van Meter, M. E., E. Diaz-Flores, J. A. Archard, E. Passegue, J. M. Irish, N. Kotecha, G. P. Nolan, K. Shannon and B. S. Braun (2007). "K-RasG12D expression induces hyperproliferation and aberrant signaling in primary hematopoietic stem/progenitor cells." Blood **109**(9): 3945-52.
- Wieman, H. L., J. A. Wofford and J. C. Rathmell (2007). "Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking." Mol Biol Cell **18**(4): 1437-46.
- Willert, K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates, 3rd and R. Nusse (2003). "Wnt proteins are lipid-modified and can act as stem cell growth factors." Nature **423**(6938): 448-52.
- Wright, D. E., E. P. Bowman, A. J. Wagers, E. C. Butcher and I. L. Weissman (2002). "Hematopoietic stem cells are uniquely selective in their migratory response to chemokines." J Exp Med **195**(9): 1145-54.
- Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-84.
- Xu, Q., J. E. Thompson and M. Carroll (2005a). "mTOR regulates cell survival after etoposide treatment in primary AML cells." Blood **106**(13): 4261-8.

- Xu, R. H., H. Pelicano, H. Zhang, F. J. Giles, M. J. Keating and P. Huang (2005b). "Synergistic effect of targeting mTOR by rapamycin and depleting ATP by inhibition of glycolysis in lymphoma and leukemia cells." Leukemia **19**(12): 2153-8.
- Yang, L., L. Wang, H. Geiger, J. A. Cancelas, J. Mo and Y. Zheng (2007). "Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow." Proc Natl Acad Sci U S A **104**(12): 5091-6.
- Yaniv, I., J. Stein, D. L. Farkas and N. Askenasy (2006). "The tale of early hematopoietic cell seeding in the bone marrow niche." Stem Cells Dev **15**(1): 4-16.
- Yilmaz, O. H., R. Valdez, B. K. Theisen, W. Guo, D. O. Ferguson, H. Wu and S. J. Morrison (2006). "Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells." Nature **441**(7092): 475-82.
- Yuan, J., Y. Shan, X. Chen, W. Tang, K. Luo, J. Ni, B. Wan and L. Yu (2005). "Identification and characterization of RHEBL1, a novel member of Ras family, which activates transcriptional activities of NF-kappa B." Mol Biol Rep **32**(4): 205-14.
- Zeng, H., R. Yucel, C. Kosan, L. Klein-Hitpass and T. Moroy (2004). "Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells." Embo J **23**(20): 4116-25.
- Zhang, J., J. C. Grindley, T. Yin, S. Jayasinghe, X. C. He, J. T. Ross, J. S. Haug, D. Rupp, K. S. Porter-Westpfahl, L. M. Wiedemann, H. Wu and L. Li (2006). "PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention." Nature **441**(7092): 518-22.
- Zlotnik, A. and O. Yoshie (2000). "Chemokines: a new classification system and their role in immunity." Immunity **12**(2): 121-7.

## CURRICULUM VITAE

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### Education

Indiana University, Indianapolis, IN  
Doctor of Philosophy, Department of Microbiology and Immunology  
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Purdue University, Fort Wayne, IN  
Bachelor of Science, Biology, With Distinction  
Bachelor of Arts, Philosophy  
May 2002

### Current Education

Indiana University School of Medicine, Indianapolis, IN  
Doctor of Medicine  
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### Teaching Experience

Teaching Assistant for Undergraduate Microbiology Laboratory (J210), Fall 2005.  
Director: Dr. Steven Larsen

### Fellowships

Fellow on T32 NIH Hematopoiesis Training Grant, 2004-2008

### Honors

Tutor, IU School of Medicine (2003-2004)  
Graduation with Distinction (2002)  
Member of Phi Kappa Phi National Honors Society (2002)  
Member of Beta Beta Beta Biological Honors Society (2001)  
Emil R. Seidel Scholarship, IPFW (2001-2002)  
Dr. Beaumont S. Cornell Scholarship, IPFW (2001-2002)

### Research Awards

Honorable Mention at Sigma Xi Research Competition, June 2006, Indianapolis, IN.

Best Graduate Student Poster at Indiana University Cancer Center's Cancer Research Day, May 2006, Indianapolis, IN.

## Publications

Campbell, T.B. and H.E. Broxmeyer. (2008) CD26 inhibition and hematopoiesis: a novel approach to enhance transplantation (Review). *Frontiers in Bioscience* 13:1795-805.

Campbell, T.B., G. Hangoc, Y. Liu, K. Pollok and H.E. Broxmeyer. (2007) Inhibition of CD26 in human cord blood CD34<sup>+</sup> cells enhances their engraftment of nonobese diabetic/severe combined immunodeficiency mice. *Stem Cells and Development* 16:347-54.

Li, G., S. Abediankenari, Y.J. Kim, T.B. Campbell, S. Ito, B. Graham-Evans, S. Cooper & H.E. Broxmeyer. (2007) TGF- $\beta$ 1 combined with M-CSF and IL-4 induces generation of immune inhibitory cord blood dendritic cells capable of enhancing cytokine-induced ex-vivo expansion of myeloid progenitors. *Blood* 110(8):2872-9.

Broxmeyer, H.E., G. Hangoc, S. Cooper, T. Campbell, S. Ito & C. Mantel. (2007) AMD3100 and CD26 Modulate Mobilization, Engraftment, and Survival of Hematopoietic Stem and Progenitor Cells Mediated by the SDF-1/CXCL12-CXCR4 Axis. *Ann N Y Acad Sci* 1106:1-19.

Broxmeyer, H.E., C.M. Orschell, D.W. Clapp, G. Hangoc, S. Cooper, P.A. Plett, W.C. Liles, X. Li, B. Graham-Evans, T.B. Campbell, G. Calandra, G. Bridger, D.C. Dale & E.F. Srour. (2005) Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* 201:1307-18.

## Abstracts at Scientific Meetings

"Rheb2 overexpression affects mouse hematopoietic progenitor and stem cell behavior." International Society for Experimental Hematology, Boston, MA, July 2008.

"Overexpression of Rheb2 has diverse effects on hematopoietic progenitor and stem cell functions." American Society for Hematology meeting, Atlanta, GA, December 2007.

"Overexpression of Ras homologue enriched in brain like-1 (RhebL1) leads to positive effects on mouse hematopoietic progenitor cells." American Society for Hematology meeting, Orlando, FL, December 2006.

"Investigating the role of Ras homologue enriched in brain like-1 (RhebL1) in murine hematopoiesis." International Society for Experimental Hematology, Minneapolis, MN, September 2006.

"The role of CD26 in human umbilical cord blood CD34<sup>+</sup> cell engraftment of NOD/SCID mice." IU Cancer Center's Cancer Research Day, Indianapolis, IN, May 2006.

"The role of CD26 in human umbilical cord blood CD34<sup>+</sup> cell engraftment of NOD/SCID mice." American Society for Hematology meeting, Atlanta, GA, December 2005.