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THE TETRASPANIN CD82 REGULATES HEMATOPOIETIC STEM CELL FITNESS AND BONE MARROW RETENTION

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**TETRASPANIN CD82 REGULATES HEMATOPOIETIC
STEM AND PROGENITOR CELL FITNESS AND BONE
MARROW RETENTION**

BY

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B.S., Biology, Chaminade University of Honolulu, 2012

DISSERTATION

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Requirements for the Degree of

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ABSTRACT

The maintenance of the hematopoietic stem and progenitor cell (HSPC) population is critical to sustaining the adult blood and immune system throughout an organism's lifespan. The bone marrow microenvironment plays a key role in the regulation of HSPC maintenance and functions. Previous work from our lab has identified the tetraspanin CD82 as an important modulator of HSPC interactions with the bone marrow niche. However, the mechanisms as to how CD82 contributes to the maintenance, trafficking and retention of HSPCs with the niche remained unclear. First, we investigated how CD82 promotes HSPC quiescence, homing and engraftment using a global CD82 knock out (CD82KO) mouse model. Our data demonstrate that CD82 promotes the maintenance of the long-term HSC (LT-HSC) population within the bone marrow through increased HSC quiescence. Additionally, we demonstrate that CD82KO HSPCs display reduced bone marrow homing and engraftment, identifying a key role for CD82 in these processes. We go on to demonstrate that Rac1 is hyperactivated in the CD82KO HSPCs and inhibition directed to Rac1 restored HSPC homing and migration. While HSPCs primarily reside within the bone marrow microenvironment, they also traffic into the blood under steady state conditions and upon treatment with mobilizing agents. We also identified CD82 as a novel regulator of HSPC mobilization utilizing the CD82KO mouse model. Our data demonstrate that CD82 promotes bone marrow retention, finding increased blood mobilization in the CD82KO mice. Further studies identified the S1PR₁ as a key component of CD82-mediated mobilization. Additionally, intravenous treatment with anti-CD82 antibodies resulted in enhanced HSPC mobilization in animal models. Taken together, these data provide evidence that CD82 is a critical regulator of HSPC quiescence, homing, engraftment and mobilization. More importantly, these studies identify CD82 as a potential novel molecular target to enhance HSPC transplantation therapies for the treatment of hematological and non-hematological disorders.

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Chapter 1 – Introduction

1.1 Hematopoietic stem cells

1.1.1 History of hematopoietic stem cells

Research over the past 70 years has led to significant advances in the field of hematopoietic stem cells (HSCs) resulting in improved treatments for hematological malignancies. The need for hematopoietic research became evident after residents of Hiroshima and Nagasaki were exposed to a continuous low lethal dose of radiation due to the atomic bomb drop in 1945. This exposure led to diseases that researchers now describe as hematopoietic failure. Development of a treatment for hematological failure began in 1949, where Jacobson et al. demonstrated mice exposed to a lethal dose of irradiation, where lead plates shielded the spleen, resulted in increased survival (Jacobson et al., 1949). This group later showed that mice were also protected when the femurs were shielded with a lead plate (Jacobson et al., 1950). Additional studies found that infusion of bone marrow or spleen cell suspensions into lethally irradiated mice also increased survival (Lorenz et al., 1951). These studies suggested that the spleen and bone marrow were critical organs for survival, but the population of cells that were responsible for radioprotection remained unknown. Future, transplantation studies provided evidence that the bone marrow and spleen contained a specialized population of cells that possess self-renewal properties (Barnes and Loutit, 1953; Main and Prehn, 1955). Work by other researchers demonstrated that transplanted bone marrow could be detected within the marrow of recipient mice, which further supported the fact that these specialized cells were responsible for survival (Ford et al., 1956; Nowell et al., 1956). However, it wasn't until 1961 when Till and McCulloch demonstrated through transplant studies that a single cell can have the capacity to self-renew and differentiate into more than one type of blood and immune cell (Till and Mc, 1961). Ultimately, in 1963 Becker, Till and McCulloch, were able to determine that this specialized group of cells with long-term self-renewing properties were hematopoietic stem cells (HSCs) (Becker et al., 1963)

1.1.2 Classification of hematopoietic stem and progenitor cells

Hematopoietic stem and progenitor cells (HSPCs) are a specialized population of cells that have the capacity to self-renew and differentiate to produce all blood and immune cells throughout an organism's life. The HSPC self-renewal and differentiation hierarchy is depicted in Figure 1.1. The hierarchy of HSPC differentiation starts with a long-term hematopoietic stem cell (LT-HSC), which has the capacity to self-renew or differentiate into a short-term hematopoietic stem cell (ST-HSC), which then gives rise to multi-potent progenitors (MPPs). MPPs differentiate into a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), which ultimately give rise to terminally differentiated blood and immune cells toward their restricted lineage type. Each of these cell types will be further described below.

1.1.3 Long-term hematopoietic stem cells

LT-HSCs are the most primitive cells, which have the ability to self-renew and repopulate the blood and immune system throughout an organism's lifespan. Currently, there is not a single surface marker that exclusively identifies LT-HSCs. Rather, a combination of surface markers are used to isolate distinct hematopoietic populations. The combination of surface markers used to identify human hematopoietic stem cells is Lineage-CD34+CD38-CD90+CD45RA- (Weissman and Shizuru, 2008). The first surface marker used to identify LT-HSCs was CD34, which enriches for the HSC population, but still contains other cell types such as lymphocyte progenitors (Civin et al., 1984; Strauss et al., 1986). CD34 is a membrane glycoprotein, which is expressed on approximately 1-4% of bone marrow cells (Saeland et al., 1992). Early studies have shown that CD34+ cells were able to establish hematopoiesis in lethally irradiated baboons (Andrews et al., 1992). In combination with the CD34+ marker, CD90+ and lineage negative (Lin-) markers further purify the LT-HSC population. Lineage negative (Lin-) cells lack surface markers found on terminally differentiated blood and immune cells. In addition, the surface marker CD90 is a GPI-linked glycoprotein that is also expressed on human HSCs (Wisniewski et al., 2011). A study showed that CD34+CD90+Lin- human cells harvested from different

hematopoietic organs had long-term multi-lineage repopulating (LTMR) potential in lethally irradiated SCID-hu mice (Whitlock et al., 1987).

Additional markers include the CD38 antigen which is expressed on >90% of CD34+ cells, however, the CD34+CD38- population has been shown to have multi-lineage reconstitution capabilities after transplantation into an immunodeficient mouse (Bhatia et al., 1997). Moreover, another LT-HSC marker is the surface glycoprotein CD45RA. CD45RA, which is an isoform of CD45, identifies B cells, naïve T cells, common myeloid progenitor (CMP), granulocyte myeloid progenitor (GMP) and myeloid erythroid progenitor (MEP) cells, however has low surface expression on LT-HSCs (Galy et al., 1995; Manz et al., 2002). One study demonstrated that the LT-HSCs identified by the markers, Lineage-CD34+CD38-CD90+CD45RA- had improved long-term engraftment potential when compared to the CD90- population (Majeti et al., 2007). Collectively, these studies provide evidence that human LT-HSCs can be identified by a combination of the following surface markers: Lineage-CD34+CD38-CD90+CD45RA-.

The hematopoietic differentiation lineages for a murine and human model are identical, however, the markers used to identify each population of cells are different. Initial irradiation studies where the bone marrow and spleen were blocked by lead shields, ultimately demonstrating the hematopoietic system was important for survival, were performed in mice. From this time, researchers heavily used animal models (mostly mice) to further investigate the function of hematopoietic stem cells. Therefore, the use of surface markers to identify mouse LT-HSCs and ST-HSCs have been important for the advancement of hematopoietic research. For the purpose of experiments explained in my dissertation, the surface markers used to identify mouse LT-HSCs and ST-HSCs are as follows: LT-HSCs are lineage-Sca1+ckit+CD34-CD135-CD48-CD150+, whereas ST-HSCs are lineage-Sca1+ckit+CD34+CD135-CD48-CD150+ (Weissman and Shizuru, 2008). Like human LT-HSCs, the mouse LT-HSC and ST-HSC populations are also negative for lineage markers. However, mouse HSPCs are commonly referred to as the “KLS” or “LSK” population, which is

negative for lineage and double positive for surface markers sca-1 and c-kit. To further distinguish between the LT-HSC and ST-HSC populations, additive markers are also used: CD34, CD135 and SLAM markers CD48 and CD150 (Kiel et al., 2005; Yilmaz et al., 2006). In contrast to human LT-HSC markers, mouse CD34 expression status is differentially expressed in which LT-HSCs are CD34- and ST-HSCs are CD34+. While the combination of markers described is commonly used to identify mouse LT- and ST-HSCs, researchers are constantly trying to detect additional markers to further purify these cell populations.

1.1.4 Hematopoietic progenitor cells

Hematopoietic progenitor cells lack self-renewal properties but are important for the differentiation of lineage restricted blood and immune cells. The following surface markers are used to identify human hematopoietic progenitors: Lineage-CD34+CD38-CD90-CD45RA- (Weissman and Shizuru, 2008). The human progenitor markers are identical to the LT-HSC population, except CD90 is negatively expressed due to the lack of long-term multi-lineage repopulation (LTMR) properties (Whitlock et al., 1987). Hematopoietic progenitor cell populations still fall within the Lin- population due to the lack of surface expression markers found on terminally differentiated blood and immune cells. These progenitor cells can differentiate into two different lineages of blood and immune cells that follow either a myeloid or lymphoid track (Weissman and Shizuru, 2008). The common myeloid progenitor lineage consists of megakaryocyte-erythroid progenitors (MEPs): which differentiate into erythrocytes and platelets and granulocyte-macrophage progenitors (GMPs): which differentiate into granulocytes, macrophages, and dendritic cells. The common lymphoid progenitor lineage differentiates into dendritic cells, T cells, B cells and natural killer cells. The overall differentiation of these blood and immune cells is tightly regulated in order to maintain homeostatic conditions.

In mice, the following markers are used to identify multipotent progenitors (MPP): Lineage-Sca1+ckit+CD34+CD135+CD48-CD150-. MPPs are part of the LSK population, which also contains LT-HSCs and ST-HSCs. However, to further

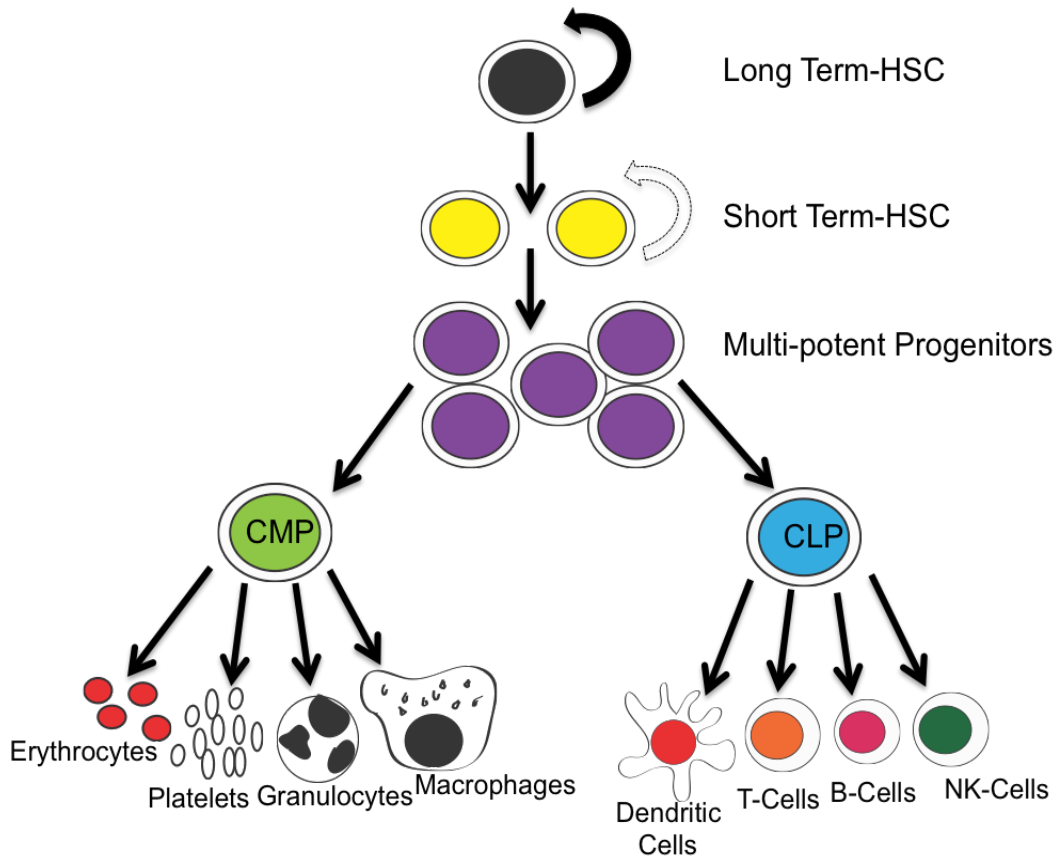


Figure 1.1: Diagram of hematopoiesis. Depicted is the hematopoietic stem and progenitor (HSPC) cell self-renewal and differentiation hierarchy. The HSPC population is collectively made up of the LT-HSCs, ST-HSCs and MPPs. The hematopoietic progenitor population termed HPCs is similar to HSPCs but does not consist of the LT-HSC and ST-HSC populations. Long-term HSCs have the capacity to self-renew or differentiate into short-term HSCs, which then give rise to multipotent progenitor cells. Multipotent progenitor cells then differentiate into common myeloid or common lymphoid progenitors, which then have the capacity to ultimately differentiate into blood and immune cells.

characterize the MPP population, SLAM markers are used. Downstream of MPPs, progenitors differentiate into CMP or CLP lineages, which then give rise to terminally differentiated cells based on their restricted lineage. Murine common myeloid or lymphoid progenitors give rise to the same lineage restricted blood and immune cells as human progenitors described in the section above. Although the markers used to identify murine HSPCs are different from human markers, the use of such surface markers for *in vivo* transplantation studies have been critical to providing a better understanding for the function of HSPCs and improving the efficacy of stem cell transplants for humans.

The expansion of HSPCs *in vitro* remains a major obstacle for researchers. HSPCs can be cultured on feeder cells with a variety of supplements but the quiescent properties are difficult to maintain. Currently, a combination of the surface markers are used to identify each of these specialized populations of HSPCs in culture through using positive and negative selection antibodies and flow cytometry. Additionally, various colony forming unit (CFUs) assays are used to identify different population of progenitors, where the morphology and number of each colony identifies and quantifies progenitors that are present within each population of HSPCs. However, the key functional assays used to identify HSCs depend on *in vivo* studies using mice. Isolated HSCs transplanted into lethally irradiated mice are used to measure the repopulation capacity, in which long-term survival of a mouse indicates the presence of a LT-HSC (Liu et al., 2012a). All it takes is one LT-HSC to engraft into a lethally irradiated mouse in order for repopulation to occur (Abe et al., 2010). Collectively, the identification of LT-HSCs through the use of surface markers has proven to be important for the improvement of bone marrow transplants.

1.1.5 Clinical use of hematopoietic stem cells

HSCs are commonly used in the clinic for treatment of a variety of hematologic and non-hematologic malignancies such as leukemia, lymphoma and neuroblastoma (Hatzimichael and Tuthill, 2010). Hematopoietic stem cell transplants require intravenous administration of autologous, allogenic or

syngeneic HSCs for re-establishment of the hematopoietic system of patients undergoing myeloablative treatment (Copelan, 2006). These transplantation methods each have benefits and weaknesses but the treatment regime heavily depends on the patient's age and disease. Autologous transplantation occurs by transplanting HSCs from patient's own bone marrow or blood to treat self. Autologous HSCs are usually harvested when the patient is in remission or in a state of low minimal residual disease. But, the risk of harvested HSCs containing malignant cells still remains. One benefit of autologous transplants is bypassing the likelihood of engrafted cells subsequently attacking the host tissues due to contaminating T lymphocytes, known as graft-versus-host disease (Ferrara et al., 1999). Although the patient is protected from graft-versus-host disease with autologous transplantation, the benefit of allogenic transplantation is the graft-versus-leukemia or graft-versus-tumor response. The graft-versus-leukemia or graft-versus-tumor response is beneficial because transplanted cells are able to recognize and attack malignant cells, which is important for achieving and maintaining remission (Kolb, 2008; Porter, 2011). To get around this issue, allogenic transplantation occurs by transplanting harvested HSCs from one individual (not genetically identical) to treat another individual. For a successful transplantation to occur, the patient and donor must have a close match between 6-10 major human leukocyte antigens (HLA) markers expressed on white blood cells (Anasetti et al., 2001). An ideal donor would match all HLA markers of the host because the more diverse the donor and host are, the higher the chance for graft-versus-host disease. The third type of transplantation is syngeneic transplantation, which occurs by transplanting HSCs from an identical twin (Fefer et al., 1986). This type of transplantation is rare, but very similar to an autologous transplant in which graft-versus-host disease does not occur in addition to graft-versus-leukemia or graft-versus-tumor. The benefit of syngeneic transplantation is the absence of donor malignant cells, which is a huge caveat of autologous transplants. Additionally, the population of transplanted cells can be increased since the donor is healthy and has not undergone any myeloablative treatment.

HSCs used for transplants are harvested from three different sources:

bone marrow, peripheral blood and umbilical cord blood for transplantation (Hatzimichael and Tuthill, 2010). Historically, bone marrow is the primary source for collecting a large volume of HSCs. Bone marrow is collected using a combination of large bore needles and heparinized syringes, which are punctured into the posterior iliac crest or sternum. The ideal amount of transplanted marrow is about $1-2 \times 10^8$ /kg nucleated cells in order to establish long-term engraftment (Bahceci et al., 2000). After collection, the marrow is filtered to remove any debris or clots before intravenous injection into the recipient. More recently, the use of peripheral blood stem cells (PBSCs) for transplantations has become a favored option. Unlike bone marrow, PBSCs harvest is significantly less laborious and invasive for the patient. A donor is treated for about a week with a mobilizing drug, granulocyte colony-stimulating factor (G-CSF) or AMD3100 to allow HSCs within the bone marrow to be released into the peripheral blood (Bensinger et al., 1995; Schmitz et al., 1995). However, due to low numbers/counts of circulating PBSCs, a donor must often undergo a few rounds of apheresis to obtain enough cells. Apheresis is a process by which circulating stem cells are removed and filtered from the blood via a centrifugation-based machine. Evidence shows that infused PBSCs engraft much quicker than bone marrow derived cells which could be due to higher numbers of CD34+ cells and lymphoid progenitors (Korbling et al., 1995). The third source of transplantable stem cells can be enriched from umbilical cord blood. The advantage of cord blood transplant is the increased enrichment of HSCs compared to other sources. Since cord blood tissue is relatively naïve, the chances of GVHD by the resident immature immune cells are decreased, which is beneficial for recipients who lack a suitable donor. Currently the major problems with cord blood transplants are: poor engraftment, high non-relapse mortality and poor survival (Rocha et al., 2009). In addition, other drawbacks include limited amounts of tissue available and the high cost for this type of transplant (Ballen, 2017).

At this time, HSCT is an active research area with continuous new advances being made to improve the efficacy of harvest and transplantation

methods. The use of HSCT for the treatment of hematological malignancies is quite successful with only about 4% of patients dying within the first 100 days of transplantation (Gribben et al., 2005). Although HSCs are commonly used to treat various hematological disorders and solid tumors, two main issues remain: 1) HSCs are limited in number when harvested from a donor and 2) the likelihood of HSCs effectively engrafting into the recipient bone marrow to restore hematopoiesis is low. These issues are currently being researched in order to identify key molecules and methods to improve the efficacy of HSC harvest and transplants, which will ultimately improve the life of many individuals suffering from non-hematological and hematological diseases.

1.2 Hematopoietic stem cell: self-renewal and differentiation

1.2.1 Asymmetric vs. symmetric divisions

Within the self-renewal and differentiation process, HSCs undergo either asymmetric or symmetric cell division (Ho, 2005). Asymmetric cell division occurs when a cell produces a daughter cell that retains intrinsic stem cell properties and the other initiates differentiation (Caocci et al., 2017). This process is critical for the maintenance of long-term hematopoiesis especially in the event of a hematopoietic stem cell transplant to replenish the bone marrow after myeloblastic treatment. Both murine and human hematopoietic progenitor cells were shown to undergo asymmetric divisions of about 20% from a single progenitor cell (Leary et al., 1984; Leary et al., 1985). In an independent approach, human cord blood HSCs were sorted and cultured into single cell suspensions to determine the cell division fate. Under the described culture conditions, asymmetric division was confirmed from a single cell in which differentiation into another cell type was detected (Mayani et al., 1993). Using a time-lapse camera system to monitor the replicative capacity of human HSCs, one group demonstrated asymmetric divisions of CD34+ HSCs. They demonstrated one daughter cell remained quiescent or divided very slowly while the other multiplied quickly into progenitors and terminally differentiated cells (Huang et al., 1999). HSCs are known to divide slowly, whereas differentiating

cells undergo rapid proliferation. Interestingly, asymmetric division is more frequent among CD34+CD38- cells, compared to CD34+CD38+ cells suggesting that some populations have a greater replicative capacity (Huang et al., 1999). The mechanism by which HSCs undergo self-renewal or differentiation commitment is still unclear. Studies measuring the mitotic index and colony formation of HSC division demonstrate that extracellular soluble molecules do not influence asymmetric division. However, surface expression of CD53, CD63, CD133, CD71, CD62L and CD34 was detected on HSCs that undergoing asymmetric division (Beckmann et al., 2007; Giebel and Beckmann, 2007). The expression of these surface proteins could influence the regulation of asymmetric division in HSCs (Beckmann et al., 2007; Giebel and Beckmann, 2007).

Symmetric cell division occurs when a cell produces two daughter cells that retain intrinsic stem cell or differentiation properties (Ho, 2005). This process is important for HSC self-renewal in order to maintain homeostasis of the primitive HSC pool. In the context of HSC transplantation, HSCs undergo symmetric division in order to replenish the depleted malignant hematopoietic system (Keller, 1992). The symmetric division of primitive HSCs is also important to maintain the rare pool of HSCs that contain limitless self-renewal properties (Ho, 2005).

HSC self-renewal in asymmetric and symmetric divisions is tightly regulated through intrinsic and extrinsic signaling for the maintenance and reconstitution of the stem cell pool. The self-renewal of HSCs is regulated by a variety of signaling pathways such as Notch, Wnt, bone morphogenetic protein (BMP), mTOR and Hedgehog (Bhardwaj et al., 2001; Butler et al., 2010; Karlsson et al., 2007; Reya et al., 2003). The activation of these signaling pathways results in the up-regulation of self-renewal genes such as β -Catenin, SMAD and STAT3/5 (Zon, 2008), which is critical for the maintenance of the HSC pool. The balance between asymmetric and symmetric division is important for HSC pool maintenance. Nevertheless, over time and with increased age the HSC pool becomes depleted due to aberrant activation of HSCs often leading to downstream hematological pathologies (Rossi et al., 2005). HSC aging is defined

by a decrease in HSC self-renewal, impaired bone marrow homing and engraftment and skewing of myeloid lineage differentiation (Warren and Rossi, 2009). This decline is due to a decrease in red blood cell production and often leads to an increase in myeloid cell differentiation (Rossi et al., 2008). Similar to humans, HSCs from old mice display decreased bone marrow homing and engraftment compared to HSCs from young mice (Morrison et al., 1996). The decline in homing and engraftment in mice is due to an increase in cycling of the old HSCs compared to the less active young HSCs. Another hallmark of HSC aging is myeloid skewing, which is defined as an increase in myeloid production and a decrease in lymphoid differentiation (Elias et al., 2017). A global gene profile of LT-HSCs harvested from young and old mice determined that lymphoid specific fate genes were down regulated and myeloid differentiation genes were up regulated in aged mice (Rossi et al., 2008). Myeloid skewing is not only influenced by intrinsic factors, but also extrinsic cues from the bone marrow microenvironment. For example, one study demonstrated that HSC localization within the bone marrow microenvironment can dictate lineage fate (Pinho et al., 2018). This study demonstrated that HSCs in contact with megakaryocytes resulted in myeloid bias, whereas, arteriole localization resulted in lymphoid bias. Collectively, these studies provide evidence that HSC self-renewal and differentiation processes are greatly influenced by age and a combination of intrinsic and extrinsic factors.

1.3 Intrinsic regulation of hematopoietic stem cells

1.3.1 Introduction to intrinsic signals

HSCs are regulated by intrinsic signals that promote sustained self-renewal and differentiation processes. Blood contains the highest turnover rate in the body with the daily production of 10^{11} - 10^{12} new blood cells in a healthy individual (Lamprea et al., 2017). Two signaling pathways that regulate HSC self-renewal and differentiation are the Notch and Wnt/ β -catenin signaling pathways.

1.3.2 Notch signaling

Notch is a highly conserved signaling pathway that functions to regulate HSC self-renewal and fate determination (Bigas and Espinosa, 2012; Kopan and Ilagan, 2009). Notch is heavily characterized for its role in T cell activation and differentiation, however, its role in HSCs is less clear due to conflicting studies (Bigas and Espinosa, 2012; Calvi et al., 2003; Karanu et al., 2000; Mancini et al., 2005; Varnum-Finney et al., 2011). Notch is a transmembrane protein that is activated through ligand-mediated interaction from cell-to-cell contact (Lampreaia et al., 2017). The receptor and ligand engagement results in two cleavage processes mediated by first the metalloproteinase, TACE, and second by the γ -secretase complex and APH1 to form the intracellular Notch receptor domain (N-ICD). The N-ICD domain translocates to the nucleus to bind to the DNA binding protein, RBP-J, which ultimately induces gene expression changes in HSC regulatory genes. Notch ligands and receptors have been identified in the bone marrow and on HSPCs (Calvi et al., 2003; Duncan et al., 2005; Milner et al., 1994). Studies have shown that a constitutively active form of Notch1 N-ICD results in increased human HSPC self-renewal capacity (Carlesso et al., 1999). In addition, *in vitro* exposure of primitive HSCs to Notch ligands promotes self-renewal (Calvi et al., 2003; Karanu et al., 2000; Varnum-Finney et al., 2003). *Itch* is an E3 ligase that negatively regulates Notch Signaling by inducing Notch receptor degradation (Rathinam et al., 2011). *In vivo* studies show that mice transplanted with *Itch*-deficient HSPCs resulted in expansion of the stem cell pool. These data further suggest that Notch signaling plays a role in HSC maintenance. In contrast, the deletion of the Notch ligand Jagged and Notch1 receptor in the bone marrow had no effect on hematopoietic pool (Mancini et al., 2005). Similarly, the loss of either Notch 1 or Notch 2 had no effect on HSC number in mice (Varnum-Finney et al., 2011). These conflicting results suggest that Notch signaling may be important for HSC self-renewal and maintenance, but other factors are likely to be involved and further studies are required.

1.3.3 Wnt/ β -catenin signaling

The Wnt/ β -catenin signaling pathway is evolutionarily conserved and important for HSC self-renewal and differentiation (Angers and Moon, 2009). Wnt proteins are secreted glycoproteins that bind to the N-terminal domain of the G-protein coupled receptor, Frizzled (Komiya and Habas, 2008). The Wnt signaling pathway is activated upon Wnt binding to the Frizzled receptor complex with low-density lipoprotein co-receptors. Upon activation, the cytoplasmic phosphoprotein Dishevelled forms a protein complex with GSK-3, Axin, APC and Ck1, which results in the accumulation of β -catenin within the cytoplasm. β -catenin undergoes nuclear translocation to the cytoplasm to bind the transcription factors LEF/TCF and activate gene expression of target genes. Wnt signaling is important for regulating the hematopoietic system during the fetal and adult stages of development (Bigas et al., 2013; Lento et al., 2013). Both the ligand and receptors of the Wnt/ β -catenin pathway have been confirmed in the bone marrow and on HSCs (Van Den Berg et al., 1998). Multiple studies have demonstrated the importance of Wnt/ β -catenin signaling in maintaining the HSC stem cell pool. Mouse HSPCs transduced with constitutively active β -catenin resulted in an increase in self-renewal and differentiation (Reya et al., 2003). In addition, activation of β -catenin via GSK-3 β inhibitors resulted in HSC expansion (Trowbridge et al., 2006). Together, these studies provide evidence that the Wnt/ β -catenin pathway is critical for the maintenance and expansion of the HSC pool.

1.4 Extrinsic regulation of hematopoietic stem cells

1.4.1 Bone marrow microenvironment

Extrinsic regulation of HSCs occurs within the bone marrow microenvironment through a combination of cell-cell interactions from direct contact or autocrine or paracrine signaling (Lin et al., 2015). Schofield hypothesized in 1978 that stem cell behavior is determined by the types of cells it interacts with (Schofield, 1978). HSCs primarily reside in the bone marrow, but can also be found in other hematopoietic organs such as the blood, spleen, and

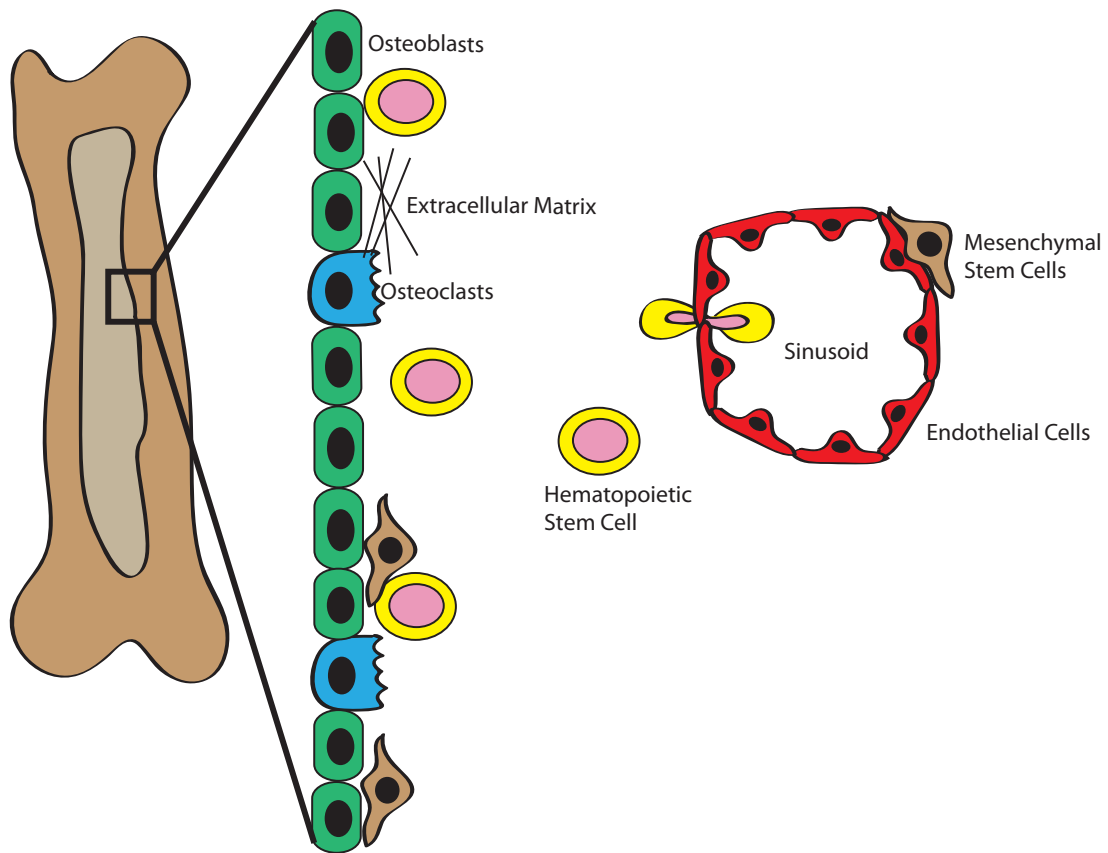


Figure 1.2: Components of the hematopoietic stem cell niche. The bone marrow microenvironment is also termed an endosteal niche. The main cellular components of this niche consist of osteoblasts, osteoclasts and extracellular matrix, which maintains the HSC population through extrinsic stimuli. In addition, the vasculature niche, also known as the sinusoid, is made up of endothelial cells. HSCs are able to migrate between the endosteal and vasculature niche by extravasating through endothelial cells.

liver. The bone marrow is a complex microenvironment that consists of different cellular components to regulate HSC function and maintenance, including osteoblasts, osteoclasts, stromal cells, mesenchymal stem cells (MSCs), adipocytes and extracellular matrix (ECM) (Morrison and Scadden, 2014). The different components of the bone marrow microenvironment are depicted in Figure 1.2. Currently, it is believed that immature HSCs primarily reside within the endosteal region, which is the inner surface of the long bone that is enriched in mature osteoblasts (Balduino et al., 2005; Balduino et al., 2012; Nilsson et al., 2005; Taichman et al., 2010). Early studies demonstrated that human CD34+ hematopoietic progenitor proliferation is stimulated by secretion of granulocyte colony stimulating factor (G-CSF) by osteoblasts (Taichman et al., 2010). The importance of osteoblasts for HSPC maintenance has been demonstrated through a variety of *in vivo* studies. One key study using a transgenic mouse engineered to increase osteoblast number demonstrated an increased percentage of Lin-sca-1+c-kit+ (LSK) cells within the bone marrow compared to control mice (Calvi et al., 2003). Additionally, another group showed that increased spindle-shaped N-cadherin+CD45- osteoblasts (SNO) cells resulted in increased numbers of long-term HSCs *in vivo* (Zhang et al., 2003), which further supports a role for osteoblasts in HSC maintenance. Another study ablated bone marrow osteoblasts, which resulted in a 3 to 10-fold decrease in HSPC number due to reduced bone marrow cellularity (Visnjic et al., 2004). This reduction in bone marrow cellularity resulted in an increase in extramedullary hematopoiesis in the spleen. Conversely, osteoblastic activation increased bone marrow cellularity, which reduced HSC activity and function (Schepers et al., 2012). Collectively, these studies provide evidence that osteoblastic homeostasis within the bone marrow is important for HSC bone marrow retention and activity.

Mesenchymal stem cells are multipotent stromal cells in the bone marrow that provide additional HSC support (Smith and Calvi, 2013). Mesenchymal stem cells are a heterogenous population of cells that can give rise to the osteogenic lineage. These cells reside perivascularly but traffic to the endosteal surface of the bone to differentiate into osteoblasts (Morrison and Scadden, 2014). One

study confirmed increased localization of nestin GFP+ mesenchymal stem cells around blood vessels throughout the bone marrow by immunostaining of mouse femoral sections (Mendez-Ferrer et al., 2010). Mesenchymal stem cells were also shown to be physically associated with HSCs using the same method (Mendez-Ferrer et al., 2010). In addition, the co-transplantation of mesenchymal stem cells with HSPCs increased bone marrow engraftment and enhanced self-renewal (Ahn et al., 2010; Masuda et al., 2009). These data provide evidence that mesenchymal stem cells are important regulators of HSC function.

In addition to the endosteal/osteoblastic niche, the vascular niche, shown by the sinusoid in Figure 1.2 has been shown to play an equally important role in HSC regulation. The bone marrow is highly vascularized and HSCs can be found adjacent to the vasculature (Kiel et al., 2005). Endothelial cells function to promote HSC maintenance within the bone marrow (Morrison and Scadden, 2014). Studies demonstrate that endothelial cells with nuclear β -catenin are located adjacent to HSCs and are important for the emergence of HSCs (Ruiz-Herguido et al., 2012). In addition, *in vitro* culture of endothelial cells with HSCs promoted long-term reconstituting HSC expansion in culture (Cardier and Barbera-Guillem, 1997; Ohneda et al., 1998). The ablation of endothelial cells with the use of an anti-VE-cadherin antibody resulted in hematopoietic failure *in vivo* (Avecilla et al., 2004). Collectively, these studies demonstrate the importance of the vasculature for the maintenance of the HSC pool.

The extracellular matrix (ECM) within the bone marrow acts as a supportive tissue for the maintenance of HSCs (Discher et al., 2009). ECM creates a dynamic and complex environment that regulates HSC behavior. Adhesion of HSCs to ECM inhibits proliferation and prevents apoptosis, which results in long-term survival of quiescent HSCs (Krause, 2002). The bone marrow microenvironment is thought to be comprised of collagen VI, collagenIV, fibronectin, laminin and tenascin-C (Klein et al., 1993; Klein et al., 1995; Nilsson et al., 1998). Integrins on the plasma membrane of HSCs are key receptors that mediate ECM interactions within the bone marrow microenvironment. Integrins are heterodimeric proteins that mediate HSC adhesion, migration and

downstream signaling for HSCs (Legate et al., 2009). Evidence shows that integrins inhibit cell proliferation through the activation of cell cycle inhibitors, p21 and p27 (Cheng et al., 2000a; Cheng et al., 2000b). Therefore, the adhesion of HSCs to ECM via integrins is thought to be important for HSC quiescence.

Adipocytes have also been found to be important for HSC maintenance within the bone marrow (Anthony and Link, 2014). Adipocyte number within the bone marrow increases with age, which directly influences the number and function of HSCs within the bone marrow (Naveiras et al., 2009). A study showed that mice with adipocyte rich bone marrow had decreased HSC numbers compared to controls with low adipose content (Naveiras et al., 2009)). Therefore, these studies demonstrate that adipocytes can play an inhibitory role in HSC activity. When taken together, the diverse components of the bone marrow microenvironment are critical regulators of HSC maintenance and function.

1.5 Hematopoietic stem cell quiescence

1.5.1 Introduction to hematopoietic stem cell quiescence

HSCs primarily reside within the bone marrow microenvironment in a quiescent state. It is thought that quiescence or slowly cycling is necessary to maintain primitive HSCs, in which a high cycling state is thought to be important for the effective expansion of progenitor populations (Pietras et al., 2011). About 20-30% of the HSC population is within the quiescent phase of the cell cycle, which means this population only cycles once every 150-200 days (Foudi et al., 2009; Wilson et al., 2008). In contrast, more actively cycling hematopoietic progenitor populations cycle once every 20-30 days (Foudi et al., 2009; Wilson et al., 2008). In mice, about 90% of LT-HSCs remain within the G0 phase of the cell cycle, with only 6% of this population entering into the active cycling phases each day (Kiel et al., 2007a). HSC quiescence is not only important for protecting the stem cell pool from mutations accumulated via active cycling, but also for sustaining the HSC pool (Li, 2011). However, when quiescence is disrupted, the HSC pool can undergo premature exhaustion, which can eventually lead to

hematopoietic failure (Cheng et al., 2000b; Wilson and Trumpp, 2006). In the event of an infection or blood loss, the demand of hematopoiesis increases, which results in HSCs cycling out of a quiescent state from inflammatory cytokine exposure (Baldrige et al., 2010; Essers et al., 2009; Morrison et al., 1997). HSC quiescence in the bone marrow microenvironment is regulated by both extrinsic and intrinsic mechanisms. The key players that initiate and regulate HSC quiescence will be explained within this section.

1.5.2 Regulation of hematopoietic stem cell quiescence

The osteoblastic niche is critical for extrinsic regulation of HSCs through the promotion of c-kit, Tie2/Ang-1, and TPO/MPL signaling (Li, 2011). Osteoblasts secrete factors such as stem cell factor (SCF), angiopoietin (Ang-1) and thrombopoietin (TPO) to mediate the interaction between HSCs and the bone marrow microenvironment to promote HSC quiescence (Czechowicz et al., 2007; Thoren et al., 2008). The disruption of the interaction between c-kit receptor on HSCs and its ligand, stem cell factor (SCF) on osteoblasts resulted in a decrease in HSC quiescence (Kiel et al., 2007a). In addition, Tie2/Ang-1 signaling mediates HSC quiescence through the activation of the PI3K/AKT signaling pathway that results in increased gene expression of cell cycle inhibitor, p21 (Visnjic et al., 2004). Moreover, TPO and the myeloproliferative leukemia virus proto-oncogene (MPL) signaling promotes HSC quiescence through an increase in β 1 integrin-mediated adhesion to osteoblasts (Yoshihara et al., 2007). As before mentioned, HSC quiescence is promoted through integrin mediated HSC adhesion to ECM by activating gene expression of cell cycle inhibitors, p21 and p27 (Cheng et al., 2000a; Cheng et al., 2000b; Legate et al., 2009) and mediated by the Wnt/ β -catenin and Notch signaling pathways (Described in Section 1.3).

Molecules that mediate the interaction between osteoblasts and HSCs to promote quiescence are N-cadherin and TGF- β (Haug et al., 2008; Li, 2011; Li and Zon, 2010; Sitnicka et al., 1996). N-Cadherin and integrin β 1 are both targets of Tie2/Ang-1 and TPO/MPL signaling, promoting HSC quiescence (Li, 2011). Overexpression of N-cadherin in HSPCs induced slow cell division, which

protected the HSPCs from myelosuppression (Hosokawa et al., 2010a). Conversely, the knock down of N-cadherin using shRNA resulted in reduced bone marrow lodgment and adhesion of HSPCs *in vivo*, which significantly reduced long term engraftment (Hosokawa et al., 2010b). These findings prompted the authors to conclude that the inhibition of N-cadherin may affect the ability of HSPCs to effectively adhere to the bone marrow microenvironment to maintain quiescence. However, the role of N-cadherin in regulating HSC function still remains controversial; the basis being that deletion of N-cadherin in osteoblasts does not affect HSC numbers or function, which suggests N-cadherin is not important for the regulation of HSCs (Bromberg et al., 2012; Greenbaum et al., 2012).

TGF- β is described as a potent inhibitor of HSC growth and critical regulator of HSC quiescence (Sitnicka et al., 1996). TGF- β is thought to mediate HSC quiescence through the increased expression of cell cycle inhibitors, p21 and p57 (Cheng et al., 2001; Dao et al., 1998), further illustrating the importance of HSC quiescence and regulation of cell cycle-mediated transcription factors and inhibitors. In addition, there are other transcription factors that mediate HSPC quiescence such as, Gfi1, which is a zinc finger transcription repressor that mediates HSC quiescence through the up regulation of p21 (Hock et al., 2004; Zeng et al., 2004). In addition, the transcription factor pre-B cell acute lymphoblastic leukemia (Pbx1) is preferentially expressed in LT-HSC population, promoting quiescence via the TGF- β pathway (Ficara et al., 2008). Moreover, LT-HSCs also express high levels of p53, which in combination with p21, results in increased HSC quiescence (Dumble et al., 2007; Lacorazza et al., 2002). As mentioned before, cell cycle inhibitors also play key roles in the regulation of cell cycle maintenance of HSCs. The up-regulation of cell cycle inhibitors, such as p21, p57 and p27, results in a decrease in cell-cycle activation, which promotes HSC quiescence. The cell cycle inhibitor, p21 is a regulator of the G1 checkpoint. The absence of p21 in mice led to increased HSC numbers due to enhanced HSC proliferation (Zhang et al., 2003; Zhang et al., 1997). In addition, the loss of p21 in mice also impaired HSC transplantation due to impaired self-renewal

potential (Zhang et al., 2003). Furthermore, the cell cycle inhibitor p57 is also important for regulation of cell cycle dynamics. TGF- β mediates the up regulation of p57 expression, promoting cell cycle arrest within HSCs (Scandura et al., 2004) Mouse studies demonstrated that p57 and p27 null HSCs had reduced engraftment capacity and increased proliferation (Scandura et al., 2004; Zou et al., 2011). Together, the transcription factors and cell cycle inhibitors described are important for the regulation of HSC quiescence within the bone marrow microenvironment.

1.6 Hematopoietic stem cell fitness

1.6.1 Introduction to hematopoietic stem cell fitness

Hematopoietic stem cell fitness is dictated by the combined ability of HSCs to migrate, adhere and self-renew, differentiate and maintain quiescence within the bone marrow microenvironment (Heazlewood et al., 2014). HSC fitness is a multi-step process that requires a combination of adhesion and signaling molecules to facilitate a successful engraftment for the restoration of long-term hematopoiesis. HSC homing is the first step in this process, which is regulated by the chemokine CXCR4/CXCL12 signaling axis. In addition, Rho-related small GTPases such as Rac1 have been shown to play a role in regulating HSPC adhesion, migration and homing (Chen et al., 2016; Gu et al., 2003; Liu et al., 2011b; Yang et al., 2001). These homing molecules are important for engraftment of HSCs within the bone marrow to establish interactions with surrounding supportive cells. The interactions between HSCs and the bone marrow microenvironment is necessary for the establishment of LT-HSCs to repopulate blood and immune cells.

1.6.2 Hematopoietic stem cell homing

The active process by which HSCs migrate towards the bone marrow from the vasculature is defined as homing. The homing process also occurs under normal physiological conditions in which HSCs circulate at low levels within the vasculature (Lapidot et al., 2005). HSC homing is a dynamic process that involves complex communication of HSCs between chemokines, chemokine

receptors, and adhesion molecules (Caocci et al., 2017). The role of the chemokine receptor CXCR4 and the corresponding ligand, CXCL12, in HSC homing will be described more in depth in the next section labeled “CXCR4/CXCL12 signaling”. In addition to chemokine signaling, adhesion molecules such as integrin complexes ($\alpha4\beta1$ (VLA-4) and $\alpha6\beta1$) and selectins (E-endothelial selectin and P-endothelial selectin) not only facilitate adhesion within the bone marrow, but also mediate rolling and tethering of HSCs within the blood vessels to enable trans-endothelial migration (Nabors et al., 2013). Other molecules that mediate the interaction between HSCs and the endothelium to facilitate homing are intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM- 1) (Frenette et al., 1998; Mazo et al., 1998). In combination, these molecules are critical for effective bone marrow homing which ultimately results in successful HSC engraftment

1.6.2.1 CXCR4/CXCL12 signaling

The primary homing receptor for HSCs is the chemokine receptor, CXCR4, which is a G_i -coupled protein receptor highly expressed on the surface of HSCs. The ligand for CXCR4, CXCL12, is highly expressed and secreted by stromal cells (expressed by osteoblasts and endothelial cells) within the bone marrow microenvironment, thereby promoting chemotaxis of HSCs to the bone marrow. Intracellular GTPases have been shown to promote HSC chemotaxis through the activation of CXCR4 upon CXCL12 ligand binding (Marchese and Benovic, 2001; Papayannopoulou et al., 2003). Downstream signaling of the CXCR4 receptor leads to the activation of ERK, JAK/STAT and MAPK pathways (Roland et al., 2003). The interaction between CXCR4-CXCL12 regulates not only HSC homing, but also promotes HSC quiescence through the inhibition of cell cycle progression, which is critical for HSC maintenance (Nie et al., 2008). The importance of CXCL12 and CXCR4 for HSC homing was identified using a combination of knock out mouse models and transplant studies. HSPCs isolated from a CXCL12KO mouse showed a significant decrease in total bone marrow engraftment in a competitive repopulation study (Tzeng et al., 2011). Whereas, the loss of CXCR4 on HSPCs led to a significant decrease in bone marrow

homing (Nie et al., 2008). Interestingly, bone marrow engraftment was rescued with the re-expression of CXCR4, providing evidence that CXCL12/CXCR4 signaling axis is critical for HSC homing and bone marrow maintenance.

1.6.2.2 Rac 1 and hematopoietic stem and progenitor cells

Additional signaling stimulated downstream of CXCR4 includes the Rho family of GTPases. GTPases act as molecular switches that cycle between a GTP (active) and GDP (inactive) state. Upon activation through extracellular signals, Rac1 converts from its active form via guanine nucleotide exchange factors (GEFs) and to its inactive form via GTPase-activating proteins (GAPs) (Bosco et al., 2009). Rac1 binding to the effector protein p21 activating kinase (PAK) leads to the stimulation of downstream processes such as cytoskeletal rearrangements, which further impacts cellular behaviors such as migration and adhesion (Ridley, 2001).

Among the family of Rho GTPases, Rac1 is known to play a clear role in HSPC migration and homing (Cancelas et al., 2005; Dorrance et al., 2013; Gu et al., 2003; Liu et al., 2011b; Shang et al., 2011; Williams et al., 2008; Yang et al., 2001). Rac1 has been shown to associate with CXCR4, in which inhibition of Rac1 induces a conformational change of CXCR4 resulting in blocked receptor internalization and impaired CXCL12 activation (Zoughlami et al., 2012). Using a conditional Rac1KO mouse both resulted in impaired HSPC engraftment and decreased adhesion to fibronectin (Gu et al., 2003). Interestingly, the deletion of both Rac1 and Rac2 resulted in a significant increase in CXCR4 expression, but migration to CXCL12 was decreased (Gu et al., 2003). These studies show the importance of Rac1 for HSPC homing and engraftment using KO studies. The dynamic process of HSPC circulation is also regulated by GTPase activity, which mediates the tethering of HSPCs within the blood vessels to enable trans-endothelial migration (Mazo and von Andrian, 1999; Sahin and Buitenhuis, 2012). Rac1KO in HSCs resulted in a defect in long-term engraftment due to a decrease in bone marrow homing (Cancelas et al., 2005). One study demonstrated that endogenous Rac1 hyperactivation could decrease HSPC bone marrow homing through the manipulation of R-Ras expression (Shang et

al., 2011). The collective studies suggest Rac1 expression and activity are important for the tight control of HSPC homing and engraftment.

1.6.3 Hematopoietic stem cell repopulation

HSC repopulation, also termed engraftment, is a critical step in hematopoietic stem cell transplants. Similar to the HSC homing process, HSC repopulation also requires a combination of signals from adhesion molecules to mediate interactions between HSCs and the bone marrow microenvironment (Mazo and von Andrian, 1999; Sahin and Buitenhuis, 2012). The cellular components within the bone marrow microenvironment, such as osteoblasts and endothelial cells, mediate the proper signals needed to promote engraftment and downstream hematopoiesis (Anthony and Link, 2014). The expression of surface markers can also influence engraftment potential of HSCs. HSCs that were CD34⁺ had greater engraftment potential compared to CD34⁻ HSCs when transplanted in a lethally irradiated NOD/SCID mouse (Gao et al., 2001). Molecular analysis comparing the expression of adhesion and homing genes associated with HSPC homing showed an increase in VLA-4 and VLA-5 in the Lin-CD34⁺ fraction compared to the Lin-CD34⁻ fraction (Manfredini et al., 2005). These data suggest that integrins on CD34⁺ cells have a role in increasing engraftment potential compared to CD34⁻ cells. However, additional adhesion molecules used to identify primitive HSCs maybe involved with long-term repopulation potential and would be beneficial to further increase engraftment.

1.6.4 Hematopoietic stem cell adhesion

The molecules that facilitate HSC maintenance within the bone marrow and vasculature consist of adhesion molecules such as integrins, selectins and cadherins. These adhesion molecules facilitate HSC homing and engraftment in order to re-establish hematopoiesis. Integrin complexes, $\alpha 4\beta 1$ (VLA-4) and $\alpha L\beta 2$ (LFA-1) play an important role in HSC adhesion to the vasculature to aide in trans-endothelial migration (Peled et al., 2000). Additionally, integrin complexes $\alpha 4\beta 1$ (VLA-4) and $\alpha 6\beta 1$, are important for homing of HSCs to the bone marrow and spleen (Papayannopoulou et al., 1995; Qian et al., 2006). Moreover,

adhesion and chemotaxis of CD34+ HPCs on fibronectin were found to be mediated by $\alpha 5\beta 1$ (Carstanjen et al., 2005).

Selectins play an important role in rolling and tethering of HSCs within the vasculature to mediate trans-endothelial migration. There are three different selectins: P-selectin, E-selectin and L-selectin, in which only P- and E-selectin have been shown to have a role in mediating HSC homing. *In vitro* studies, demonstrated that CD34+ human cells rolling was induced on surfaces coated with P- and E- selectins, but not L-selectin (Xia et al., 2004). *In vivo* transplantation studies further confirmed that bone marrow engraftment of HSPCs were dependent on P- and E- selectins (Frenette et al., 1998).

The cadherins, N-cadherin and VE-cadherin, are mediators of adhesion within the vasculature and bone marrow compartment. Interestingly, the inhibition of VE-cadherin on endothelial cells increase CD34+ HPC trans-endothelial migration (van Buul et al., 2002). This suggests VE-cadherin also mediates endothelial cell permeability to allow HPCs to traffic into the bone marrow. N-cadherin has been shown to mediate the interactions of HSCs with osteoblasts within the bone marrow (Kiel et al., 2007b; Zhang et al., 2003). However, a few *in vivo* studies show that deletion of N-cadherin in osteoblasts does not alter HSC activity, therefore, the role of N-cadherin still remains controversial (Bromberg et al., 2012; Greenbaum et al., 2012).

1.7 Hematopoietic stem cell mobilization

1.7.1 Introduction to hematopoietic stem cell mobilization

Mobilization is the active process HSCs undergo to migrate from the bone marrow into the peripheral blood (Mohty and Ho, 2011). Under physiological conditions, HSCs circulate at low levels within the vasculature to survey for infection or injury (Massberg et al., 2007). The nervous system regulates circadian mobilization, which results in oscillated release of HSCs throughout the day (Mendez-Ferrer et al., 2009). It is well known that HSC bone marrow retention and mobilization are mediated by CXCR4 and CXCL12. In addition, the bioactive lipid receptor, sphingosine-1-phosphate receptor (S1PR) promotes

HSC trafficking in combination with CXCR4 signaling. Within the clinic, HSC mobilization is often the chosen method used to isolate HSCs for transplants. For a recent period (January 1, 2015 to Decemeber 31, 2016), The Seattle Cancer Care Alliance at the Fred Hutchinson Cancer Research Center reported that the majority of transplants were performed with stem cells taken from the peripheral blood (<https://bloodcell.transplant.hrsa.gov/>). Mobilizing drugs such as AMD3100 and granulocyte-colony stimulating factor (G-CSF) are used individually or in combination to mobilize HSCs into the peripheral blood, with G-CSF being the most commonly used for transplantation.

1.7.2 Molecules that regulate hematopoietic stem cell mobilization

1.7.2.1 CXCR4/CXCL12 signaling

The interaction between CXCR4 expressed on HSCs and CXCL12 expressed in the bone marrow microenvironment promotes HSC retention. CXCR4 expression on HSCs promotes quiescence within the bone marrow critical for HSC maintenance (Nie et al., 2008). As previously described, the CXCR4 receptor has also been shown be an important regulator of HSC homing and engraftment. However, CXCR4 signaling has been shown to play an equally important role in HSC mobilization (Nie et al., 2008; Tzeng et al., 2011). The blockade of CXCR4 on HSCs with the use of clinically used drugs, AMD3100 and G-CSF, promote mobilization. These mobilizing drugs target CXCR4 through direct or indirect mechanisms in order to promote mobilization. These mechanisms will be further discussed within this section.

CXCL12 is abundantly expressed on endothelial cells and osteoblasts within the bone marrow microenvironment. However, disruption between the interaction of CXCR4 and CXCL12 results in the release of HSCs into the vasculature. CXCL12 expression is both intrinsically and extrinsically regulated with the bone marrow. The Wnt signaling was shown to transcriptionally regulate CXCL12 expression in bone marrow stromal cells (Tamura et al., 2011). Extrinsically, G-CSF treatment leads to the proteolytic cleavage of the N-terminus of CXCL12 and promotes the release of HSCs into the blood (Levesque et al., 2003). CXCL12 levels are also regulated by proteases, which could also promote

mobilization. Recent evidence shows that chronic variable stress can also contribute to the down regulation of CXCL12, which results in HSC release (Heidt et al., 2014). The pharmacological induction of HSC mobilization is used to increase the release of HSCs in the blood for the use of hematopoietic stem cell transplants.

1.7.2.2 Clinical drugs used to mobilize hematopoietic stem cells

1.7.2.2.1 Granulocyte colony stimulating factor (G-CSF)

G-CSF is important for the production of neutrophils and its effects are mediated upon binding to a single homodimer of the G-CSF receptor (Fukunaga et al., 1990). Under physiological conditions, G-CSF levels are undetectable, however the levels increase upon infection. G-CSF was first used to treat patients with neutropenia, which is a condition characterized by a decrease in neutrophil production as a result of chemotherapy treatment (Bendall and Bradstock, 2014). G-CSF treatment not only stimulated production of neutrophils, but also induced mobilization of HSCs into the blood. The induction of HSC mobilization by G-CSF treatment is an indirect response by HSCs. Studies have shown that HSC release is due to the secretion of neutrophil associated extracellular proteases such as matrix metalloproteinase 9 (MMP9), which led to the degradation of bone marrow retention molecules (Petit et al., 2002). G-CSF treatment also affects the bone marrow microenvironment by decreasing osteoblast formation by blocking mesenchymal stem cell differentiation (Ferraro et al., 2011; Semerad et al., 2005). Osteoblasts highly express CXCL12, therefore the decrease in this bone marrow component results in HSC mobilization. Today, G-CSF is routinely given to patients receiving chemotherapy or those being treated to donate HSCs for transplantation. After administration of G-CSF, the peak HSC mobilization occurs at 4-5 days (Uy et al., 2008). Although G-CSF is highly effective in mobilizing HSCs, about 5-20% of patients fail to mobilize a sufficient amount of cells (Mohty and Ho, 2011). Therefore, other mobilizing drugs such as AMD3100 can be used to increase the efficacy of HSC mobilization.

1.7.2.2.2 AMD3100

AMD3100 (Plerixafor) was first discovered to have antiviral properties by having potential inhibitory effects on the replication of the human immunodeficiency virus (HIV) (De Clercq et al., 1992). Soon after, AMD3100 was found to be a selective antagonist for the chemokine receptor, CXCR4. Upon AMD3100 treatment, the interaction between CXCR4 and CXCL12 is blocked, inducing the mobilization of HSCs from the bone marrow into the blood. In combination with G-CSF, AMD3100 treatment resulted in a dramatic increase in the mobilization of CD34+ cells in comparison to just AMD3100 treatment alone (Broxmeyer et al., 2005). In comparison to G-CSF where the peak HSC mobilization occurs on a matter of 4-5 days, AMD3100 treatment occurs within 10-16 hours (Uy et al., 2008). AMD3100 and G-CSF treatment often requires multiple sessions in order to obtain the necessary amount of HSCs for the use of autologous and allogenic transplantations. In the context of autologous stem cell transplants, one clinical study showed that combination treatment of AMD3100 and G-CSF resulted in increased stem cell mobilization, which required less apheresis sessions (Uy et al., 2008). This situation would be ideal for patients who often fail with G-CSF mediated mobilization alone.

1.7.2.3 Sphingosine 1-phosphate receptor 1 (S1PR₁)

In addition to CXCR4, the S1PR is important for HSC trafficking and mobilization. S1PR, like CXCR4, is classified as part of the G-protein-coupled-7-transmembrane receptor family that modulate cell chemotaxis (Bendall and Basnett, 2013). S1P receptors 1-5 all have unique roles in mediating cellular processes that are not only restricted to hematopoietic stem cells (Blaho and Hla, 2014). S1PR₁ is expressed on HSCs and is described as an important mediator of HSC mobilization and trafficking (Golan et al., 2012; Juarez et al., 2012). Combined treatment of AMD3100 and FTY720 (S1PR agonist), which binds and targets the receptor for degradation, resulted in decreased numbers of mobilized HSPCs compared to control treated animals (Golan et al., 2012). These data demonstrate that S1PR₁ plays an important role in HSPC mobilization. HSC mobilization was enhanced by administration of the S1P analog, SEW2871

(Juarez et al., 2012) and further increased used in combination with AMD3100. This study demonstrates that high S1P levels in the vasculature sets up a gradient for HSC mobilization that induces release from the osteoblastic niche and trans-endothelial migration.

S1P is the ligand for the S1PR and is found at high concentrations within the blood (Schwab et al., 2005). S1P levels within the blood are maintained by endothelial cells (Ito et al., 2007; Liu et al., 2011a). The levels of S1P remain at low concentrations within tissues and bone marrow in order to provide a chemotactic gradient for HSC migration to the blood (Massberg et al., 2007). Levels of S1P are regulated by the balance between type 1 sphingosine phosphate-1 kinase (SphK1) and S1P lyase which degrades S1P levels (Pebay et al., 2007). Interestingly, S1P is thought to be a stronger chemoattractant than CXCL12/CXCR4-mediated attraction for HSC homing, engraftment and mobilization (Golan et al., 2012; Juarez et al., 2012).

1.8 Tetraspanins and hematopoietic stem cells

1.8.1 Introduction to tetraspanins

The tetraspanin family of proteins function as scaffolds at the plasma membrane to regulate a large array of cellular processes such as morphology, migration, fusion and signaling (Hemler, 2003; Maecker et al., 1997; Wright et al., 2004). The first members of tetraspanins were identified in human and schistosomes (Hotta et al., 1988; Wright et al., 1990). There are 33 known tetraspanins in humans, 37 in *Drosophila* and 20 in *C. elegans* (Adell et al., 2004; Boucheix and Rubinstein, 2001; Todres et al., 2000). The expression of tetraspanins across different species suggests this family of proteins contain an evolutionary conserved structure (Garcia-Espana et al., 2008). Some tetraspanins such as CD9, CD81 and CD82 are ubiquitously expressed (Maecker et al., 1997), whereas, other tetraspanins such as CD37 and CD53 are restricted to hematopoietic cells (Maecker et al., 1997; Schwartz-Albiez et al., 1988). The differential expression of tetraspanins in various tissues translates into specific functions within different cell types.

Tetraspanins protrude only 4-5 nm above the transmembrane, which often results in them being overlooked by biochemical and immunological detection (Hemler, 2005). A simplified schematic of the tetraspanin molecular structure is depicted in Figure 1.3. Tetraspanins are characterized by four transmembrane domains, which consist of two extracellular loops and two intracellular tails. Tetraspanins range from 200-350 amino acids, in which 13-31 amino acids are in the first extracellular loop (EC1) and 69-132 amino acids are in the second extracellular loop (EC2) (Hemler, 2005; Stipp et al., 2003). EC2 contains a region with three alpha helices and a variable region, which is important for tetraspanin protein-protein interactions (Hemler, 2003; Stipp et al., 2003). EC2 also contains a conserved CCG motif and two cysteines that also consist of two di-sulfide bonds (Hemler, 2003; Stipp et al., 2003). Proteins are only characterized as a tetraspanin if they contain 4-6 conserved extracellular cysteines residues and polar residues within the transmembrane domains (Hemler, 2005; Stipp et al., 2003). A new crystal structure of full length CD81 provided evidence that tetraspanins also consist of a cholesterol-binding pocket created by the four transmembrane domain structure (Zimmerman et al., 2016). This structure also provided evidence that tetraspanin function can be mediated by an open or closed conformation due to the binding of cholesterol at the binding pocket. For example, cholesterol binding regulates CD81 function by mediating the export of CD19 to the surface of 293T cells (Zimmerman et al., 2016), however, it is still unknown how cholesterol binding affects the function of other tetraspanins.

Tetraspanins contain post-translational modifications that mediate the unique function of each transmembrane protein. The post-translational modification sites on tetraspanins include palmitoylation, N-glycosylation, and ubiquitination. Additionally, tetraspanins have a cytoplasmic tail-sorting motif YXXΦ. The addition of palmitate to the membrane proximal cysteine residues facilitates the stability of the tetraspanin within the membrane (Levy and Shoham, 2005). Tetraspanins CD9, CD37, CD53, CD63, CD81, CD82 and CD151 were found to incorporate palmitate (Charrin et al., 2002). Tetraspanin palmitoylation promotes formation of tetraspanin enriched micro-domains (TEMs), which

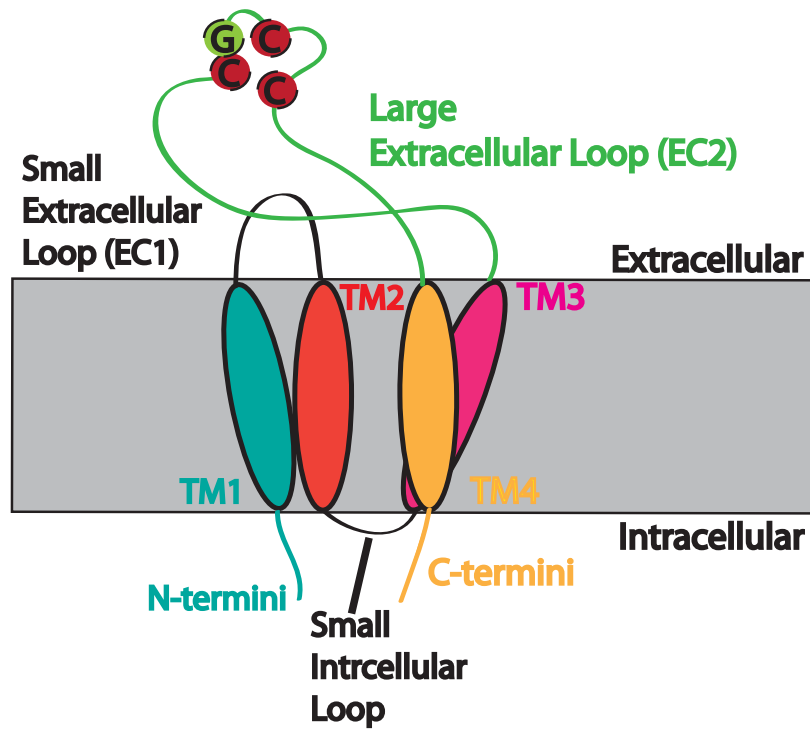


Figure 1.3: Diagram of tetraspanin molecular structure. This diagram is based off the crystal structure of CD81 published in, (Zimmerman et al., 2016). The molecular structure of tetraspanins depicts four transmembrane domains (TM1-TM4). These transmembrane domains create three loops, one small extracellular (EC1), one large extracellular loop (EC2) and one small intracellular loop. Tetraspanins also consist of two intracellular tails, N-termini and C-termini. The Cys-Cys-Gly amino acid motif and two disulfide bonds are located on EC2.

contributes to tetraspanin-dependent signaling (Charrin et al., 2002). Loss of palmitoylation results in decreased lateral associations of CD151 and CD9 (Berditchevski et al., 2002; Charrin et al., 2002; Yang et al., 2002). Palmitoylation mutants of CD82 led to the disorganization of CD82 clusters at the plasma membrane of AML cells (Termini et al., 2014). In addition, palmitoylation was also found to be important for the tight packing of the $\alpha 4$ integrin at the plasma membrane. Moreover, tyrosine phosphorylation of the nucleotide exchange factor, Vav1, has been shown to be dependent on the interaction between palmitoylated tetraspanins and cholesterol (Charrin et al., 2003). Most tetraspanins are heavily glycosylated, which contributes to the heterogeneity in size of 20 to 50kDa (Yunta and Lazo, 2003). N-glycosylation sites are expressed on the first and second extracellular loop of tetraspanins and are important for cell-cell interactions and the organization of the structure (Stipp et al., 2003). Studies from our lab and others show the importance of N-linked glycosylation sites for tetraspanin functions. Our lab demonstrated that the loss of CD82 N-glycosylation sites led to increased clustering of N-cadherin on the plasma membrane resulting in increased bone marrow homing of AML cells (Marjon et al., 2016). In addition, another group showed that N-glycosylation of CD82 regulated adhesion and motility through the interaction with $\alpha 3$ and $\alpha 5$ (Ono et al., 2000). In addition, N-glycosylation sites on CD63 mediated the down regulation of CXCR4 (Yoshida et al., 2009).

The YXX Φ cytoplasmic tail-sorting motif consists of a Tyr-Xaa-Xaa- Φ motif in which the Φ represents a bulky hydrophobic side chain amino acid (Bonifacino and Dell'Angelica, 1999). The YXX Φ sorting motif found on the C-terminal tail of specific tetraspanins is important for endocytosis (Rous et al., 2002). For example this motif has been found to be important for internalization of CD151, Tspan7 and CD82 (Liu et al., 2007; Rous et al., 2002; Stipp et al., 2003). The C-terminal tail motif is also important for endocytic trafficking, localization and lysosomal targeting (Bonifacino and Dell'Angelica, 1999). Mutations in the sorting motif of CD63 resulted in the lost of intracellular localization and cell surface trafficking (Rous et al., 2002). Tetraspanins without this sorting motif can also be

Tetraspanin	Cell Type	Function	Reference
CD9	Human CD34+ Cord Blood HSPCs Pluripotent Hematopoietic Cell Line, EML-C1 Porcine Hematopoietic Progenitors Murine HSPCs Multipotent FDCP-mix cells Human Cord Blood Human CD34+ Cord Blood HSPCs Mobilized Human Peripheral Blood Human CD34- Cord Blood HSCs	Dendritic cell marker Regulation of HSPC differentiation, proliferation and self-renewal Negative enrichment marker for Hematopoietic Progenitor Cells Marker for murine Hematopoietic Stem Cells Marker for Hematopoietic Progenitor Cells Cord blood stem cell marker Regulation of HSPC migration, homing and adhesion, HSPC marker IDB treatment increase CD9 expression, does not regulate homing Decreased engraftment in mice and sheep due to low CD9 expression	Caux C et al, 1996 and Caux C et al, 1997. Aoyama K et al, 1999 and Oritani K et al, 2000. Heinz M et al, 2002. Karlsson G et al, 2013. Bruno L et al, 2004. Zhao Y et al 2006. Leung K et al. 2011. Desmond R et al, 2011. Abe T et al, 2017.
	Human CD34+ Cord Blood HSPCs Cell Line: MO7e and Human CD34+ Cord Blood HSPCs Murine HSCs	Marker for differentiation of lymphohematopoietic stem and progenitor cells Interaction with c-kit Regulation of re-entry of HSC quiescence	Ma F et al, 2001. Anzai N et al, 2002. Lin K et al, 2011.
CD151	Mobilized Human Peripheral Blood Cell Lines: MO7e, HEL and K562	Megakaryocyte derived from HSPCs enhance T helper cell responses Interaction with integrins to mediate HSPC adhesion to various ECMs	Finkelsztejn A et al, 2015. Fitter S et al, 1999.
CD63	Cell Line: MO7e and Human CD34+ Cord Blood HSPCs Murine HSCs Mobilized Human Peripheral Blood Human CD34+ Cord Blood HSPCs Human Cord Blood and Human Peripheral Blood	Interaction with c-kit Regulation of HSC quiescence and long-term engraftment Interaction with TIMP1 mediates HSPC adhesion and migration Regulation of HSPC proliferation Marker for asymmetric HSC division	Anzai N et al, 2002. Rossi L et al, 2011. Wilik C et al, 2013. Rossi L et al, 2015. Beckmann J et al, 2006 and Giebel B et al, 2007.
	Human Cord Blood and Human Peripheral Blood Murine HSPCs Hematopoietic Cell Line: BaF/3	Marker for asymmetric HSC division Differentiation Status Marker B cell development	Beckmann J et al, 2006 and Giebel B et al, 2007. Guo G et al, 2013. Mansson R et al, 2007.
CD82	Human HSPCs from Bone Marrow, Cord Blood, Peripheral Blood Human Peripheral Blood HSPCs Murine HSCs and HSPCs	Identification of tetraspanin on hematopoietic progenitor cells Mediation of HSPC interaction with osteoblasts and HSPC quiescence Regulation of LT-HSC quiescence	Burchert A et al, 1999. Larochelle A and Gillette J et al, 2012. Hur J et al. 2016.
Tetraspanin 3	Murine HSPCs	Regulation of HSPC migration	Kwon H et al, 2015.
TSSC6	Murine Hematopoietic Cell Lines and Human Tissue Murine and Human Hematopoietic tissues Murine Hematopoietic System	Identification of tetraspanin in hematopoietic tissues Identification of tetraspanin in hematopoietic tissues No effect on hematopoiesis	Robb L et al, 2001. Nicholson R et al, 2000. Tarrant J et al, 2002.

Table 1.1: Tetraspanin regulation of hematopoietic stem and progenitor cell function. Tetraspanins have been shown to play a role in regulating hematopoietic stem and progenitor cell functions. This table lists the roles of each tetraspanin in respect to HSPC function.

localized to intracellular compartments such as endosomes, late endosomes and lysosomes by interacting with tetraspanins with this sort motif (Stipp et al., 2003).

Tetraspanin enriched micro-domains (TEMs) function as a membrane scaffold that regulates adhesion and signaling. Tetraspanins can interact with other tetraspanins, adhesion molecules, signaling molecules and Ig receptors to form TEMs (Serru et al., 1999; Szollosi et al., 1996; Termini et al., 2014). With the use of biochemical techniques and super resolution microscopy, tetraspanins have been shown to influence integrin clustering and avidity (Thoren et al., 2008). In addition, tetraspanins are known to interact with signaling molecules at the membrane in order to elicit down stream signaling. For example, Rac1 activation is mediated by CD81 expression through the interaction with the C-terminal tail (Tejera et al., 2013). In addition, PKC α has also been shown to interact with the intracellular tails of tetraspanins upon PMA stimulus to promote down stream signaling (Zhang et al., 2001). In addition, CD82-associated TEMs stabilize PKC α activation at the plasma membrane to promote down stream ERK signaling (Termini et al., 2016). Therefore, tetraspanins acts as molecular facilitators that coordinate and organize the membrane to promote down stream signaling to activate cell-mediated behaviors (Termini and Gillette, 2017). The following section will describe how tetraspanins specifically regulate HSC functions and behaviors, which are summarized in Table 1.1.

1.8.2 Tetraspanins as regulators of hematopoietic stem cell function

1.8.2.1 CD9

Tetraspanin CD9 expression has been described on hematopoietic stem and progenitor cells (HSPCs) where it was shown to regulate HSPC proliferation, migration and adhesion. The use of surface markers is critical for the identification of a pure population of HSPCs. CD9 expression was used as marker to enrich HPCs in a porcine model (Heinz et al., 2002). This study identified seven markers for negative enrichment of hematopoietic progenitor cells (HPCs), one of which was CD9. Here, HPCs with negative or low expression of CD9 fell within the side population, which is a technique used to

identify putative HSCs. In contrast, CD9 has also been identified as a positive marker for HSCs and HSPCs in the following systems: murine HSCs (Karlsson et al., 2013), bone marrow derived multipotent hematopoietic progenitor cell line (Bruno et al., 2004) and human umbilical cord blood stem cells (Zhao et al., 2006).

In addition to being used as a surface marker for HSPCs, CD9 has also been shown to regulate HSPC migration, adhesion and homing (Leung et al., 2011). This study showed that CD9 expression on human cord blood CD34+ HSPCs was modulated by SDF-1 and CXCR4 activity to increase HSPC migration and adhesion. Additionally, this group demonstrated that enhancing CD9 expression on the surface of CD34+ HSPCs with the treatment of a protein kinase C agonist, ϵ ingenol 3,20 dibenzoate (IDB), increases homing to the bone marrow. However, another group later found that IDB increases CD9 expression on CD34+ HSPCs, but does not increase HSPC homing compared to control treated CD34+ HSPCs intravenously injected into NSG mice (Desmond et al., 2011). More recently, another group characterized human CD34- HSCs isolated from cord blood and found that engraftment in mice and sheep was limited due to a decrease in CD9 and an increase in the inhibitory homing molecule, CD26 (Abe et al., 2017). Together these data suggest that CD9 can be used as a marker for HSPCs and has a role in HSPC adhesion and migration.

CD9 is also expressed on the surface of dendritic cells derived from CD34+ HPCs isolated from human cord blood (Caux et al., 1996). Human CD34+ HPCs cultured *in vitro* in the presence of the hematopoietic growth factor, granulocyte-macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor-alpha (TNF-alpha) differentiated along two independent dendritic cell pathways: 1) CD1a+ dendritic cells and 2) CD14+ dendritic cells. Day 12 of culture yielded CD14+ progenitors that differentiated into dendritic cells characterized by the expression of CD9, CD68, CD2 and factor XIIIa. Functional assays showed that CD14+ dendritic cells are unique from CD1a dendritic cells, which could be important in immune responses (Caux et al., 1997). Therefore, these studies suggest that CD9 is important for immune cell differentiation.

Finally, the expression of CD9 on stromal cells also regulates HSPC activity (Aoyama et al., 1999; Oritani et al., 2000). The pluripotent hematopoietic cell line, EML-C1, plated on stromal cells ligated with an anti-CD9 antibody blocked HSC differentiation, proliferation and self-renewal. The author speculated that the interaction of CD9 with integrin β_1 and an unknown 100kD protein leads to the inhibition of HSPC differentiation. The identification of this unknown 100kD protein could be important in determining a novel regulator for HSPC activity.

1.8.2.2 CD81

HSCs primarily reside in a quiescent state within the bone marrow microenvironment. The tetraspanin CD81 was shown to be important for the re-entry of HSC quiescence through the inhibition of the Akt signaling pathway (Lin et al., 2011). This group found that the spatial distribution of CD81 on the surface of murine HSCs was important for the re-entry of HSCs into quiescence from a highly proliferative state. The polarization of CD81 leads to the deactivation of Akt and nuclear translocation of FoxO1a, which resulted in an increase in quiescence. Therefore, CD81 is important for the regulation of HSC quiescence.

The tetraspanin CD81 has been shown to serve as a marker for the development of lymphohematopoietic stem and progenitor cells (Ma et al., 2001). Differential surface expression of CD81 and CD34 caused differentiation of CD34+ HSPCs into specific lineages of blood and immune cells. Therefore, CD81 expression can be useful for determining the differentiation status of lymphohematopoietic stem and progenitor cells

Finally, within tetraspanin-enriched micro-domains (TEMs), CD81 has been shown to interact with integrins and the c-kit receptor tyrosine kinase in human hematopoietic progenitors (Anzai et al., 2002). Using a combination of immunoprecipitation and co-localization experiments this study showed that CD81 interacts with c-kit on the surface of human CD34+ cord blood HSPCs and the human growth factor-dependent myeloid cell line, MO7e. C-kit expression on HSPCs is critical for the maintenance and regulation of HSPC processes.

Therefore, the interaction of CD81 and c-kit could be important for the regulation of HSPCs.

1.8.2.3 CD151

The tetraspanin CD151 is expressed within the hematopoietic system on activated T cells, megakaryocytes and platelets. CD151+ human megakaryocyte progenitors derived from mobilized peripheral blood were shown to enhance T helper cell responses (Finkielsztejn et al., 2015). In addition, CD151 is also expressed on the hematopoietic stem cell lines MO7e, HEL and K562 (Fitter et al., 1999). This study found that CD151 interacts with the integrins β_1 and $\alpha_{IIb}\beta_3$, which was found to mediate HSPC adhesion to various extracellular matrices (ECMs). Together these data indicate that CD151 is used as a HSPC marker and is important for the regulation of HSPC adhesion via the interaction with integrins.

1.8.2.4 CD63

HSPCs are tightly regulated within the bone marrow microenvironment through cell-cell interactions mediated by adhesion and signaling molecules. CD63 was found to be associated with c-kit on the surface of the hematopoietic progenitor cell line, MO7e (Anzai et al., 2002). The association of c-kit with CD63 could be important for the regulation of HPC functions such as adhesion, proliferation and migration. The tetraspanin CD63 was described to interact with the tissue inhibitor of metalloproteinase-1 (TIMP1) to promote cell survival in a breast cancer cell line model (Jung et al., 2006). The role of TIMP1 in HSCs was investigated using a TIMP1 knock out mouse, in which TIMP1 was shown to be important for HSC quiescence and long-term engraftment (Rossi et al., 2011). In another study, TIMP1 was found to bind to the CD63/beta 1 integrin complex on the surface of human CD34+ HSPCs to induce adhesion and migration (Wilk et al., 2013). This group also determined that homing and short term engraftment of HSPCs were also increased upon exogenous stimulation with TIMP1. The interaction of TIMP1 and CD63 has also been shown to impact HSPC proliferation through the activation of the PI3K/AKT signaling pathway (Rossi et al., 2015). This group also found that TIMP1 treatment of HSPCs led to an increase in cyclin D1 gene expression due to AKT phosphorylation. Another

study demonstrated that CD63 and another tetraspanin, CD53, were suggested to be stringent markers for asymmetric HSC division compared to the current CD133 and CD34 expression profiles (Beckmann et al., 2007; Giebel and Beckmann, 2007). Together these studies display an important role for CD63 in HSPC function and maintenance within the bone marrow microenvironment.

1.8.2.5 CD53

Tetraspanin CD53 is expressed on most immune cells and is expressed on a small population of hematopoietic stem cells. As stated above, CD53, in combination with CD63 both served as more stringent markers for asymmetric division than the current CD133 and CD34 expression profiles (Beckmann et al., 2007; Giebel and Beckmann, 2007). In addition, another study completed a comprehensive single-cell gene expression analysis of the mouse hematopoietic system, in which they found CD53 to be differentially expressed within the HSPC population (Guo et al., 2013). Moreover, another study has shown that in a HSPC cell line, CD53 is important for the development of B cells (Mansson et al., 2007).

1.8.2.6 Tetraspanin 3

The expression of Tetraspanin 3 has been confirmed on HSPCs. A recent study described Tetraspanin 3 as an important mediator of AML development and expansion (Kwon et al., 2015). This study also showed that in the normal hematopoietic system, Tetraspanin 3 expression is dependent on the expression of the RNA binding protein, Musashi 2. Interestingly, the loss of Tetraspanin 3 led to a decrease in CXCR4 activity. Together, these data suggest that Tetraspanin 3 could have a potential role in the regulation of HSPC development and migration.

1.8.2.7 TSSC6 (TSPAN32)

The expression of TSSC6 has been confirmed on HSPCs, but the function of this tetraspanin has not been fully characterized. TSSC6 has been identified in the adult hematopoietic tissue of both human and mice (Nicholson et al., 2000; Robb et al., 2001). Expression of TSSC6 was also confirmed in various hematopoietic stem cell lines (Robb et al., 2001). The role of TSSC6 in HSPCs

was assessed using a TSSC6 knock out mouse model, in which steady-state hematopoiesis was unaltered compared to control mice (Tarrant et al., 2002). In addition, there was no difference in the hematopoietic system when challenged with various stimuli. Collectively, this study suggests that TSSC6 is not necessary for hematopoietic system development, however, a specific role for TSSC6 in HSPCs has not been fully explored.

1.8.2.8 CD82

The tetraspanin CD82 is ubiquitously expressed and has been described to interact with different adhesion and signaling molecules on the surface of HSPCs. A schematic of the CD82 structure is depicted in Figure 1.4. CD82 was first described on the surface of human HSPCs isolated from peripheral blood (Burchert et al., 1999). In addition, this group found that CD82 expression is increased in leukemias such as CML, AML and CLL. However, interestingly the level of CD82 expression decreased upon differentiation of CD34+ HSPC. CD82 expression and its plasma membrane organization were also found by our group to mediate the interaction between human CD34+ HSPCs and osteoblasts (Larochelle et al., 2012). In this study, CD34+ HSPCs were also cell sorted based on their cell cycle status, which identified the distribution of CD82 in G0 cells. More recently, CD82 expression was shown to be highly expressed on LT-HSCs (Hur et al., 2016). This study also determined that CD82 is important for LT-HSC quiescence, which is mediated through the interaction of CD82 and DARC via the activation of the TGFB pathway. Together these data suggest an important role for CD82-mediated regulation of HSPC differentiation, maintenance and quiescence.

1.9 Summary and Discussion

The introduction to this thesis intends to provide an overview of key studies regarding the mechanisms known to regulate HSPC functions such as quiescence, homing, engraftment and mobilization. However, a few questions still remain within the field. Previous work from our lab identified the tetraspanin

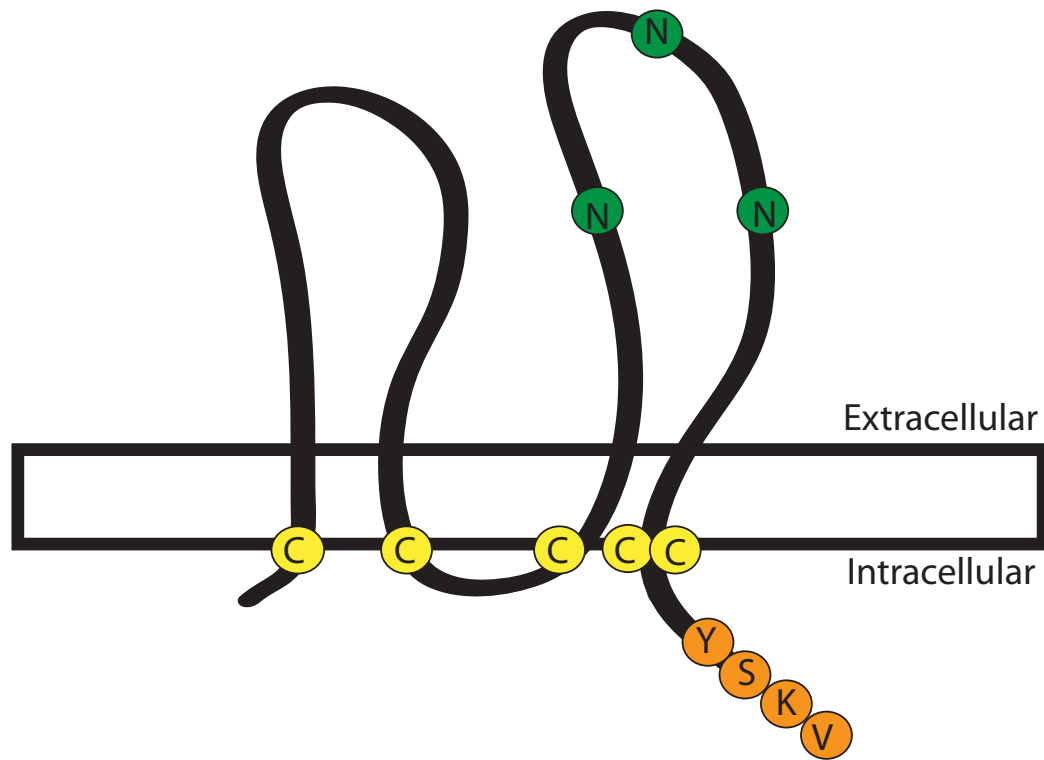


Figure 1.4: CD82 structure and posttranslational modifications. This diagram depicts the structure of CD82. CD82 spans the transmembrane four times which creates two extracellular loops and one small intracellular loop. In addition, CD82 contains two intracellular tails, a N-termini and C-termini. CD82 contains five proximal cysteine residues shown in yellow, which can be palmitoylated. The three asparagine residues shown in green are N-link glycosylated sites. The C-terminal tail of CD82 consists of a tyrosine based sort motif (YXX \emptyset) shown in orange, which contains amino acids, Tyr-Ser-Lys-Val.

CD82 as a critical regulator of HSPC function. For example, while a number of molecules involved in HSPC homing and engraftment have been extensively studied, the role of CD82 in regulating these functions is still limited. Previous antibody-based studies (Larochelle et al., 2012) suggest that CD82 is important for regulating HSPC homing, however, a role for CD82 in HSPC engraftment has never been described. We hypothesize that the CD82 scaffold promotes HSPC bone marrow homing and engraftment. In Chapter 2, we utilized a global CD82 knock out (CD82KO) mouse model to test the hypothesis that CD82 promotes HSPC quiescence, homing and engraftment. We demonstrated that the loss of CD82 resulted in decreased LT-HSCs, which we believe is due to increased CD82KO activation. In addition, we determined that the defect in CD82KO HSPC bone marrow homing was due to hyperactivation of Rac1. We were able to rescue CD82KO HSPC homing through the use of Rac1 inhibitors. In addition, we detected a defect in CD82KO HSPC engraftment in a competitive environment, which could potentially be a result of a defect in CD82KO HSPC homing.

The molecules and mechanisms that mediate HSPC mobilization has been extensively studied, however, the role for CD82 has never been described. In chapter 3, we describe a novel role for CD82 in regulating HSPC mobilization through the modulation of the sphingosine-1-phosphate receptor 1 (S1PR₁). Again, using the global CD82KO mouse and flow cytometry techniques, we hypothesized that the loss of CD82 would promote HSPC mobilization. We were able to detect enhanced mobilization of CD82KO HSPCs compared to WT HSPCs. Our data demonstrate that CD82KO HSPCs have enhanced mobilization due to increased surface expression of S1PR₁. In addition, phosphoflow signaling analysis show increased signaling of pERK and pAKT downstream of S1PR₁ within CD82KO HSPCs. Furthermore, we find a significant decrease in the internalization of S1PR₁ on the surface of CD82KO HSPCs, which could mechanistically explain the increase in surface expression we detect. Finally, through the use of a CD82 antibody we were able to increase HSPC mobilization in WT mice compared to control treatment.

Collectively, these chapters describe an important role for tetraspanin CD82 as a critical regulator of HSPC quiescence, homing, engraftment and mobilization. Chapters 2 and 3 will further describe the important role for CD82 in mediating these processes. Our work provides evidence that CD82 could be exploited to increase HSPC homing and engraftment potential. Additionally, we provide evidence that CD82 could be a valuable target to promote HSPC mobilization. Taken together, we have identified the tetraspanin CD82 as a critical regulator of HSPC fitness and function.

Chapter 2: The tetraspanin CD82 regulates bone marrow homing and engraftment of hematopoietic stem and progenitor cells.

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2.1 Abstract

Hematopoietic stem and progenitor cell (HSPC) transplantation represents a treatment option for patients with malignant and non-malignant hematological diseases. Initial steps in transplantation involve the bone marrow homing and engraftment of peripheral blood injected HSPCs. In recent work, we identified the tetraspanin CD82 as a potential regulator of HSPC homing to the bone marrow, although its mechanism remains unclear. In the present study, using a CD82 knock out (CD82KO) mouse model, we determined that CD82 modulates HSPC bone marrow maintenance, homing and engraftment. Bone marrow characterization identified a significant decrease in the number of long-term hematopoietic stem cells in the CD82KO mice, which we linked to cell cycle activation and reduced stem cell quiescence. Additionally, we demonstrate that CD82 deficiency disrupts bone marrow homing and engraftment, with *in vitro* analysis identifying further defects in migration and cell spreading. Moreover, we find that the CD82KO HSPC homing defect is due at least in part to the hyperactivation of Rac1, as Rac1 inhibition rescues homing capacity. Together, these data provide evidence that CD82 is an important regulator of HSPC bone marrow maintenance, homing and engraftment and suggests exploiting the CD82 scaffold as a therapeutic target for improved efficacy of stem cell transplants.

2.2 Introduction

Hematopoietic stem and progenitor cells (HSPCs) provide the cellular reservoir that gives rise to the highly varied blood and immune cells required to support the lifespan of an organism. As such, it is necessary that HSPCs maintain a finely tuned balance between quiescence, self-renewal, proliferation and differentiation. While key signaling pathways intrinsic to HSPCs are involved in regulating this delicate balance, HSPCs are also regulated by a variety of signals they receive from their microenvironment or niche. The bone marrow microenvironment is the primary residence for HSPCs, where they are regulated by both secreted signals and cell-cell interactions (Mendelson and Frenette, 2014; Morrison and Scadden, 2014; Morrison and Spradling, 2008). Under

physiological conditions, HSPCs are maintained in the bone marrow, but also circulate within the blood at low levels (Mazo and von Andrian, 1999; Sahin and Buitenhuis, 2012). Then from the peripheral blood, the HSPCs can migrate back to the bone marrow using a process called homing, which is the critical first step in the repopulation of the bone marrow after stem cell transplantation. Currently, allogeneic hematopoietic stem cell (HSC) transplantation is a standard treatment option for patients suffering from a variety of malignant and non-malignant hematological diseases (Gyurkocza et al., 2010). The effectiveness of this treatment requires the successful homing of donor HSPCs back to the bone marrow microenvironment, where they can engraft and repopulate the blood and immune cell lineages. Notably, only a small percentage of transplanted HSPCs have the capacity to engraft, and while graft failure is rare, it remains a significant contributor to patient morbidity and mortality (Ratajczak and Suszynska, 2016). With the ultimate goal of improving transplantation therapies, there is significant interest in understanding the molecular mechanisms that regulate the repopulation potential or fitness of HSPCs, which requires productive bone marrow homing and engraftment.

HSPC bone marrow homing is a dynamic process, which includes various adhesion and signaling molecules such as chemokines and integrins. The chemokine CXCL12 is an important chemoattractant and regulator of HSPCs. The expression and secretion of CXCL12 is abundant in the bone marrow microenvironment (expressed by osteoblasts and endothelial cells) and promotes the homing and maintenance of HSPCs within the bone marrow. The receptor for CXCL12 is the C-X-C Chemokine receptor type 4 (CXCR4) receptor, which is highly expressed on HSPCs and controls HSPC homing, mobilization and niche localization (Nie et al., 2008; Prosper and Verfaillie, 2001; Sahin and Buitenhuis, 2012). The loss of CXCR4 on HSPCs results in a significant decrease in HSPC homing to the bone marrow (Nie et al., 2008). Interestingly, the re-expression of CXCR4 restored hematopoiesis upon bone marrow engraftment. In addition, integrins such as $\alpha 4$, $\alpha 6$, and $\beta 1$ facilitate adhesion in the bone marrow, but also mediate tethering of HSPCs within blood vessels to enable trans-endothelial

HSPC migration (Mazo and von Andrian, 1999; Papayannopoulou et al., 1995; Papayannopoulou et al., 2001a). The treatment of bone marrow HSPCs with an antibody to $\alpha 4\beta 1$ resulted in decreased bone marrow homing (Papayannopoulou et al., 1995; Papayannopoulou et al., 2001a). In addition, bone marrow cells from a conditional $\alpha 4$ KO mouse showed a delay in bone marrow homing and defect in short-term engraftment (Scott et al., 2003). Similarly, HSPCs deficient in the integrin $\beta 1$ (Hirsch et al., 1996; Potocnik et al., 2000) and treatment of bone marrow cells with an $\alpha 6$ antibody (Qian et al., 2006) led to a decrease in bone marrow homing and engraftment. Together, these data highlight the critical role for integrins and the CXCR4/CXCL12 signaling axis in HSPC homing and engraftment. Thus, understanding how these signaling and adhesion pathways are regulated in HSPCs is critical for improved transplantation therapies.

Tetraspanins are a family of scaffold proteins that are known to regulate adhesion and signaling molecules at the plasma membrane (Boucheix and Rubinstein, 2001; Hemler, 2005). Tetraspanins have an evolutionary conserved structure that spans the plasma membrane four times and interact with other tetraspanins, signaling and adhesion molecules to form tetraspanin-enriched microdomains (TEMs) that are important for modulating cell migration and adhesion (Charrin et al., 2009; van Deventer et al., 2017). Previous work from our lab identified the tetraspanin CD82 as a potential regulator of HSPC adhesion and migration, demonstrating that human CD34+ HSPC bone marrow homing was diminished when CD82 was neutralized with a monoclonal antibody (Larochelle et al., 2012). CD82 was first described as a tumor metastasis suppressor in solid tumors (Bienstock and Barrett, 2001) and is expressed in both normal and malignant hematopoietic cells (Burchert et al., 1999). Using an acute myeloid leukemia (AML) cell line model, our laboratory also identified decreased bone marrow homing upon CD82 knock down (Marjon et al., 2016) and went on to show that CD82 regulates the density of the $\alpha 4$ integrin at the plasma membrane, which contributes significantly to HSPC adhesive potential (Termini et al., 2014). More recently, CD82 was shown to be highly expressed on the long-term hematopoietic stem cell population (LT-HSCs) with a potential role

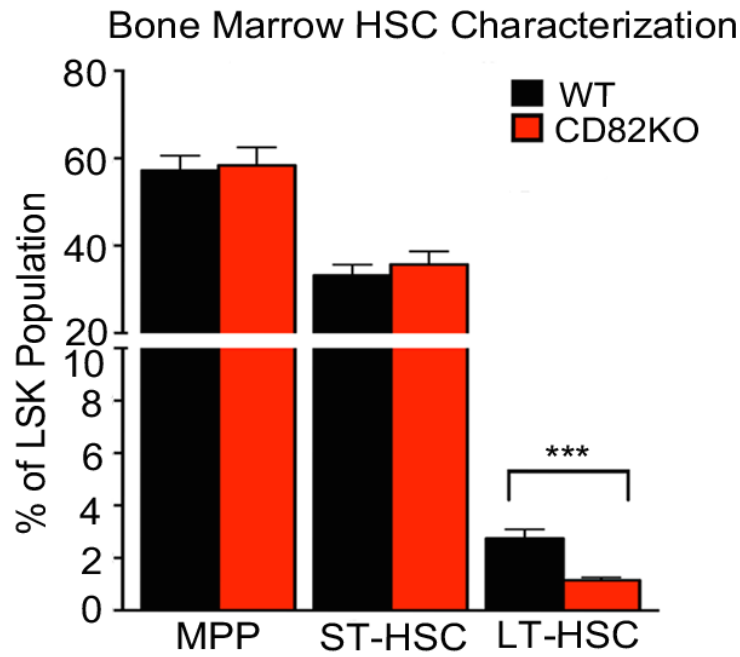
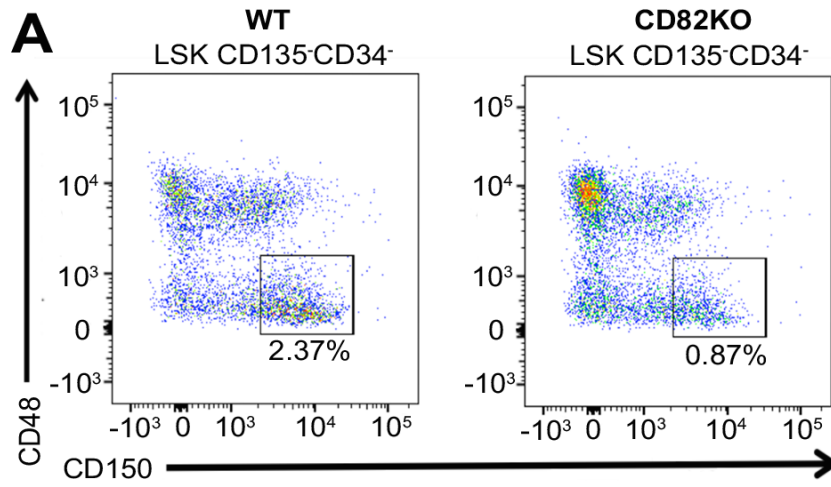
in the regulation of HSPC quiescence (Hur et al., 2016). In this current study, we set out to determine how CD82 impacts the homing and engraftment of HSPCs.

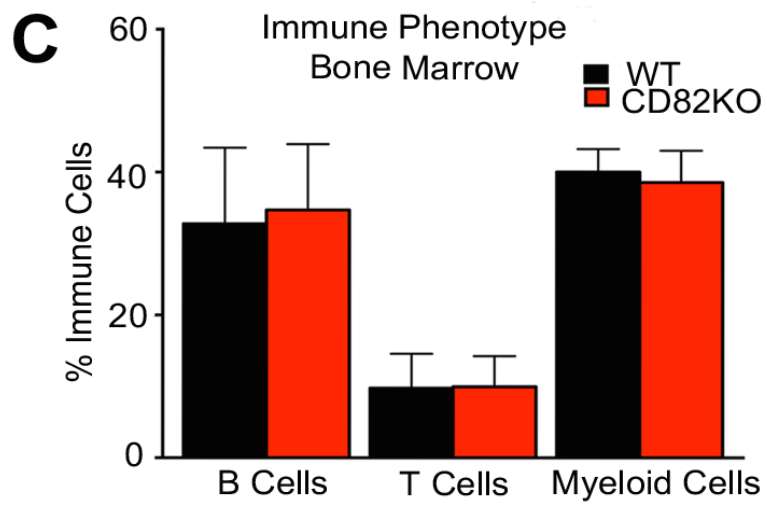
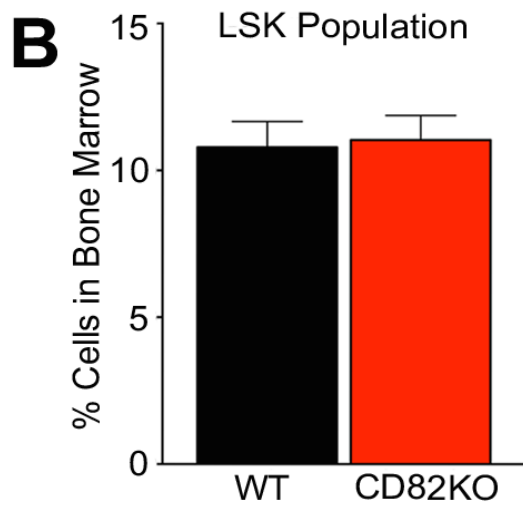
Using a global CD82 knock out (CD82KO) mouse model, we identify a reduction in the LT-HSCs localized within the bone marrow compartment, which we find results from LT-HSC activation. Moreover, measurements of HSPC fitness identified both engraftment and homing defects upon CD82 deficiency. Isolated HSPCs analyzed by confocal imaging demonstrated additional defects in migration and cell spreading. Recognizing the critical role for the Rho GTPase, Rac1, in cell migration and spreading, we analyzed the expression and activity of Rac1, finding Rac1 hyperactivation in CD82KO HSPCs. Inhibition of Rac1 hyperactivation using pharmacological inhibitors restored the bone marrow homing capacity of the CD82KO HSPCs, suggesting that CD82-mediated regulation of HSPC homing and engraftment involves the modulation of Rac1 activity.

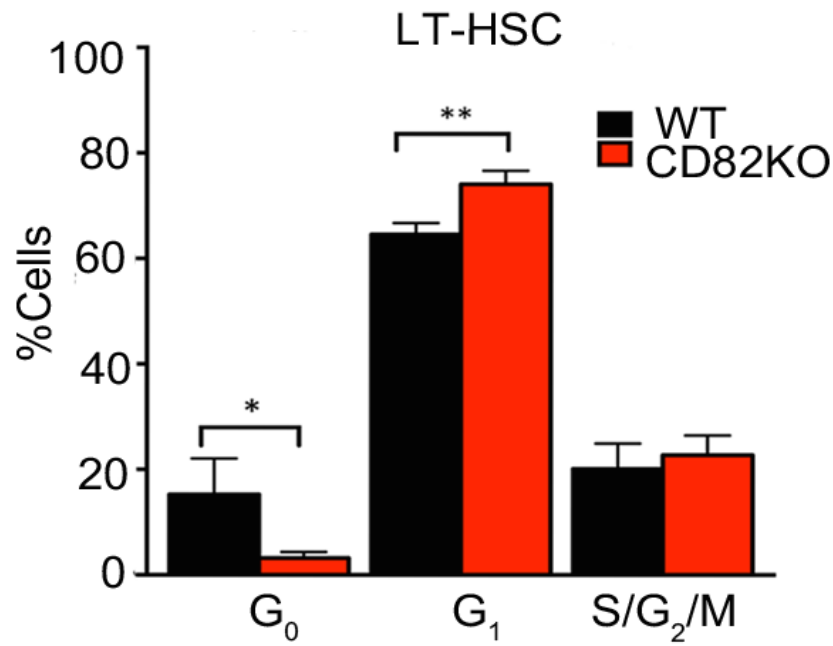
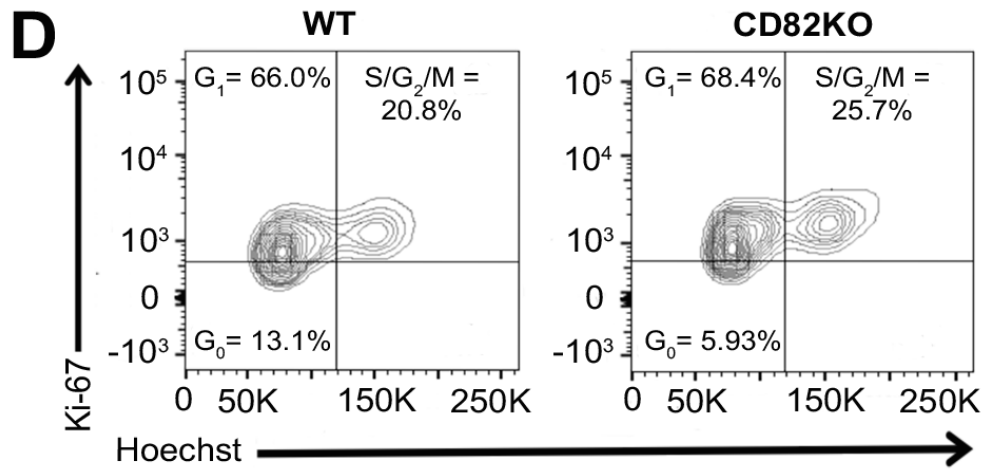
2.3 Results

2.3.1 Diminished LT-HSCs within the bone marrow of CD82KO mice.

To understand the mechanism by which the CD82 scaffold impacts HSPC regulation, we took advantage of the CD82KO mice previously described (Jones et al., 2016; Wei et al., 2014). To address the consequence of CD82KO on HSPC homeostasis, we first compared the bone marrow frequencies of HSPCs in the wild-type (WT) and CD82KO mice. Using flow cytometry, we observed a reduction in the frequency of long-term HSCs (LT-HSCs), defined as $\text{Lin}^- \text{Sca1}^+ \text{Kit}^+ \text{CD34}^- \text{CD135}^- \text{CD48}^- \text{CD150}^+$, in CD82KO, whereas short-term HSCs (ST-HSCs: $\text{Lin}^- \text{Sca1}^+ \text{Kit}^+ \text{CD34}^+ \text{CD135}^- \text{CD48}^- \text{CD150}^+$), multipotent progenitors (MPP: $\text{Lin}^- \text{Sca1}^+ \text{Kit}^+ \text{CD34}^+ \text{CD135}^+ \text{CD48}^- \text{CD150}^-$), and LSK ($\text{Lin}^- \text{Sca1}^+ \text{Kit}^+$) populations showed no significant change (Figure 1A,B). Further characterization of the immune phenotype of bone marrow isolated cells identified similar percentages of B cells, T cells and myeloid cells between the WT and CD82KO mice (Figure 1C). Together, these data suggest that CD82 functions in the







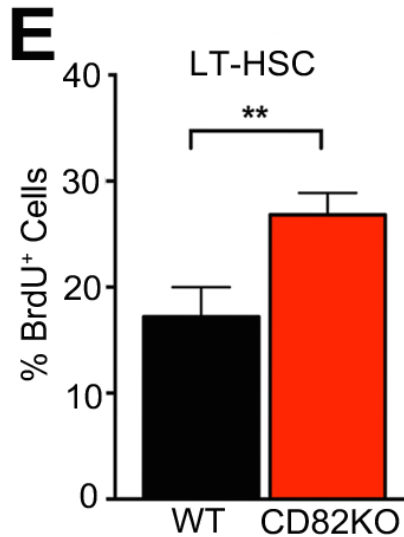


Figure 2.1: CD82 expression maintains LT-HSCs within the bone marrow.

(A) Representative flow cytometry plots of LT-HSCs gated on the LSK CD135-CD34-CD48-CD150⁺ population. Flow cytometry analysis of the percentage of MPP, ST-HSC and LT-HSCs from the bone marrow LSK population of WT and CD82KO mice. Error bars, SEM; n=8-9 mice per strain (**p<0.001). (B) Flow cytometry analysis of the percentage of the LSK population from WT and CD82KO mice. n=8 mice per strain. (C) Flow cytometry analysis of the percentage of immune cells (B cells (B220), T cells (CD3) and myeloid cells (Gr1/Mac1)) within the bone marrow of WT and CD82KO mice. n=15 mice per strain. (D) Flow cytometry plots of DNA (Hoechst) and the proliferative nuclear antigen (Ki-67) expression of the bone marrow to measure the cell cycle status of LT-HSC population from WT and CD82KO mice. Error bars, SEM; n=3 independent experiments (*p<0.05 and **p<0.01). (E) Flow cytometry analysis of BrdU expression in the LT-HSC population after 3 days of BrdU incorporation in vivo. Error bars, SEM; n=3 independent experiments (**p<0.01).

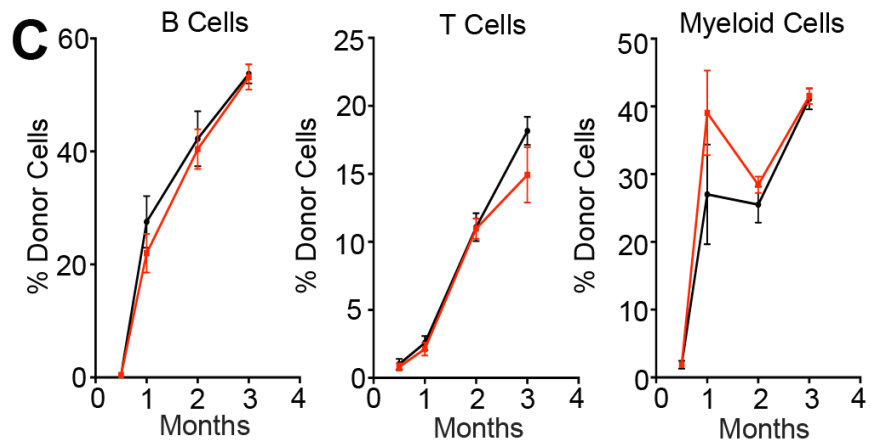
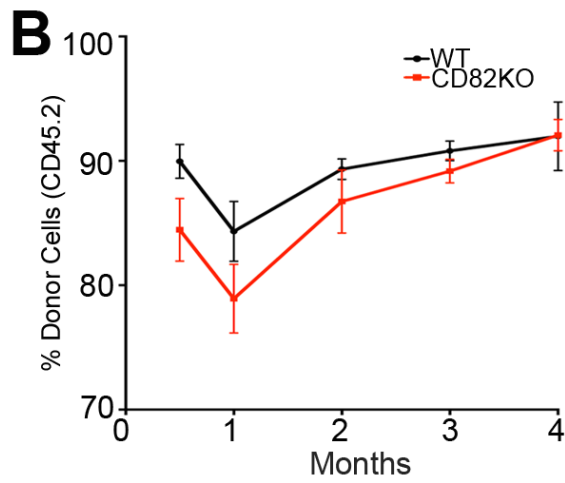
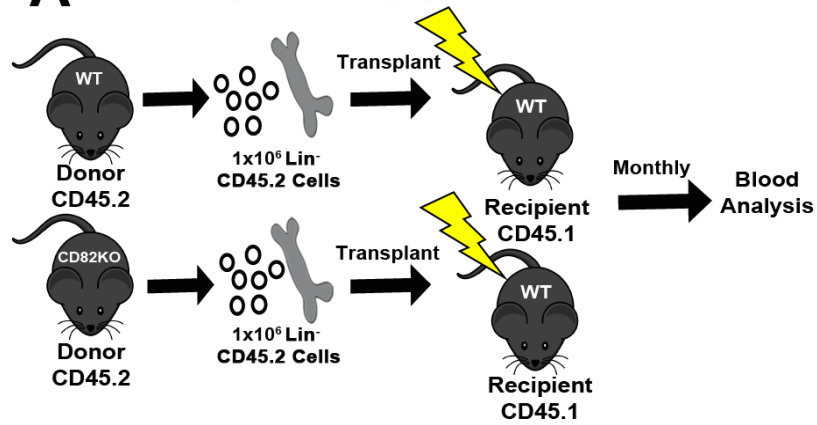
maintenance of the LT-HSC population.

To address the cause of the reduction in LT-HSCs in the CD82KO bone marrow, we first analyzed extramedullary tissues and identified no increase in the number of LT-HSCs in CD82KO mice (data not shown). Therefore, extramedullary hematopoiesis does not appear to contribute to the observed reduction in bone marrow LT-HSCs. Next, we analyzed the proliferation and cell cycle status of CD82KO LT-HSCs. Combining the Ki67 marker with DNA content analysis, we find that CD82KO LT-HSCs increase cell cycle entry (Figure 1D). We also completed bromodeoxyuridine (BrdU) incorporation assays to assess proliferation changes in vivo, identifying a significant increase in BrdU⁺ LT-HSCs within the bone marrow of CD82KO mice (Figure 1E). These data suggest that the cell cycle activation of the CD82KO LT-HSCs ultimately results in the reduction of the quiescent LT-HSC population localized to the bone marrow. Collectively, these data are consistent with a previous study using an alternative CD82KO mouse model, which described a similar reduction in the LT-HSCs, resulting from cell cycle entry (Hur et al., 2016).

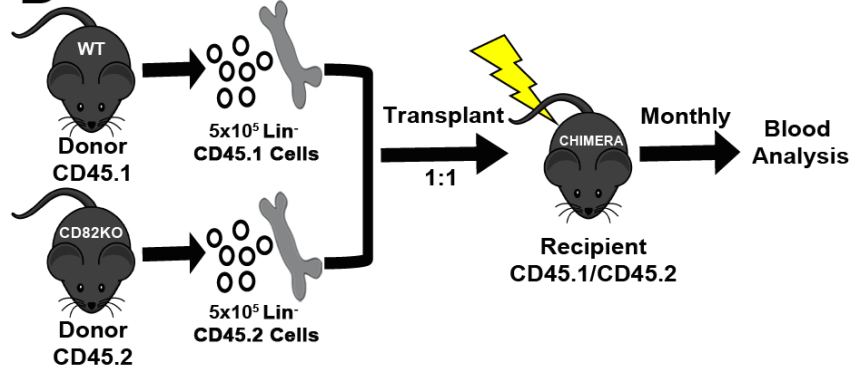
2.3.2 Reduced competitive repopulation capacity of CD82KO HSPCs.

To assess how CD82 deficiency impacts stem cell repopulation, we carried out long-term engraftment assays where we analyzed the reconstitution ability of WT and CD82KO HSPCs. Using a congenic mouse system, we transplanted donor WT or CD82KO Lin⁻ HSPCs into lethally irradiated recipients (Figure 2A). The B6.SJL-*Ptprca*^a *Pepcb*^b/*BoyJ* (CD45.1) mouse strain was used as recipients because they carry the differential pan leukocyte marker CD45.1, which can be distinguished from the WT and CD82KO donor cell populations that express the CD45.2 allele. Monthly peripheral blood analysis confirmed a similar engraftment of both CD82KO and WT donor-derived CD45.2 cells (Figure 2B). Additionally, analysis of the immune cell phenotype of the recipient mice identified no significant changes in the production of B, T, or myeloid cells (Figure

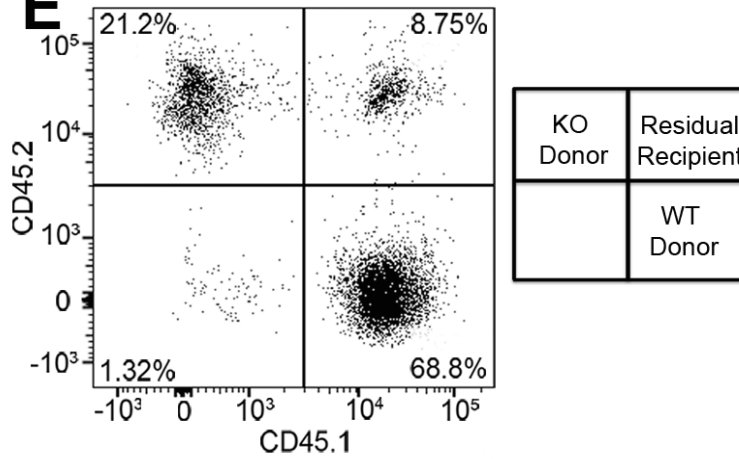
A Non-Competitive Repopulation



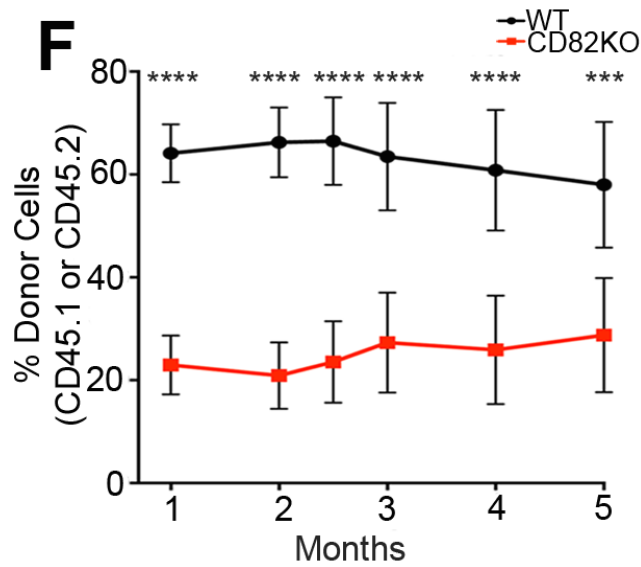
D Competitive Repopulation



E



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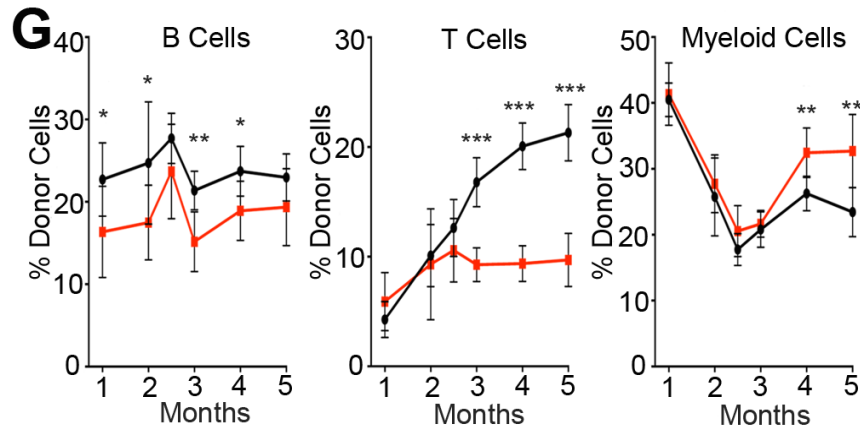


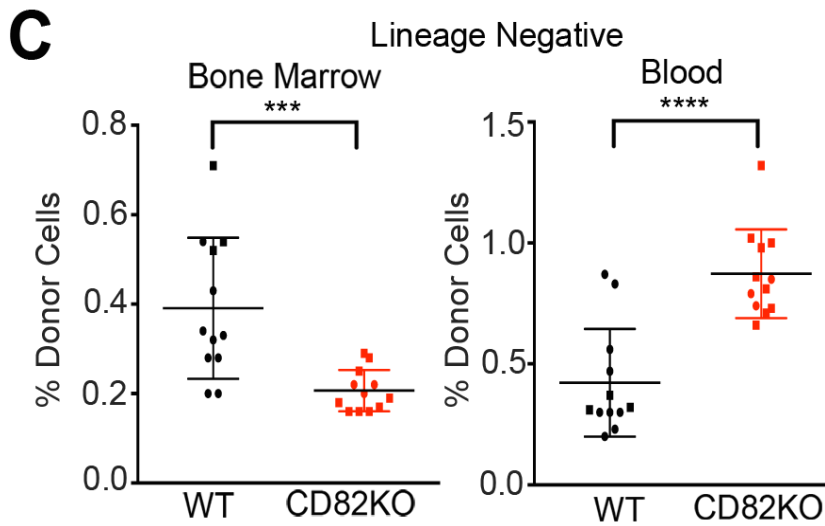
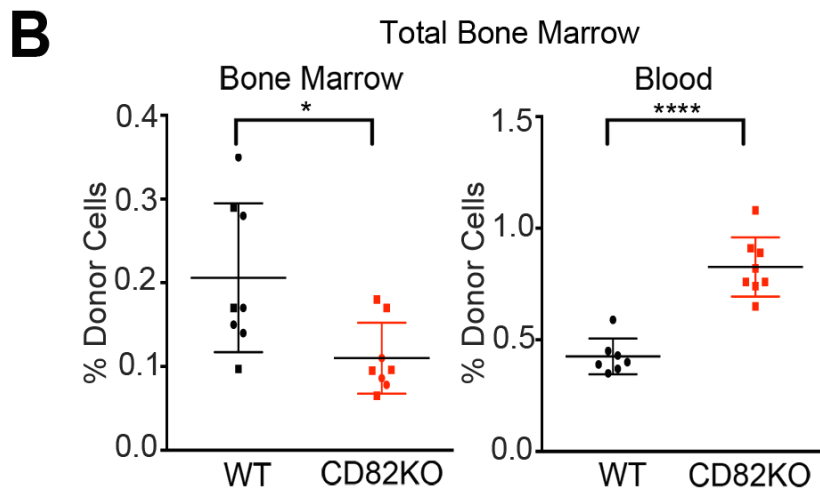
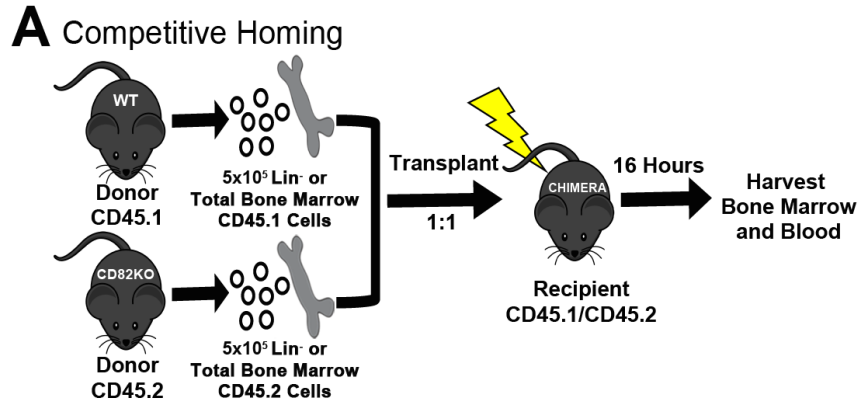
Figure 2.2: CD82KO HSPCs display decreased repopulation in a competitive environment. (A) Experimental scheme for the non-competitive repopulation experiment. (B) The percentage of donor cell repopulation of peripheral blood collected monthly from tail bleeds. Donor cell chimerism (CD45.2) status measured via flow cytometry. $n=6$ mice per strain. (C) Flow cytometry analysis of the percentage of donor immune cells (B cells (B220), T cells (CD3) and myeloid cells (Gr1/Mac1)) from donor (CD45.2) population in figure 2C. (D) Experimental scheme for the competitive repopulation experiment. (E) Representative flow cytometry plot for the competitive repopulation assay gated for donor cells (WT, CD45.1 and CD82KO, CD45.2) in the peripheral blood recipient mice. (F) The percentage of donor cell repopulation of peripheral blood collected monthly from tail bleeds. Donor cell chimerism (WT, CD45.1 and CD82KO, CD45.2) status measured via flow cytometry. Error bars, SEM; $n=7$ mice per strain ($***p<0.001$ and $****p<0.0001$). (G) Flow cytometry analysis of the percentage of donor immune cells (B cells (B220), T cells (CD3) and myeloid cells (Gr1/Mac1)) from donor population in figure 2F. Error bars, SEM; $n=7$ mice per strain ($*p<0.05$, $**p<0.01$, and $***p<0.001$).

2C). Therefore, CD82KO HSPCs have the capacity to repopulate a recipient and generate similar percentages of differentiated immune cells.

Next, we went on to assess the long-term engraftment potential of CD82KO HSPCs when transplanted into a competitive environment. Isolated Lin⁻ HSPCs were harvested from WT (CD45.1) and CD82KO (CD45.2) donors and transplanted into lethally irradiated chimeric mice (CD45.1/CD45.2) at a ratio of 1:1 (Figure 2D). The use of chimeric mice enables us to distinguish the WT (CD45.1) and the CD82KO (CD45.2) donor cells from the CD45.1/2 recipient cells by flow cytometry. After transplant, blood cell chimerism was analyzed monthly using flow cytometry to measure repopulation. Under these competitive conditions, we identify a significant decrease in the repopulation capacity of CD82KO derived cells (CD45.2) when compared to WT cells (CD45.1) (Figure 2E). These data suggest that while CD82KO HSPCs have the capacity to successfully engraft, when co-transplanted with WT HSPCs, CD82KO HSPCs display a decreased efficiency to reconstitute a recipient, indicating an overall reduction in HSPC fitness (Figure 2F). Additionally, we analyzed the immune cell differentiation potential of competitively engrafted HSPCs identifying a significant decrease of B and T cells derived from CD82KO HSPCs when compared to WT HSPCs (Figure 2G). Interestingly, we detect a significant increase in the amount of CD82KO-derived myeloid cells when compared to WT cells (Figure 2G), which suggests that CD82KO HSPCs present a myeloid skewing phenotype following competitive repopulation.

2.3.3 CD82KO HSPCs displayed reduced bone marrow homing.

Successful competitive repopulation requires the initial migration or homing of HSPCs to the bone marrow. Therefore, we next evaluated how CD82KO contributes to the early steps of HSPC repopulation by performing a competitive homing experiment (Figure 3A). Total bone marrow (Figure 3B) or Lin⁻ cells (Figure 3C) were harvested from CD82KO (CD45.2) and WT (CD45.1) mice and transplanted into lethally irradiated chimeric mice (CD45.1/CD45.2) at a ratio of 1:1. The blood and bone marrow of recipient mice were harvested 16 hours post injection to assess the chimerism status using flow cytometry.



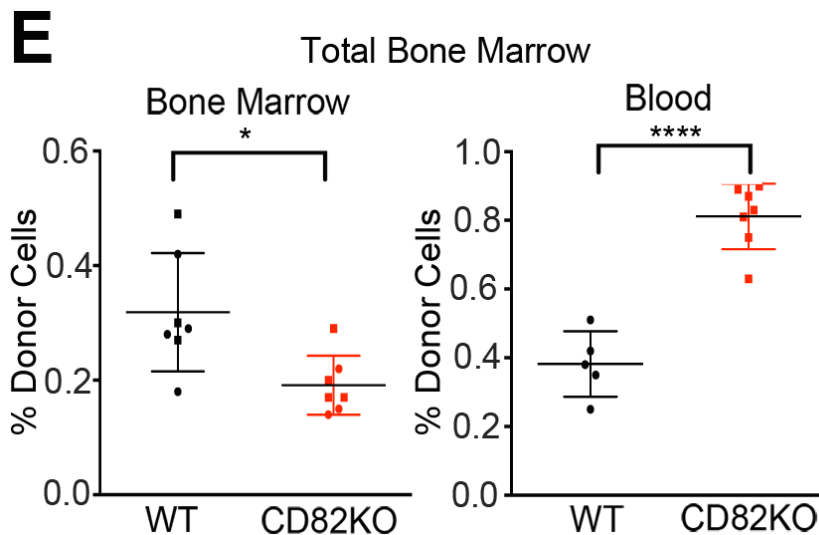
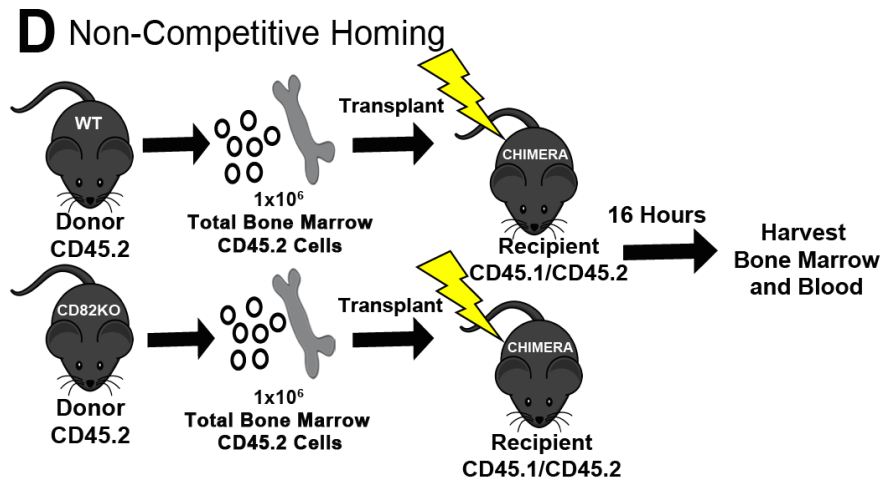


Figure 2.3: CD82KO HSPCs demonstrate decreased bone marrow homing. (A) Experimental scheme for the competitive homing experiment. (B) The percentage of total bone marrow and (C) lineage negative donor cells homed within the blood and bone marrow 16hrs post injection. Donor cell chimerism (WT, CD45.1 and CD82KO, CD45.2) status measured via flow cytometry. Error bars, SEM; n= 8-12 mice per strain (*p<0.05, ***p<0.001 and ****p<0.0001). (D) Experimental scheme for the non-competitive homing experiment. (E) The percentage of total bone marrow homed within the blood and bone marrow 16 hr post injection. Donor cell chimerism (CD45.2) status measured via flow cytometry. Error bars, SEM; n= 5-7 mice per strain (*p<0.05 and ****p<0.0001).

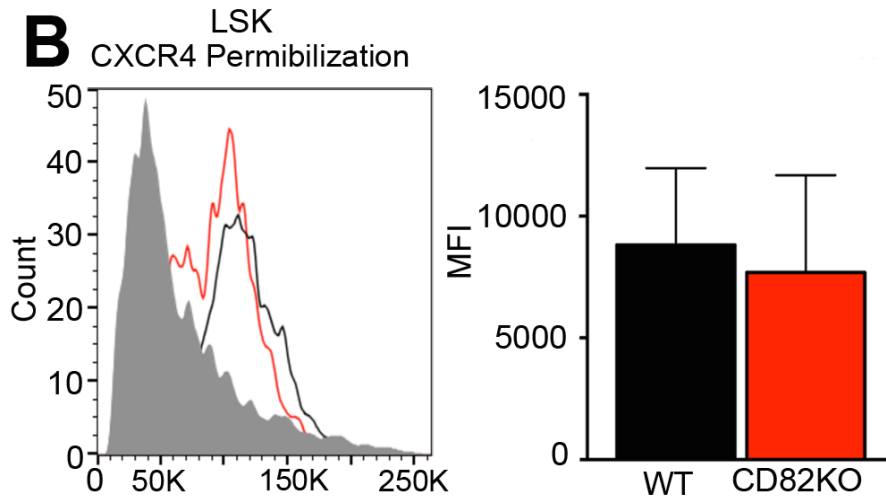
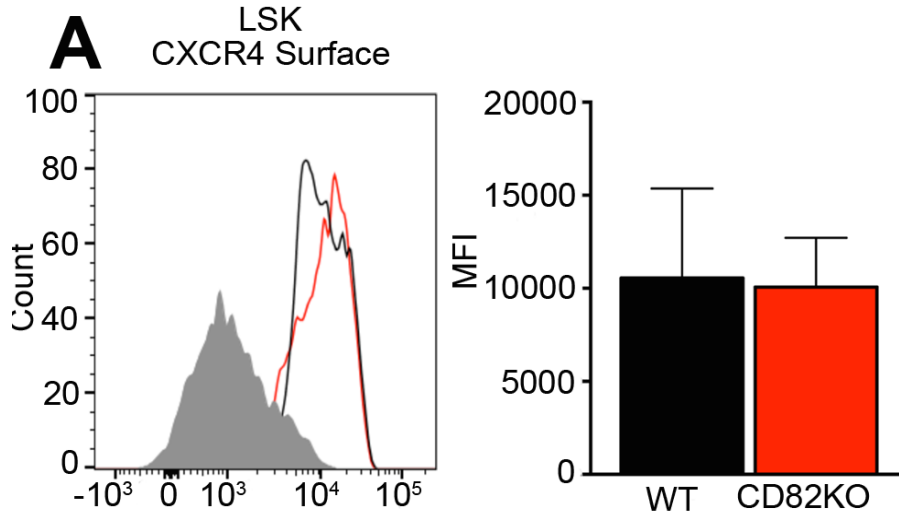
Analysis of the bone marrow indicates a significant decrease in the number of CD82KO cells that migrate to the bone marrow when compared to WT cells.

Consistent with the decrease in bone marrow homing, we measure a significant increase in the number of CD82KO cells detected within the blood when compared to WT cells. Additionally, we completed homing experiments with total bone marrow injected into a non-competitive environment (Figure 3D), identifying a similar homing defect with the CD82KO cells as that observed in the competitive environment (Figure 3E). Together, these data suggest that the reduced competitive repopulation capacity of the CD82KO HSPCs is likely due to the reduced bone marrow homing potential of these cells.

2.3.4 CD82KO does not impact CXCR4 expression or activity.

The CXCR4-CXCL12 signaling axis is a critical regulator of HSPC homing to and maintenance within the bone marrow. CXCR4 is highly expressed on HSPCs and serves as the chemokine receptor for CXCL12, which is produced by bone marrow stromal cells and controls HSPC homing, mobilization and localization (Nie et al., 2008; Prosper and Verfaillie, 2001; Sahin and Buitenhuis, 2012). To determine if the reduced homing behavior observed with the CD82KO HSPCs is due to changes in the expression of CXCR4, we measured the surface expression of CXCR4 by flow cytometry. In Figure 4A we detect no difference in the CXCR4 mean fluorescence intensity between CD82KO and WT LSKs. Recognizing that CXCR4 is internalized following activation, we also fixed and permeabilized WT and CD82KO LSKs to measure total CXCR4 expression. Similar to the surface expression, we find no difference in mean fluorescence intensity between CD82KO and WT LSK HSPCs, indicating no changes in overall CXCR4 expression (Figure 4B).

In addition to modulating the surface expression of membrane proteins, tetraspanins also have the capacity to cluster membrane-associated proteins promoting their activation (Marjon et al., 2016; Termini et al., 2016). As such, we went on to evaluate CXCR4 signal transduction downstream of CXCL12 activation with a focus on AKT and ERK signaling. Phosphoflow cytometry of phosphorylated AKT and ERK revealed no change in the basal or tonic signaling



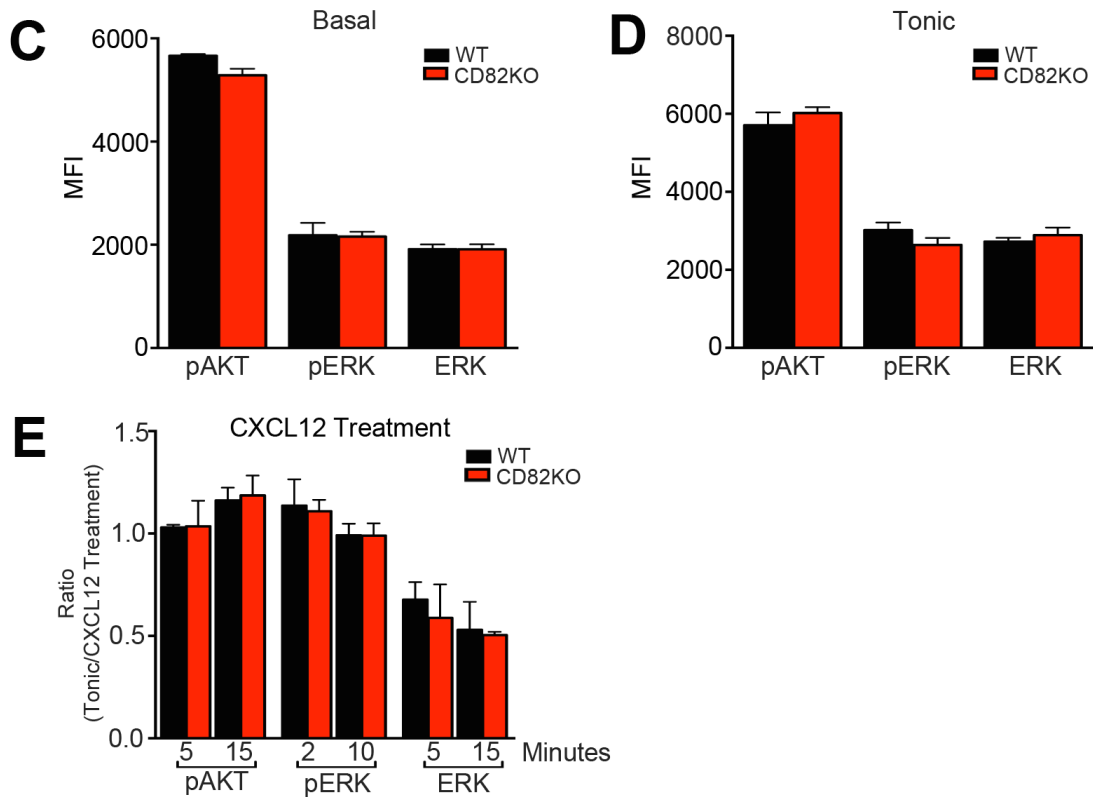


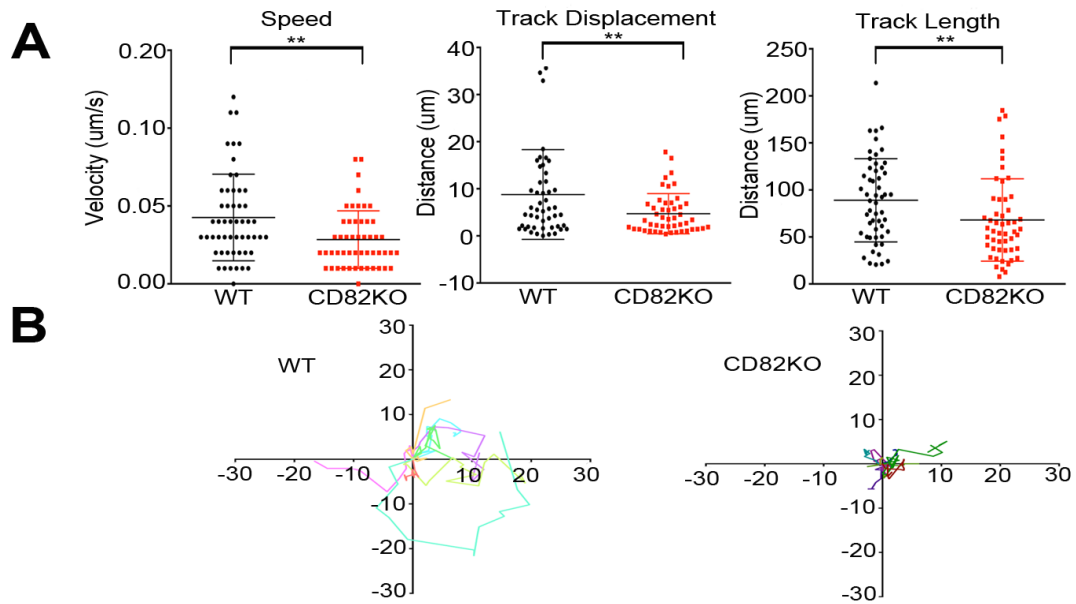
Figure 2.4: CD82KO does not impact CXCR4 expression or activity. Representative histograms of (A) Surface and (B) total (permeabilized) CXCR4 expression of bone marrow HSPCs from WT and CD82KO mice. Flow cytometry analysis measured the mean fluorescence intensity (MFI) of CXCR4 on the LSK population. Phosphoflow cytometry analysis of (C) basal and (D) tonic (1hr serum starvation) conditions to assess the mean fluorescence intensity (MFI) of pAKT, pERK and total ERK signaling of the LSK population. n= 3 mice per strain. (E) Phosphoflow cytometry analysis of SDF-1 treatment at various time points post 1hr of serum starvation to assess pAKT, pERK and total ERK signaling of the LSK population. Quantification ratio calculated by dividing tonic signaling by SDF-1 treatment conditions. n= 3 mice per strain.

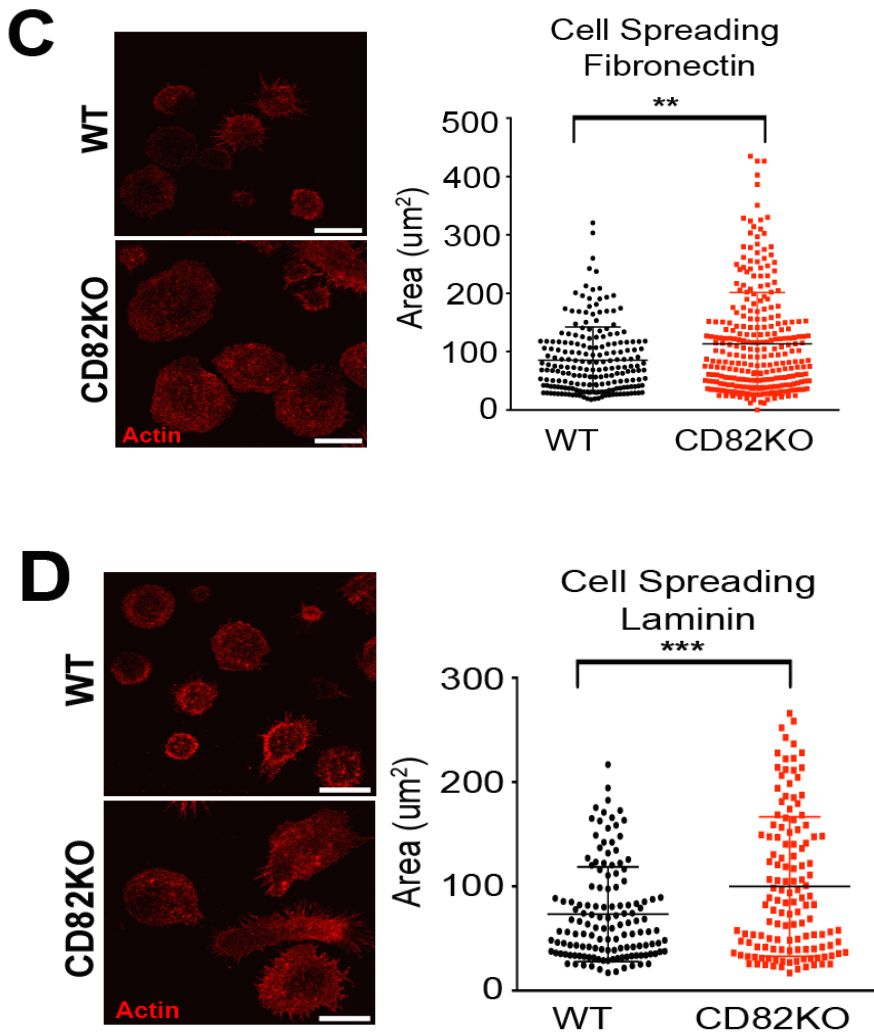
levels between WT or CD82KO LSKs (Figure 4C and Figure 4D). Moreover, following CXCL12 stimulation, we detected similar AKT and ERK phosphorylation, indicating no difference in CXCR4 activity between WT and CD82KO LSKs (Figure 4E). Taken together, the CD82KO homing defect does not result from altered CXCR4 expression or activation.

2.3.5 CD82KO HSPCs display a disruption in migratory behavior.

To further evaluate the mechanism by which CD82KO HSPCs have reduced capacity to home and engraft, HSPC migration was assessed *in vitro*. Lin⁻ HSPCs were isolated from CD82KO and WT mice and imaged by live cell confocal microscopy. The time-lapse images were analyzed using the Imaris Tracking Software to obtain measurements of track speed, displacement and length for individual HSPCs. Figure 5A illustrates that isolated CD82KO HSPCs have a significant decrease in track speed, track displacement, and track length, when compared to WT HSPCs. Moreover, single-cell trajectory rose-plots indicate that CD82KO HSPC track movements are short and consolidated at the point of origin and lack directional movement, when compared to WT HSPC tracks (Figure 5B). These data demonstrate a 2D migratory defect for CD82KO HSPCs, which is consistent with the observed disruption in homing behavior, and further implicates an important role for CD82 in HSPC migration.

Adhesive strength and cell spreading play key roles in generating the required traction for cell migration. Therefore, we next measured the cell spreading capacity of WT and CD82KO cells plated on specific extracellular matrices. Isolated Lin⁻ HSPCs were plated on either fibronectin or laminin for 4 hours before being fixed and fluorescently labeled with the cytoskeletal marker phalloidin. Confocal microscopy images were analyzed using ImageJ software to quantify the area of cell spreading. In Figures 5C and 5D, we measure a significant increase in the area of CD82KO Lin⁻ HSPCs plated on fibronectin or laminin when compared to WT HSPCs. To further assess the role of CD82 deficiency in HSPC adhesion, we quantified the surface expression of specific adhesion molecules, including integrins α_4 , α_6 , β_1 and CD44. Flow cytometry analysis of the CD82KO LSK HSPCs measured a significant decrease in mean





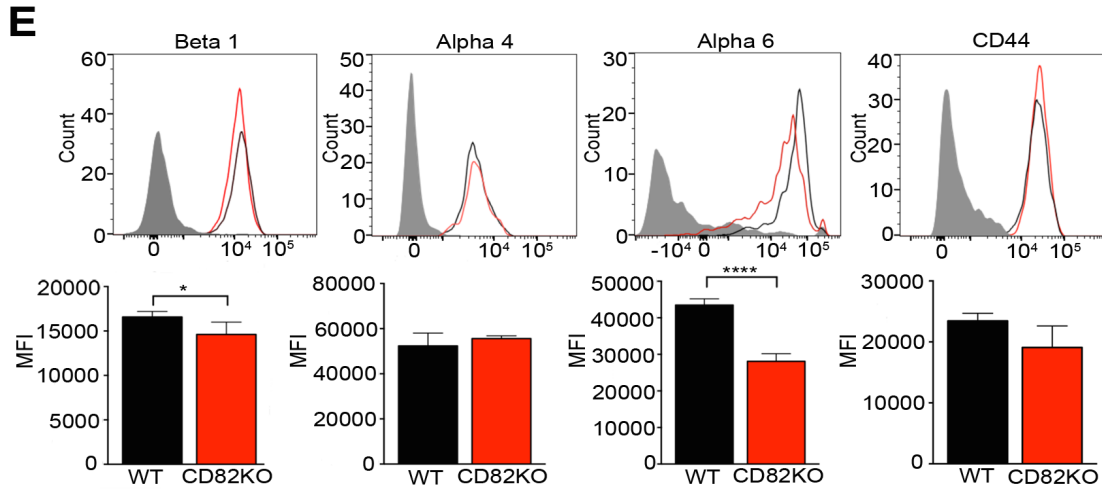
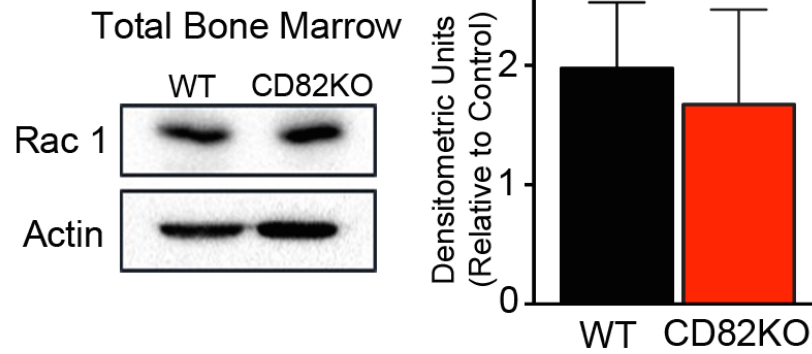
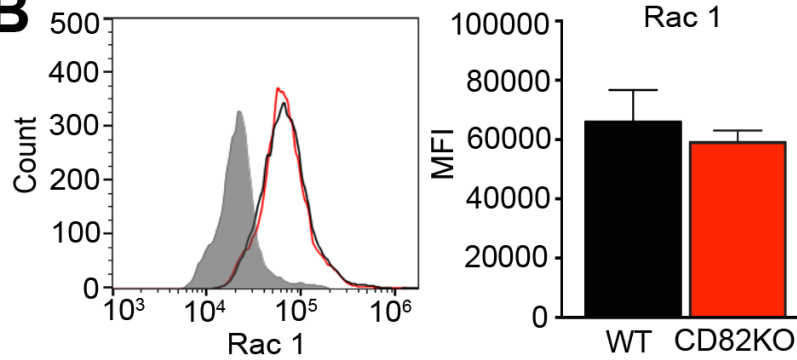
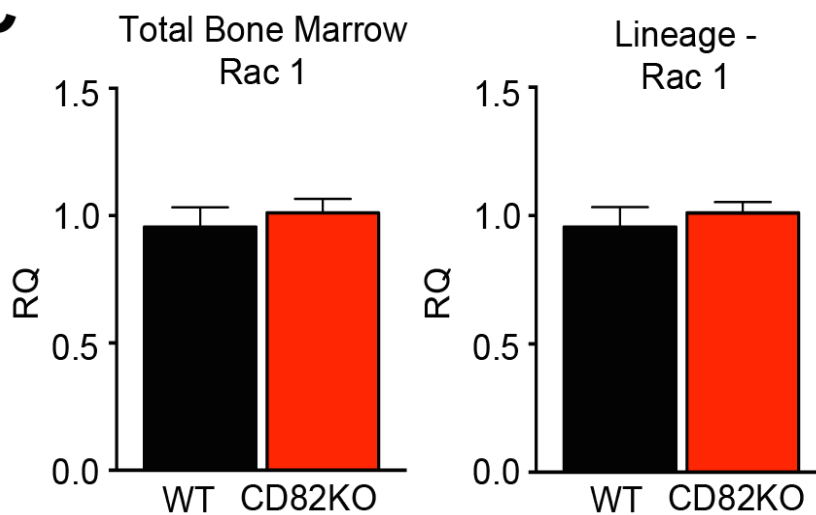


Figure 2.5: CD82KO HSPCs display decreased migration and increased cell spreading. (A) Live cell confocal imaging analysis of HSPC migration from WT and CD82KO bone marrow. IMARIS imaging software was used to assess track speed, track displacement and track length. Error bars, SEM; n= 2 independent experiments (**p<0.01). (B) Rose plots were generated using the WT and CD82KO HSPC track length coordinates. Cell spreading potential of isolated WT and CD82KO HSPCs plated on (C) fibronectin and (D) laminin. Representative images show actin staining used to quantify HSPC area using ImageJ software. Error bars, SEM; n= 2 independent experiments (**p<0.01 and ***p<0.001). (E) Representative histograms of surface adhesion molecule expression of bone marrow HSPCs from WT and CD82KO mice. Flow cytometry analysis measuring the mean fluorescence intensity (MFI) of each adhesion molecule on the LSK population. Error bars, SEM; n= 3 mice per strain (*p<0.05 and ****p<0.0001).

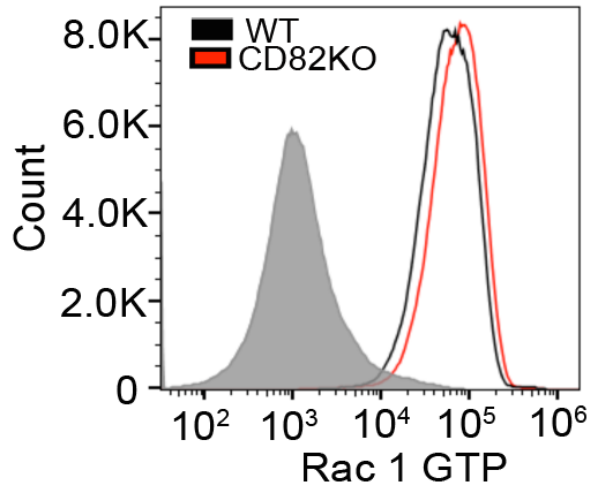
fluorescent intensity of α_6 , and a modest decrease in β_1 , with no mean fluorescent intensity changes detected for α_4 and CD44 when compared to WT HSPCs (Figure 5E). Collectively, the decreased surface expression of integrins α_6 and β_1 and the enhanced cell spreading likely contribute to the decrease in homing and engraftment potential seen with the CD82KO HSPCs.

2.3.6 CD82KO HSPCs have increased Rac1-GTPase activity.

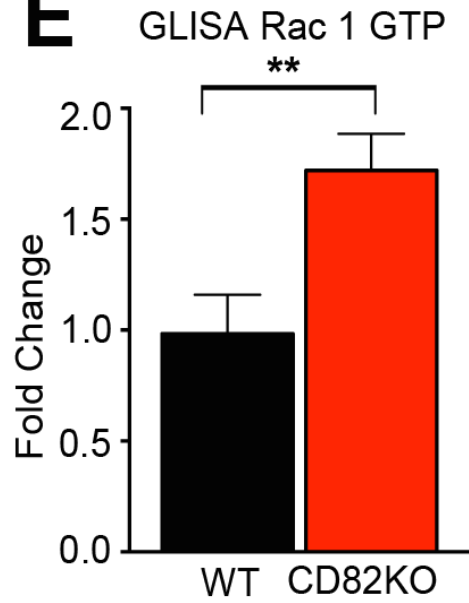
Rho GTPases play an essential role in cell spreading and cell migration. Moreover, previous studies have demonstrated that the specific RhoGTPase, Rac, can control hematopoietic stem cell activities such as marrow homing and retention (Cancelas et al., 2005; Dorrance et al., 2013; Gu et al., 2003; Liu et al., 2011b; Shang et al., 2011; Williams et al., 2008; Yang et al., 2001). Based on the increased cell spreading observed in Figures 5C,D, by the CD82KO cells, we set out to measure Rac1 expression and activity. Western blot analysis of total bone marrow suggests no change in Rac1 expression when comparing WT and CD82KO cells (Figure 6A). Additional flow cytometry analysis of the LSK cells also indicates no difference in Rac1 expression when comparing WT and CD82KO cells (Figure 6B). Similarly, we were unable to detect any changes in Rac1 gene expression between WT and CD82KO mice when analyzing either the total bone marrow or the Lin⁻ fraction of HSPCs (Figure 6C). Recognizing that Rac activity is a key contributor to cell spreading, we went on to measure changes in Rac1 activity by flow cytometry using an active Rac1-specific antibody. Figure 6D indicates that the CD82KO LSK HSPCs have increased Rac1 activity when compared to WT cells. A similar result was measured using an active Rac1 ELISA activation assay, where we identified a significant increase in the active form of Rac1 in the CD82KO Lin⁻ bone marrow lysates when compared to WT (Figure 6E). Collectively, these data suggest that while CD82 deficiency does not impact overall Rac1 expression, Rac1 hyperactivation is detected in the HSPCs upon CD82KO. Lastly, we wanted to determine whether the measured increase in Rac1 activity contributes to the disruption in cell migration and bone marrow homing observed with the CD82KO HSPCs. As such, we completed cell migration studies of the CD82KO Lin⁻ HSPCs that were

A**B****C**

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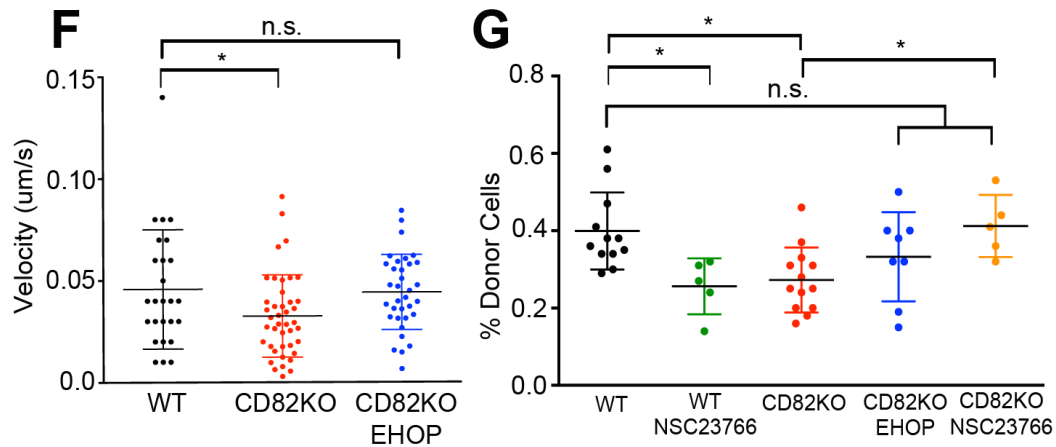


Figure 2.6: Rac1 hyperactivity in the CD82KO HSPCs contributes to diminished HSPC homing. (A) Western blot analysis of WT and CD82KO total bone marrow measuring Rac1 expression. Densitometry was used to quantify Rac1 expression relative to an actin control. n=3 independent experiments. (B) Representative histogram of surface Rac1 expression. Flow cytometry analysis measured the MFI of Rac1 on the LSK population. n=3 mice per strain. (C) Rac1 mRNA expression of total bone marrow and FACS sorted lineage negative cells using Real Time qPCR. Rac1 expression was normalized to GAPDH to obtain relative quantification (RQ) values. n= 3 independent experiments. (D) Representative histogram of Rac GTP expression. Flow cytometry analysis measured Rac GTP on WT and CD82KO lineage negative bone marrow cells. (E) Rac1 activity of WT and CD82KO lineage negative cells measured using GLISA assay. Quantification represents fold change relative to WT. Error bars, SEM; n= 3 independent experiments (**p<0.01). (F) Live cell confocal imaging analysis of HSPC migration from WT and CD82KO bone marrow. CD82KO HSPCs were treated with EHOP-016 1hr prior to migration. IMARIS software was used to assess track speed. Error bars, SEM; n=2 independent experiments, One-Way ANOVA (*p<0.05 and n.s., non-significant). (G) The percentage of total bone marrow cells homed within bone marrow 16rs post injection. Total bone marrow were treated with EHOP-016 or NSC23766 1hr prior to injection. Donor cell chimerism (CD45.2) status was measured via flow cytometry. Error bars, SEM; n= 5-13 mice per strain, One-Way ANOVA (*p<0.05 and n.s., non significant).

treated with and without the Rac1- specific inhibitor EHOP-016. Analysis of track speed indicates that inhibition of Rac1 hyperactivation restores the velocity of the CD82KO HSPCs to WT HSPC speeds (Figure 6F). Moreover, we completed the bone marrow homing experiments with WT and CD82KO total bone marrow, where we also pretreated cells with EHOP-016 or another Rac1 inhibitor, NSC23766 for 1 hr prior to injection. Bone marrow was isolated 16 hr after injection and analyzed as previously described. Again, we measure a decrease in bone marrow homing of CD82KO cells when compared to WT cells. Moreover, we find that WT cells pretreated with the Rac1 inhibitor NSC23766 display a significant reduction in bone marrow homing, consistent with previous reports establishing the importance of Rac1 in HSC migration (Cancelas et al., 2005; Dorrance et al., 2013; Gu et al., 2003; Liu et al., 2011b; Yang et al., 2001). In contrast, when Rac1 hyperactive CD82KO cells were pretreated with Rac1 inhibitors, bone marrow homing capacity was restored to WT levels (Figure 6G). Taken together, CD82 deficiency inhibits HSPC migration to the bone marrow at least in part by promoting a shift in the balance of Rac1 activity to a hyperactivated state.

2.4 Discussion

Successful clinical outcomes from transplantation depend upon the efficient bone marrow homing and engraftment of HSPCs. The current study analyzing HSPCs from CD82KO mice provides strong evidence that the tetraspanin CD82 regulates the maintenance of LT-HSCs within the bone marrow as well as both processes of homing and engraftment. Moreover, if we inhibit the activation of the Rac1 GTPase in the CD82KO HSPCs, we recover the homing defect observed upon loss of CD82. These results have led us to propose a model whereby the CD82 scaffold functions to 1) promote bone marrow niche interactions that maintain cell cycle quiescence and 2) enhance the bone marrow homing required for HSPC engraftment through the modulation of Rac1 activation.

Regulation of HSPC activation has been described for a number of tetraspanins. For example, tetraspanin CD81 was shown to be important for the re-entry of HSCs to quiescence through the inhibition of AKT signaling pathway (Lin et al., 2011). The polarized organization of CD81 on the surface of murine HSCs led to the deactivation of AKT and nuclear translocation of FoxO1a, which was important for the re-entry of HSCs into quiescence from a highly proliferative state. This study also demonstrated that CD81KO HSCs have a marked engraftment defect. Previous work by Rossi et al. also demonstrated a role for tetraspanin CD63 in the regulation of HSPC proliferation through its interaction with tissue inhibitor of metalloproteinase-1 (TIMP1) and the activation of the PI3K/AKT signaling pathway (Rossi et al., 2015). Furthermore, they found that upon TIMP1 treatment of HSPCs, cyclin D1 gene expression was increased downstream of AKT phosphorylation. Previous work from our group identified a correlation between CD82 membrane organization and cell cycle progression (Larochelle et al., 2012), where CD34+ HSPCs sorted based on the G0 cell cycle stage showed a polarized membrane distribution of CD82, suggesting that CD82 organization may also impact HSPC quiescence. More recently, a CD82KO mouse model was used to identify CD82 as an important regulator of LT-HSC quiescence (Hur et al., 2016). In this study, a cell line system was predominantly used to describe a mechanism where CD82 binds to the Duffy antigen receptor complex on bone marrow macrophages downstream of TGF β activation to modulate LT-HSC quiescence. In our own study, we use an alternative CD82KO mouse model that was previously described (Jones et al., 2016; Wei et al., 2014) and identify a similar LT-HSC defect with the loss of CD82. Our cell cycle activation studies demonstrating a loss of LT-HSC quiescence, nicely parallel the findings of the Hur et al. study. However, we then went on to characterize the fitness of the CD82KO HSPCs, further identifying homing and engraftment defects.

Once introduced into the blood stream, HSPCs have the capacity to migrate back to the bone marrow in a process that involves intercellular signaling and adhesive interactions (Caocci et al., 2017; Vermeulen et al., 1998). The

major chemokine signal responsible for this homing process is the CXCR4/CXCL12 signaling axis. As mentioned previously, CXCR4 on the surface of HSPCs migrates toward its ligand, CXCL12, produced within the bone marrow. Recognizing the critical importance of this receptor/ligand interaction in homing, we thoroughly investigated the potential influence of CD82KO on receptor expression and activation. However, we demonstrate that CD82 deficiency results in no significant impact on CXCR4 expression or activation. Therefore, the observed CD82-mediated homing defect appears to occur through an alternative mechanism, which led us to analyze *in vitro* adhesion and migration behaviors.

Tetraspanin-tetraspanin and tetraspanin-integrin interactions are known to regulate adhesion and migration in a variety of different biological systems. Focusing in on HSPCs specifically, the tetraspanin CD63 in complex with TIMP1 and the β 1 integrin were shown to modulate adhesion and migration of human CD34+ HSPCs (Wilk et al., 2013). Moreover, the tetraspanin CD9 was shown to regulate HSPC migration and adhesion, although its role in homing remains a bit unclear. In the initial study described by Leung et al., CD9 expression on human cord blood CD34+ HSPCs was shown to be modulated by SDF-1 and CXCR4 activity, resulting in increased HSPC migration and adhesion (Leung et al., 2011). Additionally, this group went on to show that enhancing CD9 expression on the surface of CD34+ HSPCs with the treatment of a protein kinase C agonist ϵ , ingenol 3,20 dibenzoate (IDB), increases homing to the bone marrow. However, in a follow up study, Desmond et al. found that while IDB increases CD9 expression on CD34+ HSPCs, it does not increase HSPC homing compared to control treated CD34+ HSPCs intravenously injected into NSG mice (Desmond et al., 2011). Our own work with CD82 demonstrated that human CD34+ HSPC pretreatment with a CD82-specific neutralizing antibody significantly reduced bone marrow homing of these cells in an animal model (Larochelle et al., 2012). In the current study, we identify a significant defect in the bone marrow homing and engraftment capacity of CD82KO HSPCs, demonstrating a clear role for CD82 in these two processes. We also detect a

myeloid skewing phenotype, which is consistent with a HSPC aging phenotype (Liang et al., 2005; Rossi et al., 2005). Interestingly, aged HSPCs are also known to have reduced bone marrow homing and engraftment capabilities (Liang et al., 2005; Morrison et al., 1996), which suggests that CD82 may impact HSPC aging.

Once injected into the peripheral blood, homing is the initial process that enables the HSPCs to traffic to the bone marrow, where the cells will ultimately engraft and repopulate the blood and immune cell lineages. Despite the clear homing defect we observe with the CD82KO HSPCs, it is interesting to note that non-competitive engraftment assays illustrate no detectable impact of CD82 expression. Only in a competitive environment do we identify a significant disruption in repopulation. These data suggest that perhaps CD82KO HSPCs have a reduced rate of migration and homing, however, when given unlimited time, as in the non-competitive engraftment, the CD82KO cells will eventually make their way to the bone marrow where they have the capacity to repopulate. Conversely, in a competitive environment, the WT HSPCs home more efficiently to the bone marrow, where they perhaps fill many of the available niche sites, reducing the engraftment capabilities of the CD82KO HSPCs with delayed bone marrow homing. These data are further supported by the *in vitro* migration data in Figure 5, where CD82KO cells display reduced 2D migration. Additionally, we find that CD82KO promotes increased cell spreading, which likely contributes to the reduction in cell migration. Previous work focusing on bone marrow-derived dendritic cells from the CD82KO mice identified a similar cell spreading defect when plated on fibronectin (Jones et al., 2016). Moreover, they went on to show altered GTPase activities in the CD82KO cells illustrating an important role for tetraspanins in the regulation of GTPase activity.

Tetraspanins can interact with Rho GTPases at the plasma membrane and mediate downstream signaling (Termini and Gillette, 2017). In fact, evidence in the literature suggests that tetraspanins are important for the regulation of both Rac1 expression and activity. For example, the overexpression of CD82 was shown to inhibit Rac1 activity resulting in actin disorganization (Liu et al., 2012b), whereas the interaction of Rac1 with the c-terminal tail of CD81 led to an overall

decrease in Rac1 expression (Tejera et al., 2013). Similarly, tetraspanins CD9 and CD151 are both implicated in the activation of Rac1 (Arnaud et al., 2015; Hong et al., 2012). The Rho family GTPases function as molecular switches that can coordinate cytoskeletal rearrangements, which ultimately impact a range of cellular behaviors including migration and adhesion. Among the Rho family GTPases, Rac is known to play a clear role in HSPCs migration and homing (Cancelas et al., 2005; Dorrance et al., 2013; Gu et al., 2003; Liu et al., 2011b; Ridley, 2001; Shang et al., 2011; Williams et al., 2008; Yang et al., 2001). Conditional knock out mouse studies for Rac1 demonstrated both impaired HSPC engraftment and reduced adhesion to fibronectin (Gu et al., 2003). Similarly, knock out of the HSPC specific Rac2 identified defects in actin cytoskeleton remodeling and $\alpha4\beta1$ -mediated adhesion (Yang et al., 2001). Interestingly, in the case of Rac2 deficiency, HSPCs showed increased migration toward a CXCL12 gradient, which the authors suggest may be the result of compensatory upregulation of Rac1 and Cdc42 activities (Yang et al., 2001). Collectively, these studies illustrate the significant impact of Rac knock out on HSPCs, but much less is known about how Rac hyperactivation alters phenotype. In a recent study, c-Kit⁺ HSPCs overexpressing a constitutively active form of Rac1 GTPase (Rac1 V12) displayed increased cell migration and adhesion (Chen et al., 2016). In contrast, a study by Shang et al, modulated endogenous Rac1 activation through manipulation of R-Ras expression and identified diminished bone marrow homing of HSPCs upon Rac1 hyperactivation (Shang et al., 2011). Our studies measuring changes in endogenous Rac1 activation identified a similar defect in bone marrow homing of HSPCs when Rac1 is hyperactive. These data suggest that once Rac1 activity goes beyond a certain threshold, cell migration is diminished. Previously, when Rac inhibitors have been used, decreased cell migration and adhesion are observed (Chen et al., 2016; Montalvo-Ortiz et al., 2012). Even in our own studies, we find that WT HSPC homing is decreased upon treatment with the Rac1 inhibitor, NSC23766. In contrast, the use of two different Rac1 inhibitors restored the bone marrow homing deficit of the CD82KO HSPCs. We speculate that upon Rac1

hyperactivation, the inhibitor reduces the Rac1 activity threshold, resulting in baseline migration and adhesion. However, when inhibitors are used to disrupt basal Rac1 activity, an overall reduction in migration and adhesion is observed (Chen et al., 2016; Montalvo-Ortiz et al., 2012). Together, these data highlight the importance of the tight regulation of a Rac1 activity threshold to maintain HSPC fitness. At this time, how the CD82 scaffold functions to regulate Rac1 activity remains unclear. While there is precedence from the literature that direct interactions can occur between tetraspanins and GTPases (Arnaud et al., 2015; Hong et al., 2012; Liu et al., 2012b; Tejera et al., 2013), we speculate that CD82 is more likely to modulate a Rac1 regulator, and thus affect Rac1 activity indirectly. For example, our own work has established a role for CD82 in the regulation of adhesion molecules $\alpha 4\beta 1$ and N-cadherin (Marjon et al., 2016; Termini et al., 2014), which can both modulate Rac activation (Arthur et al., 2002; Rose, 2006; Rose et al., 2007). Additionally, the tetraspanin CD151 was shown to facilitate interactions between the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ and several small GTPases (Hong et al., 2012). Alternatively, the modulation of specific guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) can also have a significant impact on small GTPase activity (Lawson and Burridge, 2014). Therefore, future studies will be directed at understanding how CD82 potentially modulates the expression and/or activity of key regulators of Rac1 activation.

The goal of the current study is to identify the mechanism by which CD82 regulates HSPC fitness with a focus on bone marrow homing and engraftment. Our data suggest that CD82 can not only modulate the activation of LT-HSCs, but also the overall fitness of HSPCs. Activation of LT-HSCs is likely related to how tightly HSPCs interact with specific components of the bone marrow microenvironment, although at this point, it is unclear how CD82KO impacts cellular localization within the niche. HSPC fitness is characterized in part by the successful bone marrow homing and engraft, for which our data demonstrate a key role for CD82. As such, we propose a model whereby CD82 serves to modulate the activation of Rac1, which significantly impacts the migration, adhesion and bone marrow homing behaviors of HSPCs. Finally, our detailed

insight into how CD82 contributes to the homing and engraftment of HSPCs implicates CD82 as an attractive therapeutic target to enhance the efficacy of HSPC transplantation therapies.

2.5 Materials and methods

2.5.1 Mice

C57BL/6 wild-type and B6.SJL-Ptprc^a Pepc^b/BoyJ mice were obtained from Jackson Laboratory. CD45.1/CD45.2 chimeric mice were generated by mating C57BL/6 and B6.SJL-Ptprc^a Pepc^b/BoyJ mice. CD82KO mice were generated from cre-loxP recombination (Wei et al., 2014). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico Health Science Center. Mice were housed under pathogen-free conditions in the UNM Animal Facility. The age and sex of mice were matched for each experiment.

2.5.2 Isolation and analysis of bone marrow cells

Bone marrow cells were isolated from the front and back limb bones using a mortar and pestle. Isolated bone marrow cells were passed through a 40µM strainer to remove bone fragments. Red blood cells were lysed using ACK lysis Buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA). To assess HSC populations, terminally differentiated cells were removed using a Lineage Cell Depletion Kit (Miltenyi Biotec). Isolated Lin⁻ cells were treated with Fc block (2.4G2; BD Pharmingen) prior to surface marker staining. Isolated Lin⁻ cells were stained with antibodies against surface markers using the following antibodies: mouse APC lineage cocktail (BD Pharmingen), BV605 CD117 (2B8; BD Pharmingen) Pe-Cy7 Sca-1 (D7; BD Bioscience), FITC CD34 (RAM34; BD Pharmingen), BV421 CD135 (A2F10.1; BD Pharmingen), BV510 CD48 (HM48-1; BD Pharmingen) and PE CD150 (Q38-480; BD Pharmingen). Labeled bone marrow samples were analyzed using the LSR Fortessa (BD Bioscience). For the sorting of the HSPC population, the mouse HSC isolation kit (BD Pharmingen) was used. Labeled cells were fluorescence activated cell sorted (FACS) using the iCyt Sony sy32000 Sorter to obtain the Lin⁻Sca-1⁺c-kit⁺ (LSK) population. In

addition, bone marrow immune cells were assessed using the following antibodies: PerCP-Cy5.5 CD3 (145-2C11; BD Pharmingen), BV421 B220 (RA3-6B2; Biolegend), Pe-Cy7 Ly6G (IA8; BD Pharmingen) and Pe-Cy7 CD11b (MI/70; BD Pharmingen). Labeled bone marrow samples were analyzed using the LSR Fortessa (BD Bioscience).

2.5.3 Non-competitive and competitive repopulation assay

For non-competitive repopulation assay, 1×10^6 donor Lin⁻ bone marrow cells from CD82KO or WT (CD45.2) were retro-orbitally into recipient BoyJ mice (CD45.1). For a competitive repopulation assay, 1×10^6 donor Lin⁻ bone marrow cells from CD82KO (CD45.2) and BoyJ (CD45.1) were retro-orbitally injected into recipient chimeric mice (CD45.1/CD45.2). Recipient mice underwent a total body irradiation, which was administered as a single dose of 10 Gy. Each month blood was taken from tail snips to assess chimerism and immune cell differentiation. Cells were treated with Fc block prior to staining with the following directly conjugated fluorescent antibodies: FITC CD45.1 (A20; BD Pharmingen) APC CD45.2 (104; BD Pharmingen) PerCP-Cy5.5 CD3 (145-2C11; BD Pharmingen), BV421 B220 (RA3-6B2; Biolegend), Pe-Cy7 Ly6G (IA8; BD Pharmingen) and Pe-Cy7 CD11b (MI/70; BD Pharmingen). Labeled blood samples were analyzed using the LSR Fortessa (BD Bioscience).

2.5.4 Cell cycle analysis

Lineage depleted bone marrow cells were incubated with Hoechst 33342 (Sigma-Aldrich) then labeled with PE Ki67 (16A8; BD Bioscience) in addition to LT-HSC surface markers: Lineage, Sca-1, CD117, CD135, CD34, CD48 and CD150. Cells were analyzed on the LSR Fortessa (BD Bioscience). For *in vivo* BrdU incorporation studies, one dose of 1 mg BrdU (5-bromo-2-deoxyuridine; Sigma-Aldrich) was intraperitoneally injected into mice and bone marrow was collected 3 days later. Bone marrow samples were processed using the FITC BrdU Flow kit (BD Bioscience) per the manufacturer's instructions. Processed bone marrow cells were labeled with surface markers: lineage, Sca-1, CD117, CD34, CD135 and CD150 to assess BrdU incorporation in the LT-HSC population. Cells were analyzed on the LSR Fortessa (BD Bioscience).

2.5.5 Non-competitive and competitive homing

For a non-competitive homing experiment, 1×10^6 donor bone marrow cells from CD82KO or WT (CD45.2) were retro-orbitally injected into recipient chimeric mice (CD45.1/CD45.2). For a competitive homing experiment, donor bone marrow cells from CD82KO were mixed at a ratio of 1:1 with BoyJ competition bone marrow cells into chimeric recipient mice. Competitive homing experiments were also performed using isolated HSPCs from the bone marrow as described above. Recipient mice underwent a total body irradiation, which was administered as a single dose of 10gy. Mice were euthanized 16 hours post injection to assess chimerism of the bone marrow and peripheral blood. Blood and bone marrow samples were treated with Fc block prior to labeling with directly conjugated fluorescent antibodies, FITC CD45.1 (A20; BD Pharmingen) and APC CD45.2 (104; BD Pharmingen) to assess chimerism. Samples were analyzed on the LSR Fortessa (BD Bioscience).

2.5.6 Cell surface expression

Bone marrow cells were analyzed for the surface expression of PE CXCR4 (L276F12; Biolegend), PE β 1 integrin (HM B1-1; BD Pharmingen), PE α 4 integrin (9C10; BD Pharmingen), PE- α 6 integrin (GoH3; BD Pharmingen) and PE-CD44 (IM7; BD Pharmingen) on the LSK population. In addition, we also assessed Rac-1 (Cytoskeleton) surface expression. Isolated bone marrow cells were treated with Fc block prior to staining with the LSK markers. All samples were labeled in MACs Buffer (PBS, 0.5% BSA, 2mM EDTA, pH 7.2; Miltenyi Biotec) for 30 min on ice. Samples were washed three times with MACs buffer after staining and analyzed using the LSR Foretessa (BD Bioscience). Histograms were created using FlowJo software.

2.5.7 Phosphoflow cytometry

Basal signaling activity was assessed by fixing isolated bone marrow cells with 4% Paraformaldehyde and permeabilizing with 100% methanol. Tonic signaling activity was assessed by serum starving bone marrow cells in SFEM for 1hr at 37°. Starved cells were then fixed and permeabilized. After 1hr of serum starvation, bone marrow cells were also treated with 100ng/mL SDF-1 for 2, 5, 10

and 15 minutes. After each time point, samples were fixed and permeabilized. Permeabilized samples were stained for LSK markers and the following signaling molecules: p-AKT Pacific Blue (BD Pharmingen), p-ERK FITC (Biolegend), and MEK2 PE (BD Pharmingen). Samples were analyzed on the LSR Fortessa (BD Bioscience). The ratio of SDF-1 treatment was calculated by dividing Tonic mean fluorescence intensity (MFI) by SDF-1 treatment MFI.

2.5.8 HSPC migration

The 4-chamber coverslips (Thermo Scientific) were coated with fibronectin (50 μ g/mL in PBS; Millipore). Isolated HSPCs were plated at 250,000 cells/well on fibronectin in StemSpan™ SFEM (StemCell Technologies) media supplemented with murine cytokines: IL-3, SCF and FLT-3 (20ng/mL; Peprotech) overnight at 37°C. For EHOP-016 treatment, isolated HSPCs were treated with 5 μ M EHOP-016 for 1hr at 37°C prior to plating. Each well was washed twice with PBS to remove non-adherent cells. CO₂ independent media supplemented with 0.5% FBS was added to each well for 1hr and migration was measured using a Zeiss Axiovert confocal microscope. Images were taken every 10 seconds for 50 min and were analyzed using the ImarisTrack Software (Bitplane Oxford Instruments Co.) to measure track length, track displacement and speed. The single cell trajectory rose-plots were produced from track position parameter data generated from the Imaris Tracking Software. The beginning X, Y position and ending X, Y position of each HSPC was taken into account. Using the position data of each HSPC, each track was normalized to shift each HSPC to an origin of (0,0). A total of 15 positions between the beginning and end positions were randomly selected in order to produce a track. For each condition, a total of 10 HSPCs were assessed.

2.5.9 Cell spreading

The 8-chamber glass coverslips (Thermo Scientific) were coated with 50 μ g/mL of fibronectin. Isolated HSPCs were placed in IMDM media supplemented with 10% FBS and plated at 200,000 cells/well on fibronectin for 1hr at 37°. For SDF-1 treatment, cells were treated at 200ng/mL SDF-1 for 10 min. Medium was removed from each well and washed three times. Adherent cells were fixed with

4% PFA for 15min at room temperature. Each well was washed three times and then blocked with 5% BSA in PBS for 30min at room temperature. Cells were stained with rhodamine phalloidin F-actin stain (Invitrogen) for 1hr at room temperature. Each well was washed out three times and replaced with PBS to image. Cells were imaged using the confocal microscope using the LSM 510 software. Cell spreading was analyzed ImageJ software to measure the area of each cell.

2.5.10 Western blot analysis

Total bone marrow was isolated from WT and CD82KO mice. Cells were lysed in RIPA buffer containing (150mM NaCl, 0.1% SDS, 0.5% DoC, 50mM Tris pH 8.0 and 1% IGEPAL-NP40). Cell lysates were run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blotted with a mouse monoclonal antibody against mouse Rac1. Blots were developed using ECL (Thermo Scientific).

2.5.11 Quantitative RT-PCR

Total RNA was extracted from total bone marrow and FACS isolated LSKs using the RNeasy Micro Kit (Qiagen). RNA samples were quantified using a Nanodrop 2000 (Thermo Scientific). cDNA was made using qScript cDNA synthesis kit (Quanta Bioscience) and amplified using a MyCycle Thermocycler (Bio Rad). Real time polymerase chain reaction was done using SYBR Green PCR Master Mix (Applied Biosystems). Primers for target genes were designed using Primer-BLAST (NCBI). Primers sets for each gene: Rac1 (Forward: 5'-AGA GTA CAT CCC CAC CGT CT-3' and Reverse: 5'- CAT GTG TCT CCA ACT GTC TGC-3') and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Forward: 5'-AAC TTT GGC ATT GTG GAA GG-3' and Reverse: 5'- ACA CAT TGG GGG TAG GAA CA-3') (Scoumanne et al., 2011). Target genes were amplified using the AB75000 Fast Real Time PCR System (Applied Biosystems). The endogenous control gene GAPDH was used to normalize each sample. The relative change in gene expression was calculated using $2^{-\Delta\Delta CT}$ algorithm.

2.5.12 Rac1 activity

WT and CD82KO lineage negative bone marrow HSPCs were lysed for the G protein linked immunosorbent assay. G-LISA Rac-1 Activation Assay Biochem Kit (catalog no BK128; Cytoskeleton Inc.) was performed per the manufacturer's instructions. In addition, Rac1 GTP (New East Biosciences) was assessed via flow cytometry. Lineage depleted bone marrow cells were fixed, permeabilized and treated with Fc block prior to staining with Rac1 GTP antibody. All samples were labeled in MACs Buffer for 30 min on ice. Samples were washed three times with MACs buffer after staining and analyzed using the Accuri C6 (BD Bioscience). Histograms were created using FlowJo software.

2.5.13 EHOP homing

Prior to injection, 1×10^6 donor bone marrow cells from CD82KO (CD45.2) or WT (CD45.2) were treated with $5 \mu\text{M}$ EHOP-016 (Selleckchem) or $50 \mu\text{M}$ NSC23766 (Cayman Chemical) in SFEM for 1hr at 37°C . Treated cells were then retro-orbitally injected into BoyJ recipient (CD45.1) mice. Recipient mice underwent a total body irradiation, which was administered as a single dose of 10gy. Mice were euthanized 16 hours post injection to assess chimerism of the BM and peripheral blood. Blood and bone marrow samples were treated with Fc block prior to labeling with directly conjugated fluorescent antibodies, FITC CD45.1 (A20; BD Pharmingen) and APC CD45.2 (104; BD Pharmingen) to assess chimerism. Samples were analyzed on the LSR Fortessa (BD Bioscience).

2.5.14 Statistical analysis

Statistical significance was calculated using a Student's t-test for which significance was labeled * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. One-Way ANOVA was used to calculate significance of EHOP migration and Rac-1 inhibitor (EHOP and NSC23766) homing studies for which significance was labeled * $p < 0.05$. The annotation n.s. is for non-significance. All statistical analyses were performed using GraphPad Prism 6 Software.

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Chapter 3: The tetraspanin CD82 regulates S1PR₁ mediated hematopoietic stem and progenitor cell mobilization

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Submitted

3.1 Abstract

Hematopoietic stem and progenitor cell (HSPC) egress into the blood occurs under normal physiological conditions and upon treatment with mobilizing agents. The trafficking of HSPCs from the bone marrow into the blood, or mobilization, is stimulated in the clinic to enable the isolation of HSPCs used for transplantation therapies. In this present study, we identified the tetraspanin CD82 as a novel regulator of HSPC mobilization. Using a global CD82 knock out (CD82KO) mouse model, we measured enhanced mobilization of HSPCs within CD82KO mice following AMD3100 treatment, which results from CD82KO in the HSPCs specifically. Moreover, we found that CD82KO HSPCs have increased surface expression of sphingosine 1-phosphate receptor (S1PR₁) and altered downstream signal transduction, including ERK and AKT. Using ImageStream cytometry, we identified disrupted S1PR₁ internalization in the CD82 deficient HSPCs, suggesting that CD82 plays a critical role in S1PR₁ regulation. We went on to find that the combined use of AMD3100 and anti-CD82 treatments enhanced HSPC mobilization in animal models. Together, these data provide evidence that CD82 is an important regulator of HSPC mobilization and suggests exploiting the CD82 scaffold as a therapeutic target to enhance stem cell mobilization treatments.

3.2 Introduction

Hematopoietic stem cell transplantation is a routinely performed treatment for malignant and non-malignant hematological diseases. Successful transplantation depends on a combination of factors, which include the number and fitness of transplanted hematopoietic stem and progenitor cells (HSPCs). Under static and stress conditions, HSPCs are released into the vasculature from the bone marrow, in a process termed mobilization. Transplantation therapies take advantage of this normal mobilization process by using specific treatments such as granulocyte-colony stimulating factor (G-CSF) to enhance the mobilization response, thereby increasing the number of HSPCs available in the blood for harvest. However, studies suggest that 5-25% of patients mobilize

poorly with G-CSF alone (Jantunen et al., 2012; Pusic et al., 2008). As such, identifying novel molecules and mechanisms that regulate HSPC mobilization is crucial for the improvement of transplantation therapies.

HSPC mobilization is mediated by a variety of key molecules such as chemokines, cytokines and proteolytic enzymes that promote egress into the peripheral blood. In particular, the chemokine receptor, CXCR4, which is highly expressed on the surface of HSPCs, facilitates bone marrow migration towards the chemoattractant, CXCL12. The clinical drug AMD3100 and G-CSF both target the CXCR4 receptor in order to induce mobilization (Broxmeyer et al., 2005; Uy et al., 2008). In addition to the CXCR4/CXCL12 signaling axis, the lysophospholipid Sphingosine-1-phosphate (S1P) ligand produced by mature red blood cells and activated platelets is found in high concentration within the vasculature (Schwab et al., 2005). S1P binds to the Sphingosine-1-Phosphate Receptors (S1PR₁₋₅), which are G-coupled protein receptors (GPCRs) that elicit downstream cellular activities such as migration, proliferation and cytoskeletal rearrangement (Bendall and Basnett, 2013; Blaho and Hla, 2014). In particular, the S1PR₁ was shown to mediate HSPC mobilization in combination with CXCL12 release from the bone marrow (Golan et al., 2012). Moreover, the mobilization of HSPCs was identified to be dependent on the induction of S1PR₁ towards a high S1P gradient within the blood and lymph. Together, these data suggest an important role for S1PR₁ in HSPC mobilization.

Previous work from our lab identified the tetraspanin CD82 as an critical regulator of HSPC migration and adhesion within the bone marrow niche (Saito-Reis et al., 2018). The tetraspanin family of scaffold proteins mediates a variety of cellular processes such as cell migration, adhesion and signaling via their regulation of surface molecules, including GPCRs, adhesion receptors and receptor tyrosine kinases (Termini and Gillette, 2017). However, the specific contribution of CD82 to HSPC mobilization had not been explored. Using a global CD82 knock out (CD82KO) mouse model, we find that CD82 regulates HSPC mobilization through the regulation of S1PR₁ expression and activity. Furthermore, our data indicate that antibody targeting of CD82 promotes the

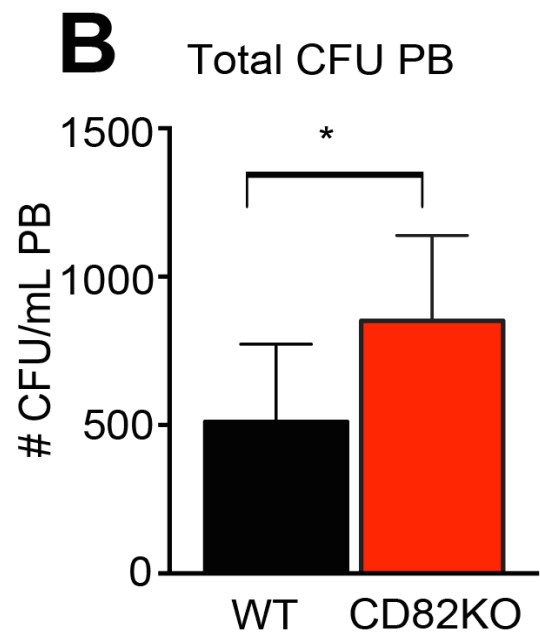
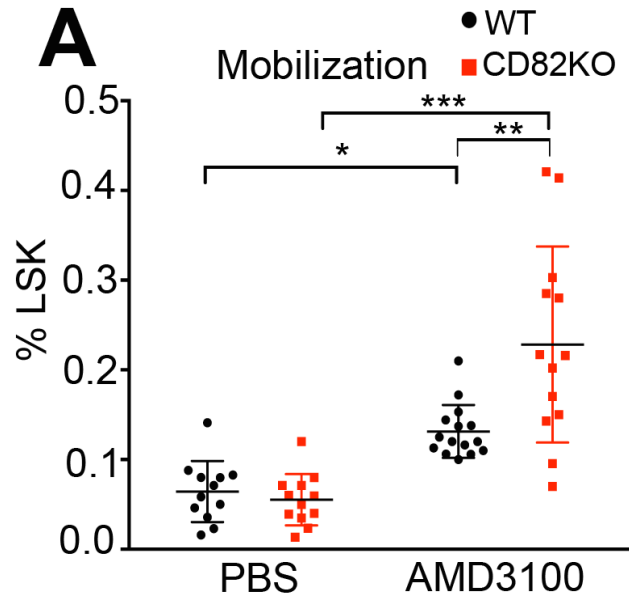
mobilization of HSPCs, suggesting that CD82 may be a novel target to enhance the release of HSPCs for improved transplantation therapies.

3.3 Results

3.3.1 Enhanced mobilization potential of CD82KO HSPCs.

To determine how the CD82 scaffold impacts HSPC mobilization, we utilized the CD82KO mouse previously described (Jones et al., 2016; Wei et al., 2014). Wild type (WT) and CD82KO mice were injected with AMD3100, a drug used clinically to mobilize HSPCs, or a vehicle control. One hour after injection, blood was harvested from the animals and the Lin⁻Sca1⁺Kit⁺ (LSK) HSPC population was identified by flow cytometry. Under control treatment, a minimal number of HSPCs were detected in the blood, with no difference identified between WT and CD82KO animals (Figure 1A). As expected, AMD3100 treatment significantly increased the amount of mobilized HSPCs measured in the blood, however an even greater increase in HSPC mobilization was detected in the CD82KO mice when compared to WT (Figure 1A). In addition, colony forming unit (CFU) assays were performed with the blood collected from WT and CD82KO mice treated with AMD3100. Similar to the flow cytometry analysis, AMD3100 treatment increased the number of CFUs measured in the CD82KO mice when compared to WT (Figure 1B), further confirming an increased mobilization potential of CD82KO HSPCs.

Since we are using a global CD82KO mouse model, we next wanted to confirm that the enhanced mobilization we observe is due to the loss of CD82 on the HSPCs rather than an effect of CD82KO within the bone marrow microenvironment. Thus, we completed bone marrow transplants of WT or CD82KO HSPCs into lethally irradiated B6.SJL-*Ptprc*^a *Pepc*^b/BoyJ (CD45.1) recipient mice. BoyJ mice maintain CD82 expression in all cells but carry the differential pan leukocyte marker CD45.1, and thus, can be distinguished from the WT and CD82KO donor cell populations that express the CD45.2 allele. The bone marrow transplants were allowed to establish for two months and then both



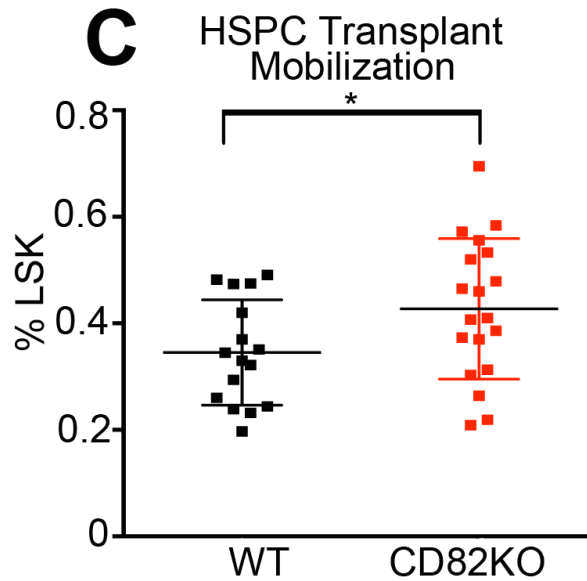


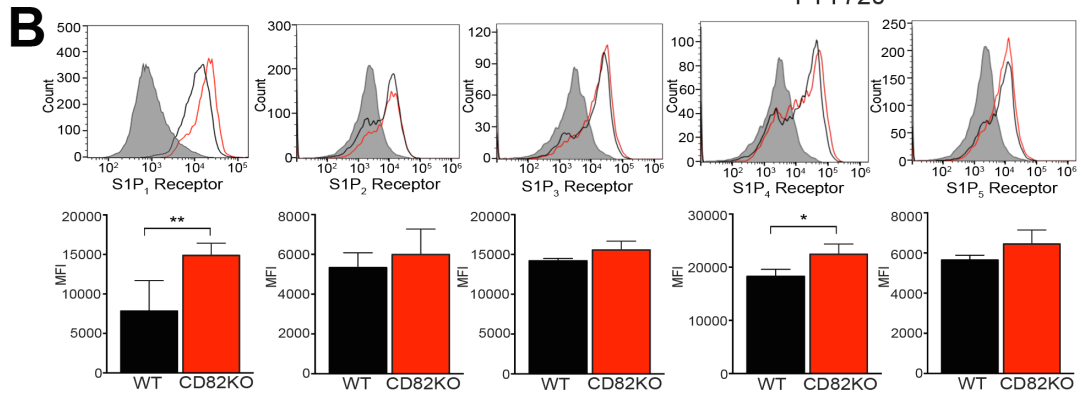
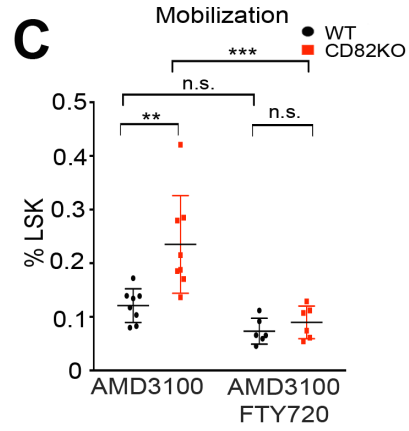
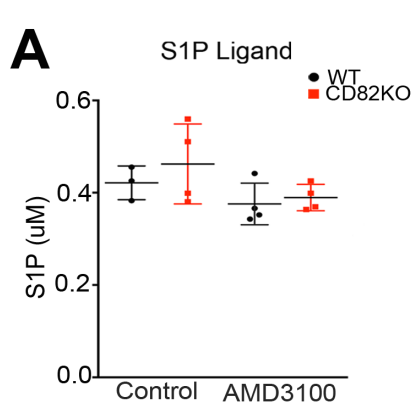
Figure 3.1: CD82KO HSPCs display enhanced mobilization.

(A) Flow cytometry analysis of %LSK (HSPCs) in peripheral blood collected from WT and CD82KO micetreated with control PBS or AMD3100 (n=12-15 mice/group). ***p<0.001, **p<0.01, *p<0.05, two-way ANOVA. (B) Peripheral blood CFU after AMD3100 induced HSPC mobilization (n=3 mice/group, in triplicate). *p<0.05, unpaired t-test. (C) Flow cytometry analysis of %LSK in peripheral blood collected after AMD3100 induced HSPC mobilization of WT and CD82KO transplanted mice. (n=16-19 mice/group). *p<0.05, unpaired t-test.

WT and CD82KO transplanted mice were treated with AMD3100 to induce mobilization. Again, we used flow cytometry to quantify the LSK cells released into the blood. Similar to our previous observation, we detect an increased mobilization of transplanted CD82KO HSPCs when compared to WT cells, indicating a cell-intrinsic defect of the CD82KO HSPCs (Figure 1C). Taken together, these data suggest the loss of CD82 enhances HSPC mobilization and implicates the CD82 scaffold as a regulator of HSPC egress.

3.3.2 CD82 regulates S1PR expression and signaling

HSPC mobilization is critically dependent upon S1PR signaling in response to the S1P ligand gradient (Liu et al., 2011a; Schwab et al., 2005). S1P is found at higher concentrations within the blood and thus can promote the egress of HSPCs from the bone marrow (Liu et al., 2011a). We quantified the S1P ligand within the plasma of WT and CD82KO mice under control and AMD3100 treatment conditions, identifying no significant difference in S1P ligand (Figure 2A). Next, we asked whether CD82 regulates HSPC egress by modulating S1PR expression. Using flow cytometry, we characterized the surface expression of the S1PR family, which consists of five receptors, S1PR₁₋₅. While we detected no difference in the surface expression of S1PR₂, S1PR₃, or S1PR₅ between WT and CD82KO HSPCs, we measured a significant increase in the surface expression of S1PR₁ and a more modest increase of S1PR₄ in the CD82KO HSPCs (Figure 2B). Since trafficking of HSPCs and their egress from extramedullary tissues was shown previously to depend on S1PR₁ expression (Massberg et al., 2007; Ratajczak et al., 2010; Seitz et al., 2005), we set out to determine if the increased expression of S1PR₁ mediates the increased mobilization of CD82KO HSPCs. The S1PR agonist, FTY720, can act upon S1PR₁₋₅, but it has the highest affinity for S1PR₁ and stimulates the down-regulation of the receptor. Therefore, we repeated the AMD3100 mobilization experiments in the presence of FTY720, which results in the internalization of S1PR₁. As indicated in Figure 1, AMD3100 treatment of animals increased the number of mobilized CD82KO HSPCs when compared to WT. However, upon FTY720 treatment, the number of mobilized WT and CD82KO HSPC is



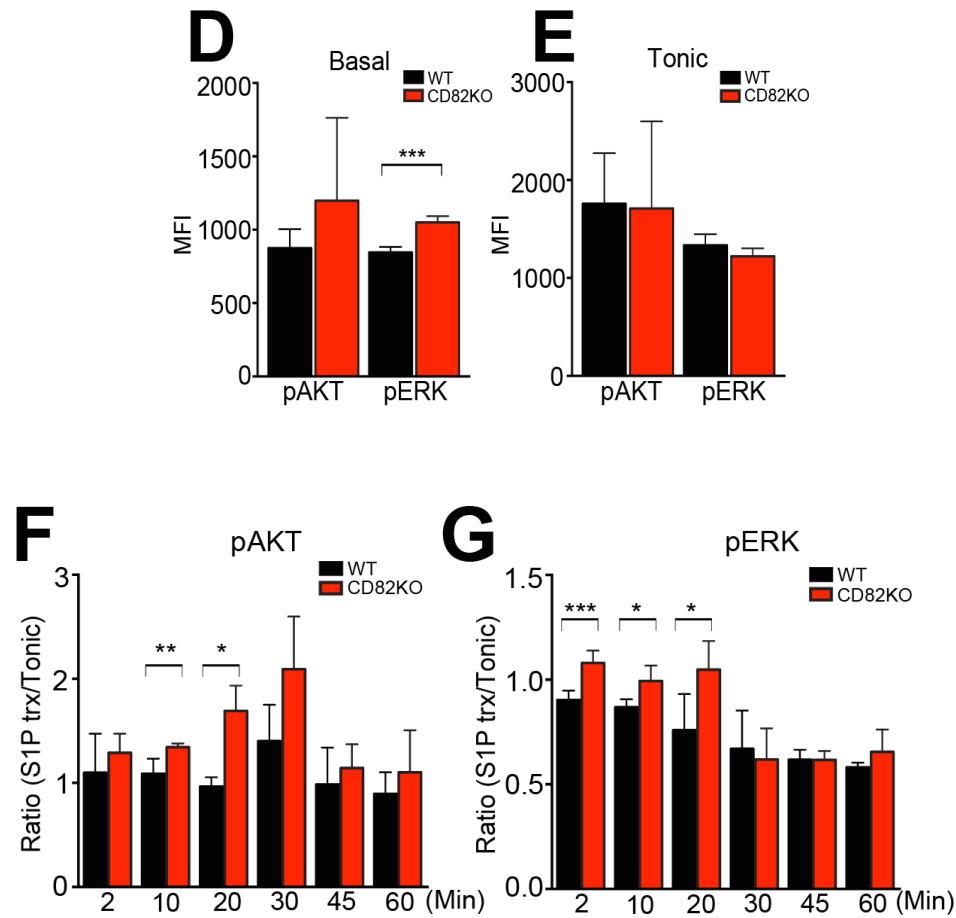


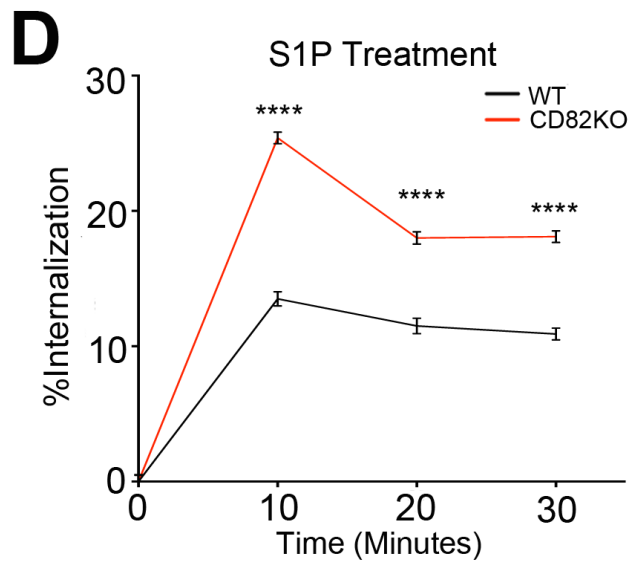
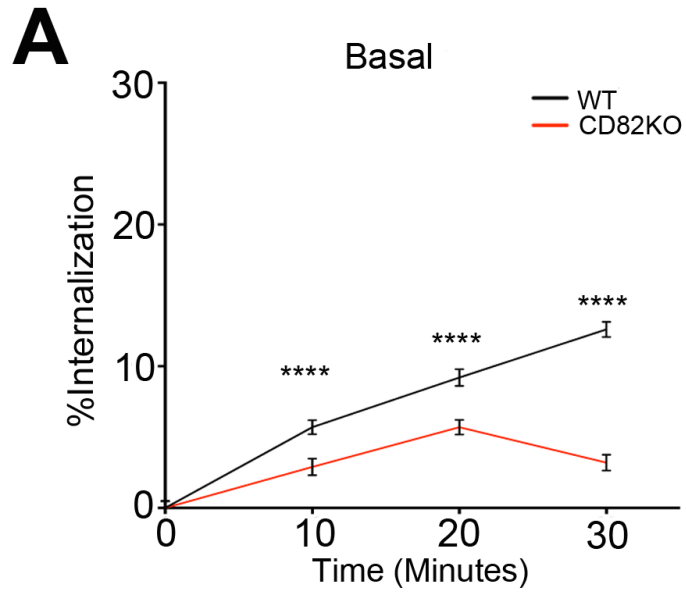
Figure 3.2. CD82KO HSPCs have increased S1PR₁ expression and signaling. (A) S1P ligand plasma levels of WT and CD82KO plasma post PBS control or AMD3100 treatment (n=3-4 mice/group). (B) Flow cytometry analysis measured the mean fluorescence intensity (MFI) of WT and CD82KO HSPC S1PR₁₋₅ surface expression (n=4 mice/group). **p<0.01 and *p<0.05, unpaired t-test. (C) Flow cytometry analysis of %LSK in peripheral blood collected from WT and CD82KO mice treated with AMD3100 or AMD3100/FTY-720 (n=6-8 mice/group). ***p<0.001, **p<0.01, n.s., non-significant, two-way ANOVA. Phosphoflow cytometry analysis of (D) basal and (E) tonic (1hr serum starvation) conditions to assess mean fluorescence intensity (MFI) of pAKT and pERK signaling of the LSK population. (n=3-4 mice/group) ***p<0.001, unpaired t-test. Phosphoflow cytometry analysis of 10μM S1P treatment at various time points post 1hr serum starvation to assess (F) pAKT and (G) pERK signaling of the LSK population. Quantification ratio calculated by dividing tonic signaling by S1P treatment conditions (n=3-4 mice/group) ***p<0.001, **p<0.01, *p<0.05, unpaired t-test

decreased compared to AMD3100 treatment with no difference in mobilization detected between WT and CD82KO mice (Figure 2C). Therefore the increased mobilization of CD82KO HSPC is inhibited by downregulation of the S1PR₁, suggesting that the increased S1PR₁ expression on CD82KO HSPCs mediates the enhanced blood mobilization.

In addition to measuring an increase in S1PR₁ and S1PR₄ expression, we also evaluated the downstream signal transduction from S1P ligand activation. S1PR₁ activates multiple intracellular signaling cascades, including the extracellular signal-regulated kinase (ERK) and the phosphatidylinositol-3-linase (PI3K)-AKT Pathways (Rosen et al., 2009). Using flow cytometry to perform phosphoflow of pERK and pAKT, we detect a significant increase in basal levels of pERK in CD82KO HSPCs when compared to WT, with no change in pAKT (Figure 2D). We also serum starved the LSK population to measure the tonic levels of pERK and pAKT expression between WT and CD82KO HSPCs, identifying no difference (Figure 2E). However, following S1P stimulation, we detected a significant increase in pERK expression in CD82KO HSPCs at early time points (2, 10, and 20 min), which returned to WT levels at 30 min (Figure 2F) and a similar increase in pAKT levels in CD82KO HSPCs at slightly later time points (10, 20 and 30 min) that returned to WT levels after 45 min (Figure 2G). Collectively, these data suggest that in addition to increased S1PR₁ expression, CD82KO HSPCs also demonstrate enhanced signal transduction downstream of ligand engagement.

3.3.3 CD82 regulates S1PR₁ internalization

We next wanted to define how CD82 regulates S1PR₁ expression and signaling. Recognizing that tetraspanins including CD82 have been reported to modulate receptor internalization, we examined internalization using ImageStream cytometry. This technique combines flow cytometry with fluorescence microscopy, enabling image-based analysis of large numbers of cells per sample. Bone marrow was isolated from both WT and CD82KO animals and antibody labeled for the S1PR₁ at 4°C to minimize endocytosis. Cells were then moved to 37°C and fixed at various time points to monitor S1PR₁ trafficking.



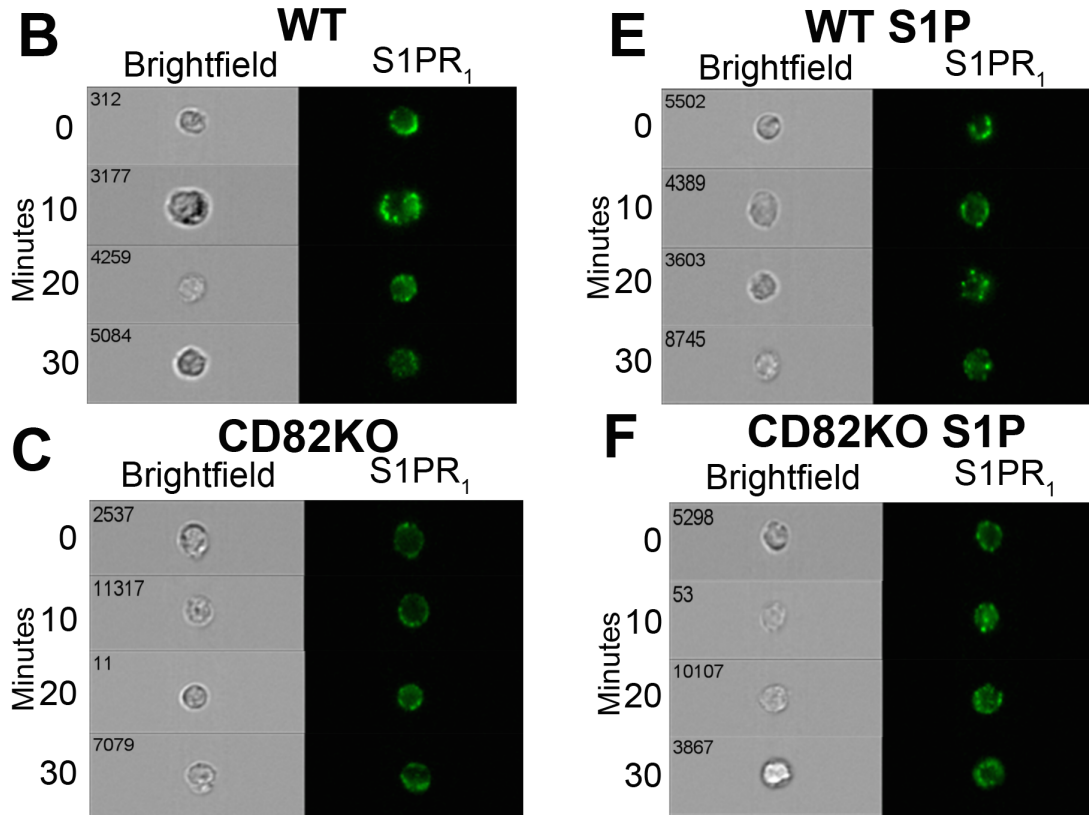


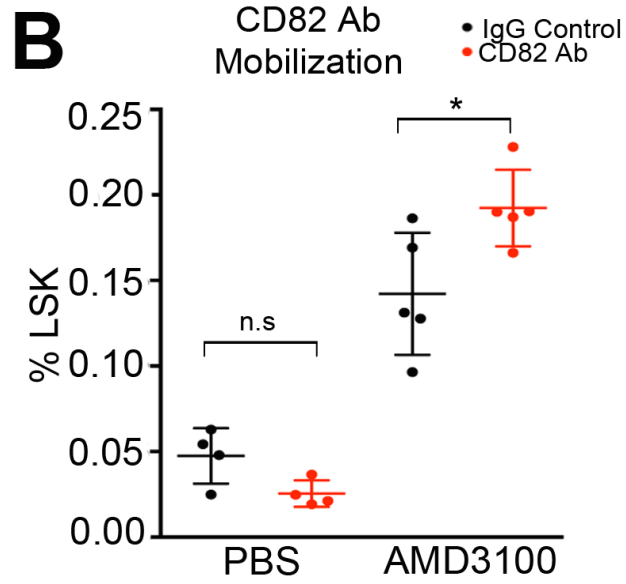
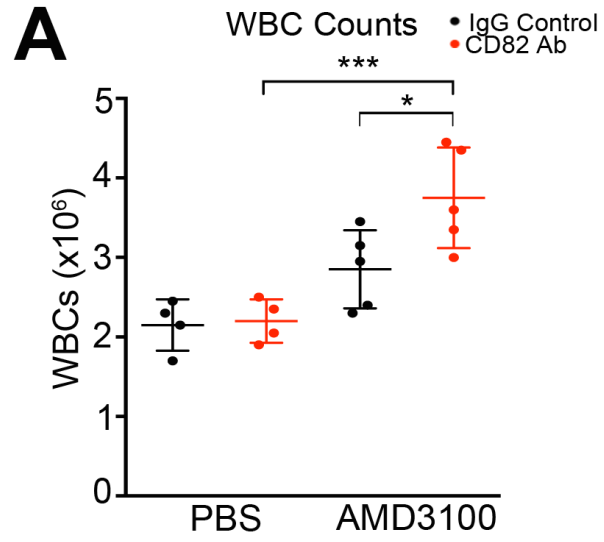
Figure 3.3: CD82KO HSPCs have decreased S1PR₁ internalization.

Amnis Image flow cytometry analysis of % internalization of S1PR1 on WT and CD82KO HSPCs during A) basal and (D) 10µM S1P treatment at various time points. ****P<0.0001, Two-Way ANOVA. Representative images of S1PR1 internalization of WT and CD82KO HSPCs under (B,C) basal and (E,F) 10µM S1P treatment at various time points.

Cells were also antibody labeled with Lin⁺ antibodies so that the Lin⁻ HSPC population of cells could be identified by the ImageStream. Using the internalization feature, we distinguished Lin⁻ cells with surface labeled S1PR₁ expression from those S1PR₁ receptors that had been internalized over the course of the 30 minute experiment. Under basal conditions, we find that CD82KO HSPCs have decreased S1PR₁ internalization compared to WT cells at times 10, 20 and 30 minutes (Figure 3A-C). These data suggest that the increased S1PR₁ surface expression and enhanced signaling measured in CD82KO HSPCs (Figure 2) is due at least in part to decreased S1PR₁ internalization. Additionally, we assessed S1PR₁ internalization upon S1P ligand treatment using a relatively high concentration of S1P (10 μ M). Interestingly, upon ligand treatment, we detected increased S1PR₁ internalization of CD82KO HSPCs compared to WT at time points 10, 20 and 30 minutes (Figure 3D-F). These findings suggest that distinct pathways may be in place to regulate the internalization of S1PR₁ with CD82 promoting endocytosis under basal signaling and perhaps attenuating internalization upon high concentrations of ligand.

3.3.4 Anti-CD82 treatment enhances HSPC mobilization

The observation that HSPCs are more readily released into the peripheral circulation of CD82KO mice led us to ask whether CD82 could be a novel target to promote HSPC mobilization. Previous studies have used antibodies to illustrate a critical role for specific integrins in HSPCS mobilization (Bonig et al., 2009; Craddock et al., 1997; Papayannopoulou et al., 1995; Papayannopoulou et al., 1998; Papayannopoulou et al., 2001b). As such, we set out to determine if pretreatment with anti-CD82 could induce HSPC mobilization in mice. WT mice were intravenously injected with either 2mg/kg of anti-CD82 (M35) (Custer et al., 2006) or control IgG for two hours and then treated with AMD3100 for 1 hour. Blood was isolated and analyzed by flow cytometry to measure potential changes in the population of peripheral blood mobilized HSPCs. While anti-CD82 alone shows no effect on white blood cell (WBC) or HSPC mobilization, Figure 4 illustrates that mice treated with anti-CD82 in combination with AMD3100 display increased WBC (Figure 4A) and LSK (Figure 4B) cell mobilization when



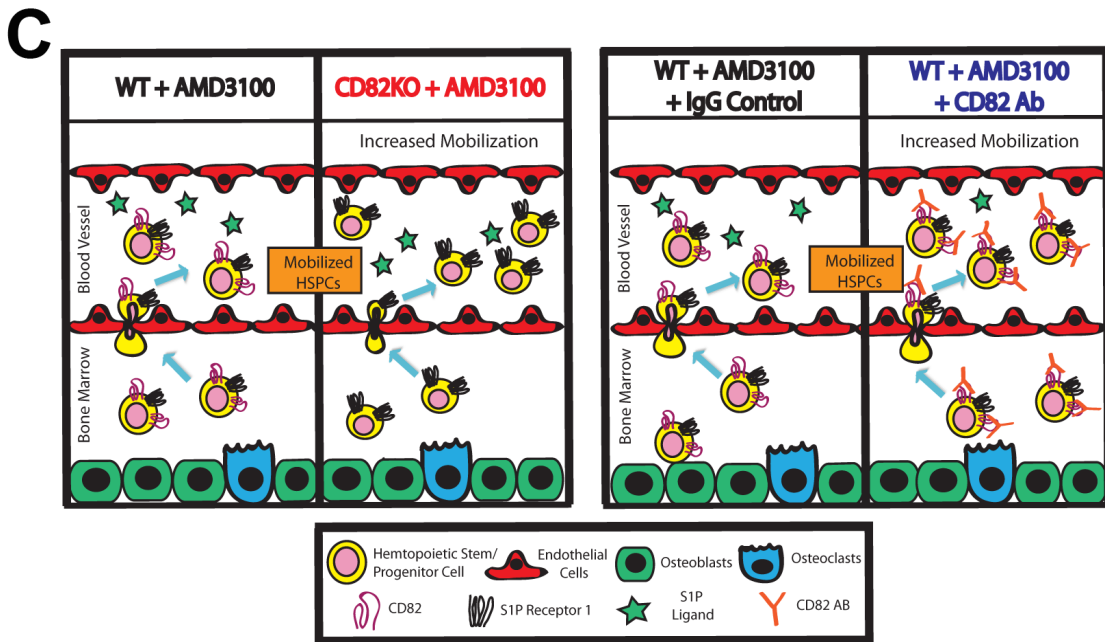


Figure 3.4. CD82 Ab treatment enhances HSPC mobilization.

(A) Counts of white blood cells within the peripheral blood of WT mice treated with IgG control or CD82 Ab upon PBS or AMD3100 treatment (n=4-5 mice/group). ***p<0.001 or *p<0.05, two-way ANOVA. (B) Flow cytometry analysis of %LSK in peripheral blood collected from WT mice treated with IgG control or CD82 Ab (n=4-5 mice/group). *p<0.05, two-way ANOVA. (C) Current model illustrates the stem cell niche, which consists of the bone marrow and the vasculature. This model depicts increased S1PR1 mediated CD82KO HSPCs mobilization upon AMD3100 treatment. In addition, AMD3100+ CD82 antibody treatment increased HSPC mobilization of WT HSPCs compared to IgG control treatment.

compared to controls. Therefore, anti-CD82 treatment stimulates an additive mobilization of HSPCs when used in combination with AMD3100 and further suggests that CD82 is a key contributor to the bone marrow retention of HSPCs. Collectively, from these data we suggest the current model (Figure 4C) where the CD82 scaffold regulates S1PR₁ internalization, resulting in enhanced S1PR₁ surface expression, signaling and increased HSPC mobilization.

3.4 Discussion

Decreased numbers of HSPCs harvested from the peripheral blood limits the success of bone marrow transplantations. In fact, standard methods for peripheral blood mobilization of hematopoietic stem cells fail to collect sufficient stem cells in 5-40% of patients (Giralt et al., 2014). Therefore, identifying unique targets to promote HSPC mobilization and increase HSPC numbers within the peripheral blood is crucial for treatment of both non-hematological and hematological malignancies. The tetraspanin family of scaffold proteins function as molecular facilitators interacting with adhesion and signaling molecules at the plasma membrane to create tetraspanin-enriched microdomains (TEMs) (Charrin et al., 2009; van Deventer et al., 2017). TEMs contribute to a number of cellular functions, including migration, adhesion and protein trafficking (Termini and Gillette, 2017; van Deventer et al., 2017). Specifically within HSPCs, tetraspanins are described to impact homing, engraftment, migration, and quiescence (Charrin et al., 2009; Hur et al., 2016; Larochelle et al., 2012; Marjon et al., 2016; Saito-Reis et al., 2018), and in the current study, we identified a novel role for the tetraspanin CD82 as a critical regulator of HSPC mobilization.

Under normal physiological conditions, HSPCs are found in circulation at very low numbers (Massberg et al., 2007). However, increased numbers of HSPCs mobilize into the blood in response to injury, infection or stress (Heidt et al., 2014; Massberg et al., 2007). Additionally, treatments such as G-CSF and Plerixafor (AMD3100), which target CXCR4, are used to induce peripheral blood mobilization of HSPCs for stem cell transplant. Using the well characterized CD82KO mice (Jones et al., 2016; Saito-Reis et al., 2018; Wei et al., 2014), we

identified an increase in the mobilization capacity of HSPCs from CD82KO mice following AMD3100 treatment. Through a combination of transplant experiments, we went on to show that CD82KO, specifically within the HSPC population, is primarily responsible for the enhanced mobilization phenotype. Previous work from our lab thoroughly evaluated the expression and signaling potential of the CXCR4 receptor in the context of the CD82KO HSPCs, finding no altered expression or signaling of CXCR4 (Saito-Reis et al., 2018). Therefore, despite its essential role in regulating bone marrow interactions, these data suggest that CXCR4 is unlikely to be a key contributor to the observed mobilization defect. Additionally, our previous work analyzed the integrin profile of CD82KO HSPCs, including the integrin $\alpha4\beta1$ (Saito-Reis et al., 2018), which also impact the bone marrow retention of HSPCs. Our findings identified no difference in $\alpha4$ integrin expression and a minor decrease in $\beta1$ integrin expression in CD82KO HSPCs (Saito-Reis et al., 2018), which suggests a modest potential for integrin involvement in CD82-mediated mobilization. These collective data led us to evaluate the role of the S1P receptor class of GPCRs.

S1P receptors are targets of the lipid signaling molecule S1P, which facilitates the egress of HSPCs from the bone marrow into the blood (Ratajczak et al., 2010; Rosen et al., 2009; Schwab et al., 2005; Seitz et al., 2005). S1P stimulates multiple cellular processes including proliferation, stress fiber formation and migration. Within the receptor family, the S1PR₁ is the most well characterized as an important mediator of HSPC mobilization (Golan et al., 2012; Liu et al., 2011a; Ratajczak et al., 2010; Schwab et al., 2005). Previous work found that treatment of mice with the S1PR₁ agonist, FTY-720, results in the rapid downregulation and degradation of the receptor and subsequently prevents HSPC mobilization (Mullershausen et al., 2009). Our data suggest that CD82KO HSPCs have an increased mobilization capacity, due at least in part to S1PR₁, since treatment of CD82KO mice with FTY-720 ablated the enhanced mobilization observed in these animals. Further analysis of the CD82KO HSPCs identified increased surface expression of S1PR₁ when compared to WT cells, suggesting that the enhanced mobilization is promoted by an increase in S1PR₁

surface expression. One potential mechanism for the increase in S1PR₁ surface expression is through disruptions in receptor internalization. For example, the tetraspanin CD63 was shown to mediate receptor internalization of the H,K-ATPase β -subunit through direct interaction (Duffield et al., 2003). Additionally, CD82 was identified to regulate epidermal growth factor receptor (EGFR) endocytosis through TI-VAMP expression (Danglot et al., 2010). Moreover, work from our own lab identified a role for CD82 in the regulation of internalization and recycling of the α 4 integrin (Termini et al., 2014). Previous studies identified a role for dynamin-2 (Willinger et al., 2014) and the clathrin-mediated endocytic pathway (Reeves et al., 2016) in the uptake of S1PR₁. Our current data also implicates CD82 to be involved in the regulation of S1PR₁ internalization. Interestingly, we find that in contrast to the decreased S1PR₁ internalization observed under basal conditions in the CD82KO HSPCs, we find that S1PR₁ internalization is enhanced upon S1P treatment. These findings suggest that distinct pathways may be in place to regulate the internalization of S1PR₁. The crystal structure of S1PR₁ provides insight into the location of the ligand pocket that is restricted between helices VII and I within the transmembrane region (Hanson et al., 2012). This study also went on to show that the N-terminal tail of S1PR₁ folds over the top of the receptor to block access to the ligand binding pocket, suggesting that ligand access to the binding pocket occurs from within the cell membrane and not the extracellular space. As such, CD82 and the TEMs that it assembles may have a unique ability to modulate ligand access to S1PR₁, which could also contribute to the increased S1PR₁ endocytosis observed in CD82KO HSPCs following S1P treatment. Whether CD82 interacts directly or indirectly with S1PR₁ still remains unclear.

PI3K/AKT and ERK are known signaling pathways stimulated downstream of S1PR₁ activation (Rosen et al., 2009). Using phosphoflow cytometry analysis, we demonstrated that upon S1P ligand binding, CD82KO HSPCs have increased pERK and pAKT signaling when compared to WT HSPCs. The increased S1PR₁ expression observed on the CD82KO HSPCs is likely a primary contributor to the increased activation of ERK and AKT detected. However, the tetraspanin scaffold

has also been described as a mechanism to modulate signaling directly at the plasma membrane (Termini and Gillette, 2017). In fact work from our own lab demonstrated that CD82 can stabilize PKC α at the plasma membrane, promoting the sustained downstream signaling of MAPK and ERK1/2 in acute myeloid leukemia cells (Termini et al., 2016). In addition to ERK and AKT signaling, PKC α and Rac1 GTPase activation have been described to signal downstream of S1PR₁. Interestingly, both PKC α and Rac1 were previously described to be modulated by the CD82 scaffold (Saito-Reis et al., 2018; Termini et al., 2016). Therefore, future studies will be required to help identify the mechanisms by which CD82 influences S1PR₁ signaling and the impact on specific downstream pathways.

Hematopoietic stem cell transplantations often fail due to insufficient numbers of harvested HSPCs (Copelan, 2006; Hatzimichael and Tuthill, 2010). Under normal physiological levels, HSPCs circulate at 0.04% within the blood (Massberg et al., 2007). Currently, within the clinic mobilizing agents are often used for harvesting HSPCs in lieu of the alternative invasive method such as bone marrow aspiration (Hatzimichael and Tuthill, 2010). Therefore, understanding the mechanisms and molecules that facilitate HSPC mobilization is critical to increase HSPCs within the blood for transplantation. Previous work has shown that HSPC mobilization can be induced through the use of antibodies. For example, intravenous treatment with antibodies targeting the integrin $\alpha 4$ resulted in enhanced HSPC mobilization (Bonig et al., 2009; Qian et al., 2006). Additionally, another study used a CD82 monoclonal antibody to mobilize AML cells into the peripheral blood, which led to increased effects of chemotherapy treatment (Nishioka et al., 2015). In this study, we demonstrate that intravenous injection of a CD82 antibody into WT mice increased HSPC mobilization, further strengthening the critical role for CD82 in HSPC mobilization. At this time, how the antibody impacts TEMs and specifically S1PR₁ signaling remains unclear. When taken together, our data provide evidence for CD82 to be used as a novel target in the clinic to mobilize HSPCs for transplantation therapies.

3.5 Materials and Methods

3.5.1 Mice

C57BL/6 wild-type and B6.SJL-Ptprc^a Pepc^b/BoyJ mice were obtained from Jackson Laboratory. CD82KO mice were generated from cre-loxP recombination (Wei et al., 2014). All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of New Mexico Health Science Center. Mice were housed under pathogen-free conditions in the UNM Animal Facility. The age and sex of mice were matched for each experiment.

3.5.2 HSPC Mobilization

Mice were subcutaneously injected with one dose of 5mg/kg AMD3100 (Sigma Aldrich). Control treated mice were subcutaneously injected with PBS. Peripheral blood was collected by a cardiac puncture 1hr post AMD3100 injection. The inhibitor for S1PR, FTY720 (Cayman Chemicals) was injected i.p. at 10mg/kg 14 hours before AMD3100 treatment. The inhibitor for the S1P lyase, 4-deoxypyridoxine (DOP) (Cayman Chemicals) was supplemented into the drinking water for 3 days at 30mg/L with 10g/L glucose. RBCs were lysed using ACK lysis buffer. The amount of mobilized HSPCs within the blood were quantified using the following directly conjugated fluorescent antibodies for mouse APC lineage cocktail (BD Pharmingen), Pe-Cy7 Sca-1 (D7; Biolegend) and PE CD117 (2B8, Biolegend). Labeled samples were then read on the LSR Fortessa (BD Bioscience) to determine the percentage of HSPCs mobilized based off of the total population.

3.5.3 Colony-forming assay

Peripheral blood collected from a cardiac puncture was collected 1hr post treatment with either PBS or 5mg/kg AMD3100. RBCs were lysed using ACK lysis buffer. Blood cells were plated at 100,000 cells/dish suspended in Isocove's Modified Dulbecco's Medium (IMDM) media and placed in MethocultTMGF M3434 (StemCell Technologies). The total amount of colonies was scored 12 days later using gridded scoring dishes (Stem Cell Technologies). Colonies were scored using a Zeiss Axioskop microscope.

3.5.4 Isolation and analysis of bone marrow cells

Bone marrow cells were isolated from the front and back limb bones using a mortar and pestle. Isolated bone marrow cells were passed through a 40 μ M strainer to remove bone fragments. Red blood cells were lysed using ACK lysis Buffer (150mM NH₄Cl, 10mM KHCO₃, 0.1mM EDTA). Isolated bone marrow cells were treated with Fc block (2.4G2; BD Pharmingen) prior to surface marker staining. Bone marrow cells were stained with antibodies against the following surface markers: mouse APC lineage cocktail (BD Pharmingen), Pe-Cy7 Sca-1 (D7; Biolegend) and PE CD117 (2B8, Biolegend). Labeled bone marrow samples were analyzed using the LSR Fortessa (BD Bioscience) or Accuri C6 Flow Cytometer (BD Bioscience).

3.5.5 HSPC engraftment mobilization

For engraftment assay, 1X10⁶ donor Lin⁻ bone marrow cells from CD82KO or WT (CD45.2) were retro-orbitally into recipient BoyJ mice (CD45.1). Recipient mice underwent a total body irradiation, which was administered as a single dose of 10 gy. One month post transplant, HSPCs were mobilized with a single dose of 5mg/kg AMD3100. Peripheral blood was collected by a cardiac puncture 1hr post AMD3100 injection. The amount of HSPC within the blood was quantified using the antibodies and procedure described in the “HSPC mobilization” section.

3.5.6 S1P Ligand ELISA

S1P ligand protein concentration from WT and CD82KO plasma were determined by enzyme-linked immunosorbent assay (ELISA) (Echelon Biosciences Inc) per the manufacturer’s instructions. Blood was collected from a cardiac 1hr post control or ADM3100 treatment. Collected blood were allowed to sit for 2hrs. Blood samples were centrifuged at 2.0 rcf for 20min to isolate plasma. Plasma samples were then assessed using the S1P ELISA kit.

3.5.7 Cell surface expression

Bone marrow cells were analyzed for the surface expression of S1PR₁ (R&D Systems), S1PR₂ (Proteintech), S1PR₃ (Alomone Labs), S1PR₄ (ThermoFisher Scientific) and S1PR₅ (Proteintech) on the HSPC population. All samples were labeled in MACs Buffer (PBS, 0.5% BSA, 2mM EDTA, pH 7.2; Miltenyi Biotec) for

30 min on ice. Samples were washed three times with MACs buffer after staining and analyzed using the LSR Fortessa (BD Bioscience). Histograms were created using FlowJo software.

3.5.8 Phosphoflow cytometry

Basal signaling activity was assessed by fixing isolated bone marrow cells with 4% Paraformaldehyde and permeabilizing with 100% methanol prior to antibody staining. Tonic signaling activity was assessed by serum starving bone marrow cells in SFEM for 1hr at 37°. Starved cells were then fixed and permeabilized. After 1hr of serum starvation, bone marrow cells were also treated with 10uM S1P for 2, 10, 20, 30, 45 and 60 minutes. After each time point, samples were fixed and permeabilized. Permeabilized samples were stained for LSK markers and the following signaling molecules: p-AKT Pacific Blue (BD Pharmingen), and p-ERK Alexa 488 (Biolegend). Samples were analyzed on the LSR Fortessa (BD Bioscience). The ratio of S1P treatment was calculated by dividing Tonic mean fluorescence intensity (MFI) by S1P treatment MFI.

3.5.9 Internalization of S1PR₁

Total bone marrow harvested from WT and CD82KO mice were labeled on ice for 1 hour using S1PR₁ (anti-EDG1 ab11424; Abcam). Cells were then labeled using an Alexa 488 anti-rabbit secondary for 45 min. In addition, cells were labeled with mouse APC anti-lineage cocktail (BD Bioscience). An aliquot of cells were fixed with 4% PFA at time point 0, which is considered 100% surface staining. Remaining cells were placed at 37° in the presence or absence of 10µM S1P ligand (Caymen Chemicals) for 10, 20 and 30 minutes. At each time point approximately 1x10⁶ cells were removed and fixed. Samples were assessed on the ImageStream multispectral imaging flow cytometer (Amnis) and data collected were analyzed using the IDEAS image-analysis software (Amnis). For each sample, 10,000 events were collected. Internalization was assessed on the lineage negative population. The internalization feature was used to determine the ratio of intensity inside the cell/intensity of the entire cell. The feature used to define internalization is an adaptive erosion mask that fits within the membrane

of the cell. Internalization is graphed by subtracting the internalization percentage from each time point to time 0.

3.5.10 Statistical analysis

Statistical significance was calculated using a Student's t-test for which significance was labeled * $p < 0.05$ and ** $p < 0.01$. Two-Way ANOVA was used to calculate significance of Mobilization studies (Control and AMD3100) and (AMD3100, AMD3100/FTY720, AMD3100/CD82Ab) for which significance was labeled * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The annotation n.s. is for non-significance. All statistical analyses were performed using GraphPad Prism 6 Software.

3.5 Acknowledgements

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Chapter 4: Conclusions, significance and future directions

4.1 Conclusions

The studies described in my dissertation provide novel information regarding how tetraspanins regulate HSPC fitness and bone marrow retention. Utilizing the global CD82KO mouse, we identified the tetraspanin CD82 as an important regulator of HSPC quiescence, homing, engraftment and mobilization, Figure 5.1.

In chapter 2, our data demonstrate that the loss of CD82 results in decreased LT-HSCs within the bone marrow of mice, due to increased HSPC cycling. In addition, we measured decreased competitive bone marrow engraftment of CD82KO HSPCs, which we determined was in part due to decreased HSPC homing. Ultimately, we found that the CD82KO HSPC bone marrow homing defect was due to Rac1 hyperactivation, which we rescued with Rac1 inhibitor treatment. Collectively, these data demonstrate an important role for CD82 in HSPC quiescence, homing and engraftment.

In chapter 3, our data demonstrate that HSPCs from CD82KO mice have enhanced mobilization from the bone marrow into the blood upon AMD3100 treatment. We determined that increased CD82KO HSPC mobilization is in part due to increased S1PR₁ surface expression. In addition, using image-based flow cytometry, we found that CD82KO HSPCs have increased S1PR₁ surface expression due to decreased receptor internalization. Additionally, phosphoflow studies with S1P ligand treatment demonstrated that CD82KO HSPCs have increased pERK and pAKT signaling when compared to WT HSPCs. Moreover, we found that anti-CD82 treatments further enhance HSPC mobilization into the blood of mice. Therefore, these data demonstrate that CD82 regulates S1PR₁-mediated HSPC mobilization and suggests CD82 may serve as a therapeutic target to promote HSPC egress.

4.2 Significance

Maintenance of LT-HSCs is critical to preserve the hematopoietic population. The LT-HSC population is primarily kept in a quiescent state within

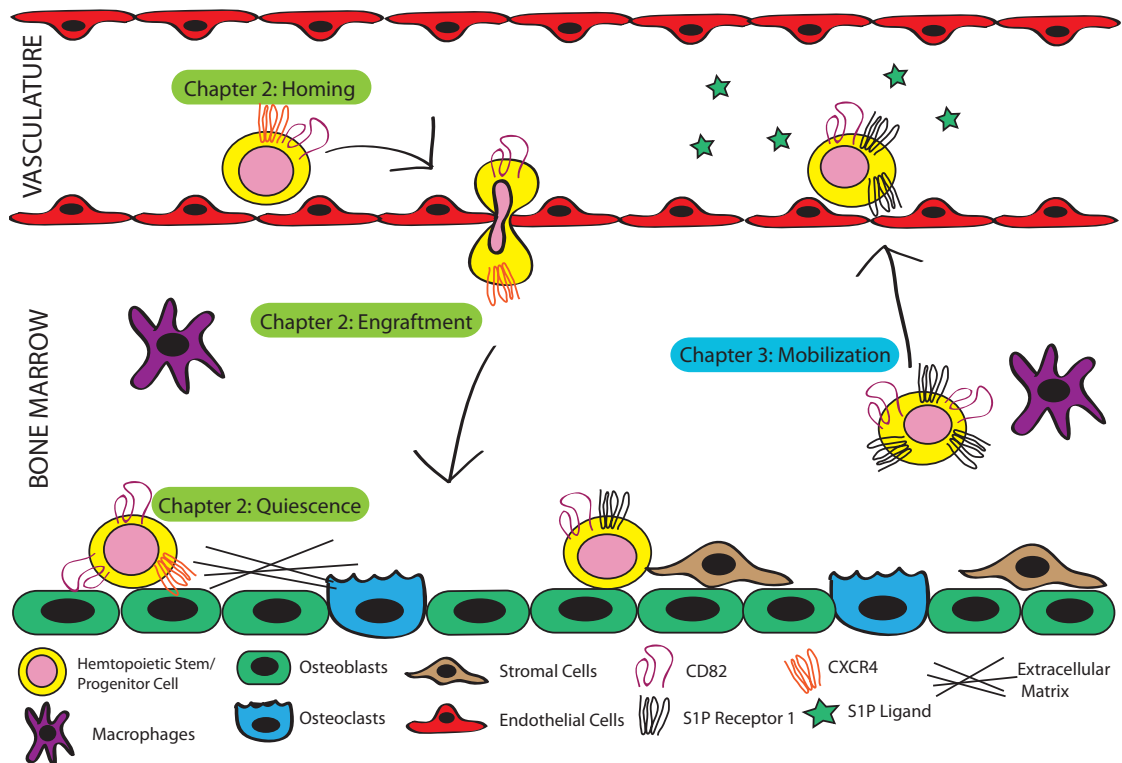


Figure 5.1: Conclusion Model. The stem cell niche consists of the bone marrow (also termed the endosteal niche) and vasculature. In Chapter 2 (shown in green) we determined that CD82 is an important regulator of HSPC quiescence, homing and engraftment. In this model, HSPCs are primarily found in the bone marrow microenvironment in a quiescent state. Additionally, HSPCs home to the bone marrow by extravasating through endothelial cells in order to enter the bone marrow niche for engraftment. Our data suggest that the loss of CD82 leads to decreased HSPC quiescence and a defect in HSPC homing and engraftment. In chapter 3 (shown in blue) we determined that CD82 is an important regulator of HSPC mobilization. In this model, HSPC mobilization occurs under normal physiological conditions and upon treatment with chemotherapy or mobilizing agents. Our data suggest that CD82KO HSPCs undergo enhanced mobilization due to increased S1PR₁ surface expression. Together these data provide evidence that CD82 is a critical regulator of HSPC maintenance and function.

the bone marrow microenvironment to prevent HSC exhaustion and ultimately bone marrow failure (Kiel et al., 2007a; Pietras et al., 2011). Therefore, understanding the key molecules involved in regulating HSC quiescence is important especially for *in vitro* and *ex vivo* studies where the HSC pool is expanded for stem cell transplants. However, the maintenance of HSCs *in vitro* and *ex vivo* is problematic due to the loss of stem like and long-term reconstitution properties, which in part is due to differentiation (Ko et al., 2017; Ogawa et al., 1997; Schuster et al., 2012; Xie and Zhang, 2015). Regulation of HSC quiescence occurs through a number of signaling pathways, which include Notch, Wnt and TGF β (Angers and Moon, 2009; Bigas and Espinosa, 2012; Bigas et al., 2013; Hur et al., 2016; Kopan and Ilagan, 2009; Larochelle et al., 2012). We and others have shown that CD82 is also an important modulator of HSPC quiescence (Hur et al., 2016; Larochelle et al., 2012; Saito-Reis et al., 2018). For example, CD82 expression on HSPCs has been shown to promote interactions with adjacent cells within the bone marrow niche, which could contribute to HSPC quiescence. More specifically, the contact site between human CD34+ HSPCs and osteoblasts was shown to have increased CD82 expression and plasma membrane enrichment of CD82 occurred in the G₀ phase of the cell cycle (Larochelle et al., 2012). Using a global CD82KO mouse model, my studies show that the CD82KO LT-HSC population is reduced in the bone marrow compared to WT mice (Saito-Reis et al., 2018). Aligned with our findings, Hur et al. also detected a significant decrease of LT-HSCs within the bone marrow using a different CD82KO mouse, which further supports the conclusion that CD82 is an important regulator of LT-HSC quiescence. However, Hur et al. suggests that LT-HSC quiescence is maintained through an interaction with DARC expressed on macrophages, which is controversial in the tetraspanin field due to the lack of evidence that confirms this interaction. The interaction between CD82 and DARC was first discovered using a yeast two-hybrid screen (Zoughlami et al., 2012), however, in combination with non-specific mouse CD82 antibodies used in the Hur et al study, this interaction still needs to be validated. Our studies went on to find that the loss of CD82KO LT-HSCs within the bone

marrow is due to increased stem cell activation and cycling. Chronic HSC activation often results in bone marrow failure (Baumgartner et al., 2018; Harrison and Astle, 1982; Harrison et al., 1990; Mauch et al., 1988; Pawliuk et al., 1996), which often leads to the need for bone marrow transplantation. Therefore, my studies suggest that CD82 expression may be a potential marker for bone marrow failure.

The fitness of HSPCs is critical for the successful reconstitution of the hematopoietic system. We define HSPC fitness as the ability of HSPCs to effectively home and engraft within the bone marrow of a lethally irradiated organism. HSPC engraftment is essential to repopulate the hematopoietic cells of an individual that has undergone chemotherapy or radiation treatment. My study is significant because we identified a role for CD82 in HSPC engraftment. We found that CD82KO HSPCs are able to effectively engraft into lethally irradiated mice when transplanted on their own. In fact, monthly blood and immune cell phenotype analysis of WT and CD82KO engrafted mice suggest CD82KO HSPCs are functional with production of B, T and myeloid cells. However, we did detect a significant defect when CD82KO HSPCs were transplanted in a competitive repopulation assay with WT HSPCs. Here, we detected a decreased amount of circulating CD82KO cells within recipient mice compared to WT cells, which demonstrates a defect in CD82KO HSPC engraftment. Moreover WT derived T and B cells were measured in the blood, however, we detected increased numbers of CD82KO-derived myeloid cells within the blood of competitively engrafted recipient mice. The myeloid skewing we observed could be due to an ageing phenotype of the CD82KO mice. Additionally, many studies have shown that the loss of CD82 alters lymphoid cell activation and responses, which could explain the decreased production of lymphoid cells detected within the competitive repopulation assay. Many studies have described an important role for CD82 as an important co-stimulatory receptor for T cell activation (Delaguillaumie et al., 2004; Delaguillaumie et al., 2002; Iwata et al., 2002; Lagaudriere-Gesbert et al., 1998; Lebel-Binay et al., 1995). Using the same CD82KO mouse model, another group showed a dysregulation of T cell IFN- γ

response (Jones et al., 2016). Moreover CD82 was shown to interact with the B cell marker CD19 in a coimmunoprecipitation assay using B cell lines, but no function has been described (Horvath et al., 1998). More studies will need to be done to determine if the homing and engraftment defects we observed are due to enhanced ageing of the CD82KO hematopoietic compartment, which is consistent with the myeloid skewing we detect. Collectively these data suggest that CD82 is important for the engraftment of HSPCs.

Efficient HSPC homing is the first step for successful HSPC bone marrow engraftment. Understanding the molecules involved in HSPC homing is critical to improving this process for successful engraftment of HSPCs into the bone marrow microenvironment. We and others have determined a role for CD82 in HSPC homing (Larochelle et al., 2012; Marjon et al., 2016; Saito-Reis et al., 2018). One study demonstrated that human CD34+ HSPCs that were pre-treated with a CD82 blocking antibody and then intravenously injected into NSG mice showed decreased bone marrow homing (Larochelle et al., 2012). Also, previous data from our lab showed that CD82 regulates bone marrow homing of AML cells through the modulation of N-Cadherin (Marjon et al., 2016) and $\alpha 4$ integrin (Termini et al., 2014) organization and expression. My study specifically assessed a role for CD82 in HSPC homing. We demonstrate that CD82KO HSPCs and total bone marrow have decreased bone marrow homing. In addition, *in vitro* studies measured increased cell spreading and decreased migration of CD82KO HSPCs. Protein analysis using GLISA and active Rac1-specific antibodies measured an increase in active Rac1 in CD82KO HSPCs. The increase in Rac1 activity could explain the increase in cell spreading measured of CD82KO HSPCs plated on fibronectin and laminin. A specific study showed increased cell spreading of CD82KO dendritic cells (Jones et al., 2016). The increase in cell spreading we detect could also contribute to the decrease in cell migration measured by *in vitro* migration studies. We were able to recover CD82KO HSPC bone marrow homing and *in vitro* migration with the use of Rac1 inhibitors, EHOP and NSC23766. Therefore, our study suggests that regulation

of Rac1 activity downstream of CD82 is critical for promoting successful HSPC homing.

Under normal physiological conditions, HSPCs are found in circulation at very low numbers (Massberg et al., 2007). However, increased numbers of HSPCs mobilize into the blood in response to injury, infection or stress (Heidt et al., 2014; Massberg et al., 2007). Our study is the first to describe a role for CD82 in HSPC mobilization. In chapter 3, we determined that CD82 modulates HSPC mobilization through the regulation of the S1PR₁. The CXCR4 and S1PR₁ receptors are heavily studied as important mediators of HSPC mobilization (Golan et al., 2012; Juarez et al., 2012; Nie et al., 2008; Tzeng et al., 2011). However, we found no difference in CXCR4 expression or activity as mentioned in chapter 2, so we focused our attention on S1PR₁. The loss of CD82 results in enhanced HSPC mobilization through the increased surface expression of S1PR₁. We determined that increased S1PR₁ surface expression on CD82KO HSPCs is due to decreased internalization of this receptor. At this time, more studies need to be conducted to determine if there is a direct or indirect interaction between CD82 and S1PR₁. Collectively, these studies present an important role for CD82 in HSPC mobilization, which could be exploited to improve the mechanisms for harvesting stem cells for bone marrow transplantations.

4.2.1 Clinical Significance

Currently, HSPCs are the gold standard treatment in the clinic for non-hematologic and hematologic diseases (Copelan, 2006). Allogenic bone marrow transplantation is the most common treatment method, which uses stem cells from a donor (Copelan, 2006; Hatzimichael and Tuthill, 2010). The different sources of hematopoietic stem cell collection are from bone marrow, peripheral blood or cord blood. However, there are two major limitations of bone marrow transplantations which include: 1) limited numbers of transplanted stem cells effectively engraft into the bone marrow and 2) limited numbers of stem cells are acquired at time of collection. Therefore, determining the molecules and

mechanisms involved in HSPC homing, engraftment and mobilization will improve the efficacy of bone marrow transplants.

In chapter 2, we determined that CD82 is an important regulator of HSPC bone marrow homing and engraftment. We found that in the absence of CD82, HSPC bone marrow homing and engraftment is decreased. Therefore, in the clinic CD82 could be used as an additional marker to predict stem cell transplant success. We hypothesize that increased CD82 expression would promote HSPC bone marrow homing, therefore, resulting in successful transplantation. According to the National Marrow Donor Program, from 1998 to 2011, the amount of transplants performed using peripheral blood stem cell transplants increased over time. Currently, collection of mobilized HSPCs within the peripheral blood is preferred compared to the alternative bone marrow puncture. Although current collection methods of mobilized HSPCs from the peripheral blood yields less stem cells compared to other collection methods, this method also allows for quicker donor recovery. Therefore, determining ways to improve HSPC mobilization into the peripheral blood will increase the amount of HSPCs for collection. In chapter 3, we determine that CD82 may be a novel target to increase HSPC mobilization into the peripheral blood for the use of bone marrow transplants. Many studies have shown that the use of antibodies to block surface receptor/molecule expression can promote HSPC mobilization (Bonig et al., 2009; Craddock et al., 1997; Nishioka et al., 2015; Papayannopoulou et al., 1995; Papayannopoulou et al., 1998; Papayannopoulou et al., 2001b; Qian et al., 2006). Additionally, our data in chapter 3 suggests that treatment of WT mice with a CD82 antibody, in combination with AMD3100, promotes HSPC mobilization compared to WT HSPCs. Therefore, these findings are promising evidence that antibodies against CD82 can enhance HSPC mobilization.

4.3 Future directions

A few unanswered questions remain about the mechanism by which CD82 mediates HSPC quiescence. We found that CD82 is an important regulator of HSPC quiescence, however, future studies are needed to determine what

signaling pathways are altered to induce CD82KO HSPC activation. Potential regulators of HSPC quiescence are the Notch and or Wnt signaling pathways, which could be altered in CD82KO HSPCs to induce cycling (Angers and Moon, 2009; Bigas and Espinosa, 2012; Bigas et al., 2013; Kopan and Ilagan, 2009). Hur et al. suggested that CD82KO HSPC activation is mediated by the TGF β signaling pathway. However, the induction of this pathway through the interaction between CD82 and DARC is still controversial. Future experiments could investigate if the Notch, Wnt or TGF β signaling pathways are responsible for the increased CD82KO HSPC activation that we observe. Therefore, identifying a mechanism for CD82KO HSPC quiescence would be essential to maintain the primitive HSC pool.

The interactions between HSPCs and the bone marrow microenvironment are critical for the tight regulation of the hematopoietic compartment. Tetraspanins are known to interact with other tetraspanins, adhesion and signaling molecules to form tetraspanin-enriched micro-domains (TEMs). Our lab showed that CD82 expression and organization on the surface of AML cells modulates α 4 integrin and N-Cadherin expression to promote HSPC and bone marrow interactions (Marjon et al., 2016; Termini et al., 2014). In chapter 2, we also identified a significant decrease in the α 6 integrin surface expression on CD82KO HSPCs, which could result in weaker interactions and allow for easier activation of HSPCs within the bone marrow microenvironment. To investigate if decreased expression of the α 6 integrin of CD82KO HSPCs results in weak bone marrow interactions, future experiments could include transduction of the α 6 to try and rescue integrin to rescue this phenotype. Collectively, these experiments would identify a role for α 6 in promoting CD82-mediated HSPC interactions with the bone marrow.

Chronic stress has been shown to promote HSPC activation, which results in pathological diseases such as bone marrow failure and more recently cardiovascular diseases (Heidt et al., 2014). Our data suggest that CD82KO HSPCs have increased activation under basal conditions, but downstream pathologies have not yet been detected. We hypothesize that increased HSPC

activation in CD82KO animals results in enhanced differentiation of inflammatory immune cells, which could contribute to atherosclerotic plaque formation. My preliminary studies show that CD82KO mice have increased aortic lipid content compared to WT mice (Supplemental Figure 3). However, future studies are needed to assess the mechanism for increased lipid content within the aortas of CD82KO mice under basal conditions. Future studies could also assess the pathogenic burden of the cardiovascular system within WT and CD82KO mice placed on a high fat diet. Furthermore, measurements of circulating inflammatory immune cells within the vasculature of WT and CD82KO mice on a normal chow or high fat diet could offer insight into a mechanism for increased aortic lipid content.

HSPCs undergo a series of self-renewal and differentiation processes that are critical for the maintenance of the hematopoietic compartment. Classic signs of HSPC aging include increased stem cell activation, decreased homing and engraftment and myeloid skewing. Based on our studies, we have detected these classic aging signs within CD82KO HSPCs. Determining the mechanism for myeloid skewing of CD82KO HSPCs is of interest because we detect this phenotype only under stress conditions (Fluorouracil (5-FU) treatment, serial transplantation and competitive engraftment). We have conducted a serial transplantation experiment in which we detected a significant decrease of the WT pool at the tertiary engraftment, which is when we expected exhaustion of the HSC pool (Supplemental Figure 1). However, at the tertiary and quaternary engraftment we detected a significant increase in the CD82KO pool, which was unexpected due to the increase in HSPC activation we measured in the CD82KO mouse (Supplemental Figure 1). Furthermore, we went on to characterize the immune cell populations from each donor pool and found that majority of the CD82KO tertiary and quaternary population were from the myeloid lineage (Supplemental Figure 2). This phenotype is consistent with myeloid skewing that we have detected with the competitive repopulation experiments described in chapter 2. Future experiments will be done to determine what could be contributing to the myeloid skewing phenotype we detect and to see if we have a

leukemic phenotype due to the hyperproliferation of myeloid cells. We have not detected any oncogenic phenotype within the CD82KO mouse model under normal physiological conditions. However, we speculate that the deletion of exon 5 and 6 of CD82 within our mouse model could contribute to this oncogenic phenotype. One study showed that a splice variant of CD82 due to the deletion of exon 7 increased tumorigenicity and invasion of gastric cancer (Lee et al., 2003). Therefore, future studies are needed to determine if the deletion of CD82 in our KO mice can contribute to oncogenesis. Additional studies would need to be conducted to determine if ageing the CD82KO mice would also contribute to disease burden. It has been shown that CD82 expression is enriched in HSPCs and expression gradually decreases towards more differentiated hematopoietic populations (Burchert et al., 1999; Hur et al., 2016). Therefore, determining if CD82 expression decreases overtime in the HSPC population in aging mice could be important to better understand the mechanism of HSPC aging.

HSPC homing is a multi-step process that is mediated by a combination of molecules such as signaling and adhesion molecules. The defect we detected in CD82KO HSPCs was due in part to hyper-activation of Rac1, where homing was recovered with the use of Rac1 inhibitors. Future studies could determine if other Rho GTPases such as Cdc42 or RhoA levels are also differentially expressed in CD82KO HSPCs. Other studies have shown that RhoA expression contributes to weak interactions of cells (Lawson and Burrige, 2014), which could potentially contribute to the decrease in CD82KO HSPCs we detect in the bone marrow. Additionally, Cdc42 has also been shown to be an important mediator of HSPC quiescence, homing and retention (Liu et al., 2011b; Williams et al., 2008; Yang et al., 2001; Yang and Zheng, 2007). Many studies have shown that the loss of Cdc42 contributes to increased stem cell activation, decreased LT-HSCs within the bone marrow, decreased homing, increased mobilization and myeloid skewing (Yang and Zheng, 2007). These defects are also consistent with what we observed with CD82KO HSPCs.

Under basal conditions, HSPCs are found within the peripheral blood at very low amounts but can be increased with mobilizing drugs. In addition to

CXCR4, the S1PR is important for HSPC mobilization. We determined that CD82 regulates HSPC mobilization through the modulation of surface S1PR₁ expression. CD82KO HSPCs have increased S1PR₁ expression, which we determined was due to decreased receptor internalization. However, it is still unclear if CD82 directly or indirectly contributes to S1PR₁ internalization. Our data indicate that under basal levels CD82KO HSPCs have decreased S1PR₁ internalization, however, upon treatment with S1P ligand, S1PR₁ internalization is enhanced when compared to WT HSPCs. One explanation for increased S1PR₁ internalization upon S1P treatment could be due to increased expression of surface S1PR₁. Increased S1PR₁ internalization in CD82KO HSPC is consistent with increased pERK and pAKT signaling upon S1P treatment. It is known that S1PR₁ signaling still occurs on early endosomes upon ligand binding and then is recycled back to the plasma membrane (Mullershausen et al., 2009; Reeves et al., 2016; Thangada et al., 2010). Therefore, the increase in CD82KO HSPC S1PR₁ signaling could be due to increased receptor recycling. In addition, the results we found are with S1P treatment, which is not specific to S1PR₁, therefore treatment with SEW2871, another S1PR₁ agonist would be critical to tease out the mechanism for downstream mediated signaling of this receptor.

In conclusion, studies described in this dissertation have provided evidence that tetraspanins are important regulators of HSPC function. More importantly we have identified additional roles for CD82 in the regulation of HSPC quiescence, homing, engraftment and mobilization. The current limitations of bone marrow transplants are 1) limited numbers of transplanted stem cells engraft into the bone marrow and 2) limited numbers of stem cells acquired at the time of collection. Our studies have provided evidence that the modulation of CD82 could be used in the clinic to address these two main limitations. For example, we provided evidence that CD82 can be exploited to promote HSPC homing and improve bone marrow transplant. Additionally, CD82 may be a therapeutic target to enhance HSC mobilization into the blood to increase HSC numbers acquired for bone marrow transplants. My collective dissertation

presents evidence of the critical role for CD82 in the regulation of HSPC fitness and bone marrow retention.

Appendices

Appendix A: Abbreviations used

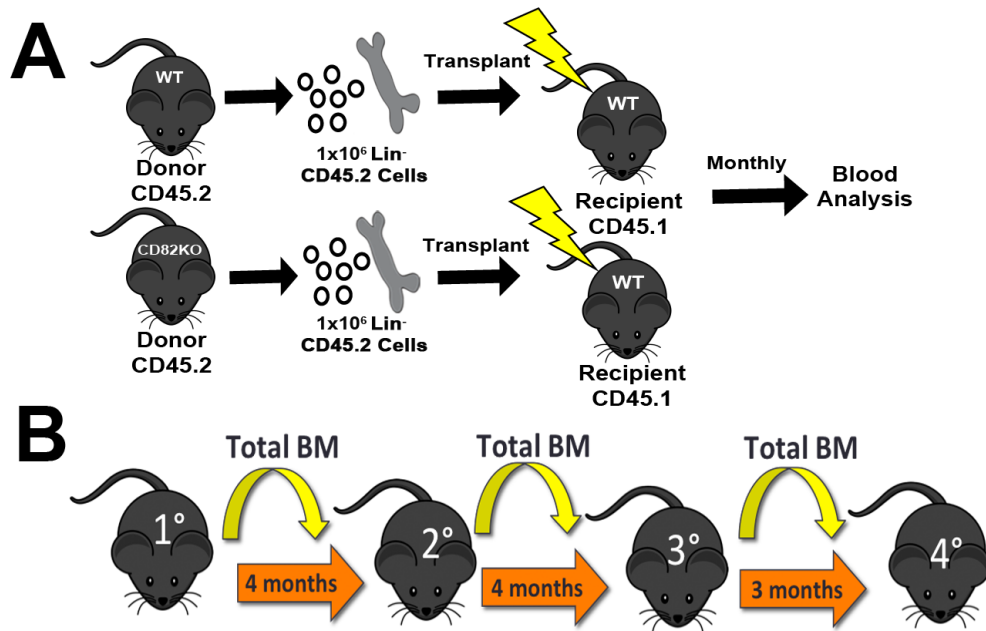
5-FU- fluorouracil
ANG-1– angiopoietin 1
AML – acute myeloid leukemia
AKT– protein kinase B
BOYJ– B6.SJL-Ptprc^a Pepc^b/BoyJ mice
BMP – bone morphogenic protein
BrdU– bromodeoxyuridine
CCG – Cys-Cys-Gly amino acid motif within large tetraspanin loop
CD82KO—Knock out of CD82
CFU – colony forming unit
CLL – chronic lymphocytic leukemia
CLP– common lymphoid progenitor
CML– chronic myeloid leukemia
CMP – common myeloid progenitor
CXCL12 – C-X-C motif ligand 12, also known as SDF-1
CXCR4– C-X-C chemokine receptor 4
DARC – Duffy antigen/chemokine receptor
EC1 – small extracellular loop of tetraspanins
EC2 – large extracellular loop of tetraspanins
ECM – extracellular matrix
EGFR – epidermal growth factor receptor
ELISA- enzyme-linked immunosorbent assay
ERK – extracellular signal-regulated kinases
E-selectin – Endothelial selectin
FACS– fluorescence-activated cell sorting
FN– fibronectin

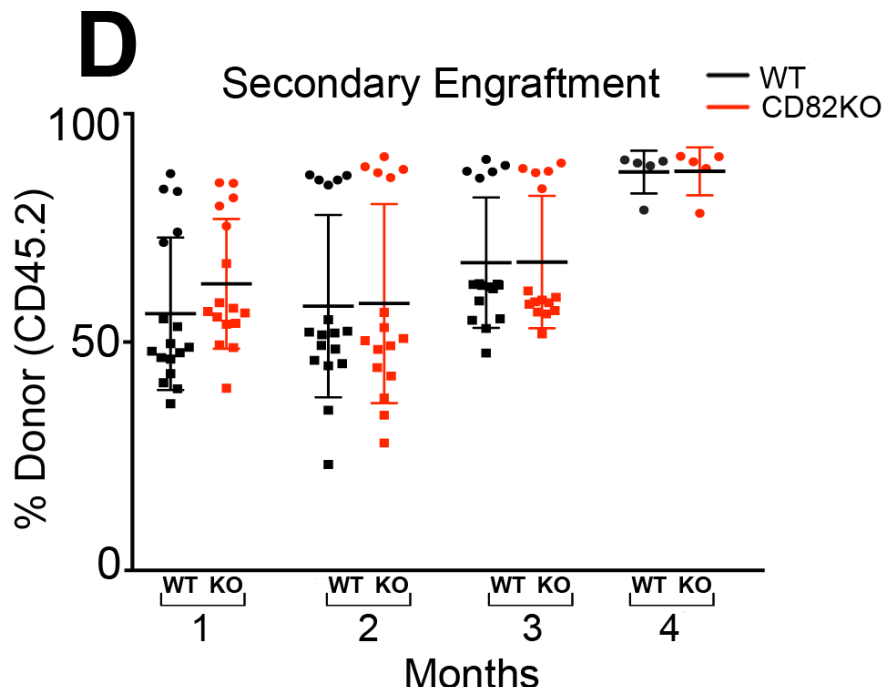
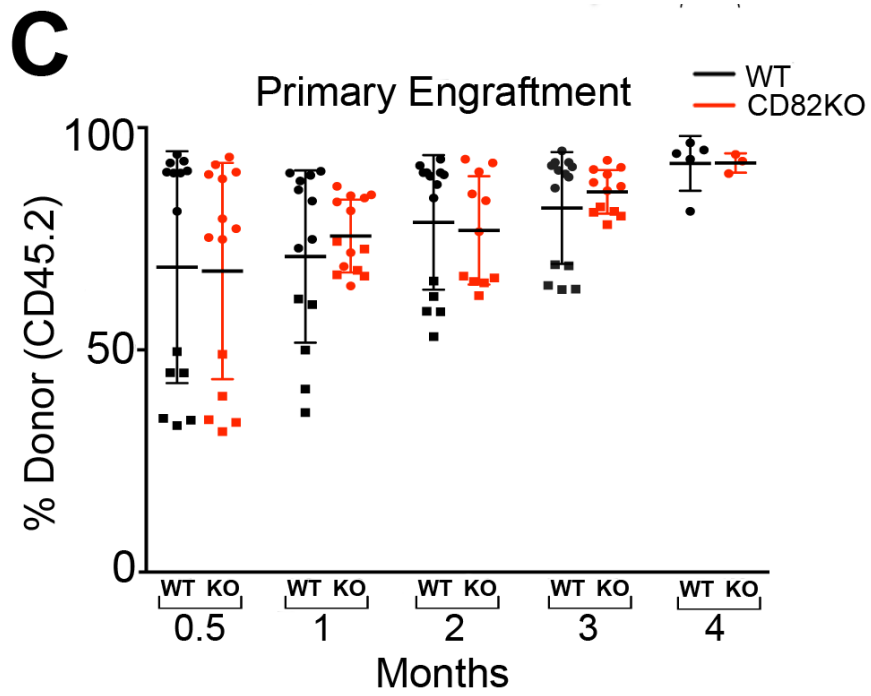
FLT-3 – FMS-like tyrosine kinase 3
FOXO1A– forkhead box protein O1a
GAP–GTPases-activating protein
G-CSF – granulocyte-colony stimulating factor
GM-CSF – granulocyte-macrophage colony-stimulating factor
GDP–guanine diphosphate
GEF–Guanine nucleotide exchange factor
GLISA–small GTPase activation assay
GMP–granulocyte-macrophage progenitor
GPCR – G-protein-coupled receptor
GTP – guanosine triphosphate
GVHD – graft-versus-host disease
GVL–graft-versus-leukemia
GVT–graft-versus-tumor
HIV–human immunodeficiency virus
HLA – human leukocyte antigen
HSPC – hematopoietic stem/progenitor cell
HSC – hematopoietic stem cell
HSCT– hematopoietic stem cell transplantation
IACUC– institutional animal care and use committee
ICAM-1 – intracellular adhesion molecule-1
IDB– ingenol 3,20 dibenzoate
JAK–janus kinase
Ki-67–nuclear antigen for proliferation
KO–knock out
Lin (-) – lineage negative
LM–laminin
LT-HSC – long-term hematopoietic stem cell
LTMR–long-term multi-lineage repopulating
L-selectin–leukocyte selectin
LSK–Lin-Sca+ckit+, HSPC population

MAPK – mitogen-activated protein kinase
MEP–megakaryocyte erythrocyte progenitor
MFI–mean fluorescence intensity
MLL – mixed-lineage leukemia
MMP-9–matrix metalloproteinase-9
MPL – myeloproliferative leukemia
MPP – multipotent progenitor cell
MSC – mesenchymal stem cell
N-CAD- N-cadherin
N-ICD – Notch intracellular receptor domain
NSG – NOD scid gamma
PAK–p21 activating kinase
PBSCs–peripheral blood stem cells
PBX1–pre B cell acute lymphoblastic leukemia
PI3K – phosphoinositide-3-kinase
PKC – protein kinase C
PMA – phorbol 12-myristate 13-acetate
P-selectin–platelet selectin
RAC1–ras-related C3 botulinum toxin substrate 1
RAC2– ras-related C3 botulinum toxin substrate 2
SCF – stem cell factor
SDF-1 – stromal cell-derived factor-1, also known as CXCL12
SFEM–serum free expansion medium
S1P–sphingosine 1-phosphate
S1PR–sphingosine 1-phosphate receptor
SNO – N-cadherin+CD45-
SPHK-1– sphingosine phosphate 1 kinase 1
STAT5 – signal transducer and activator of transcription 5
ST-HSC –short term hematopoietic stem cells
Tcf/Lef – T-cell factor/lymphoid enhancer factor
TEM – tetraspanin enriched microdomain

TIE2–angiopoietin receptor
TIMP1–tissue inhibitor of metalloproteinase
TGF – transforming growth factor
TNF–tumor necrosis factor
TPO – thrombopoietin
VCAM-1 – vascular cell adhesion molecule 1
WT–wild type

Appendix B: Supplemental Data





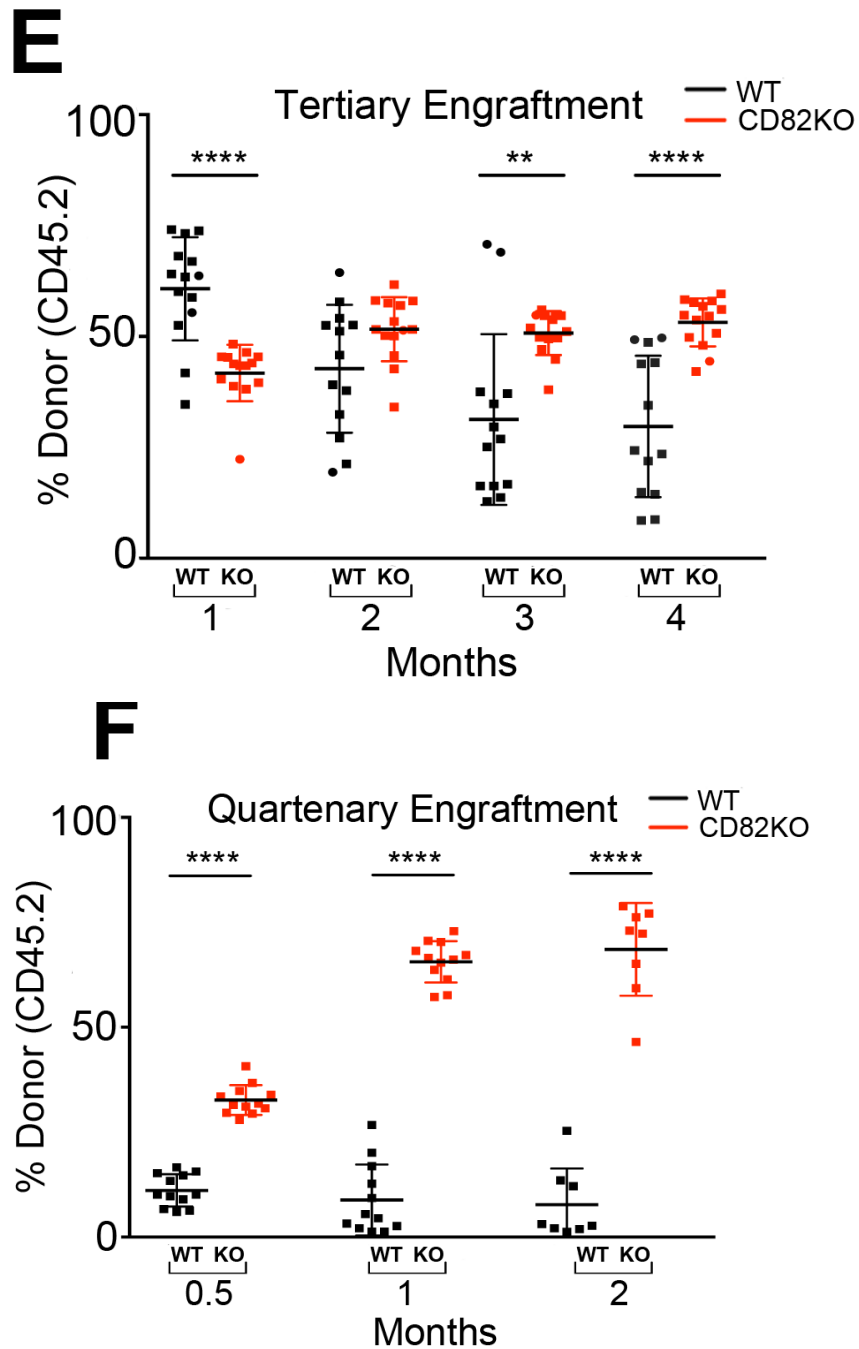
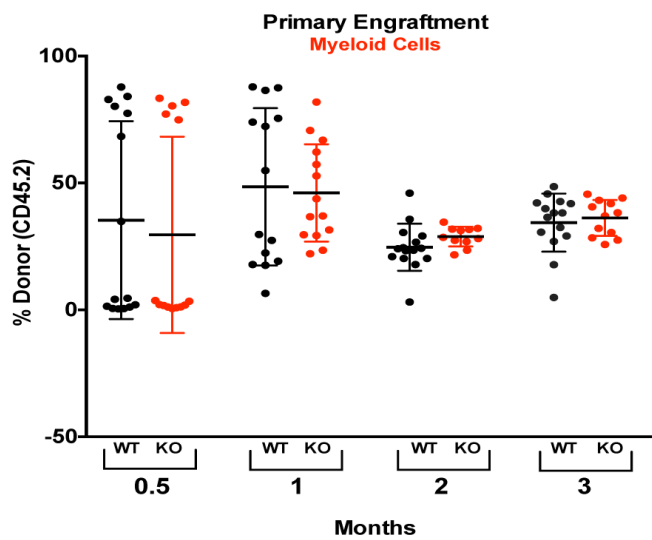
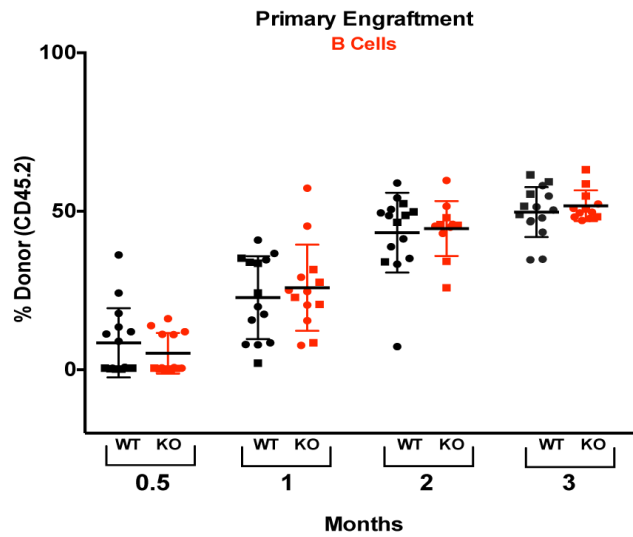
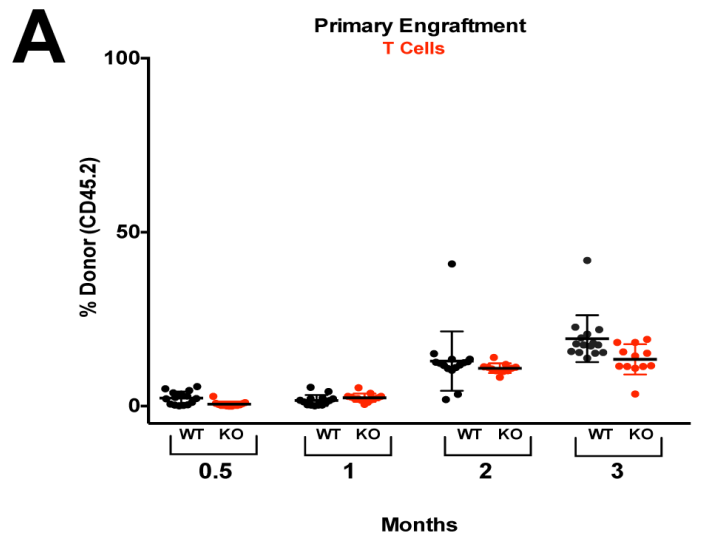
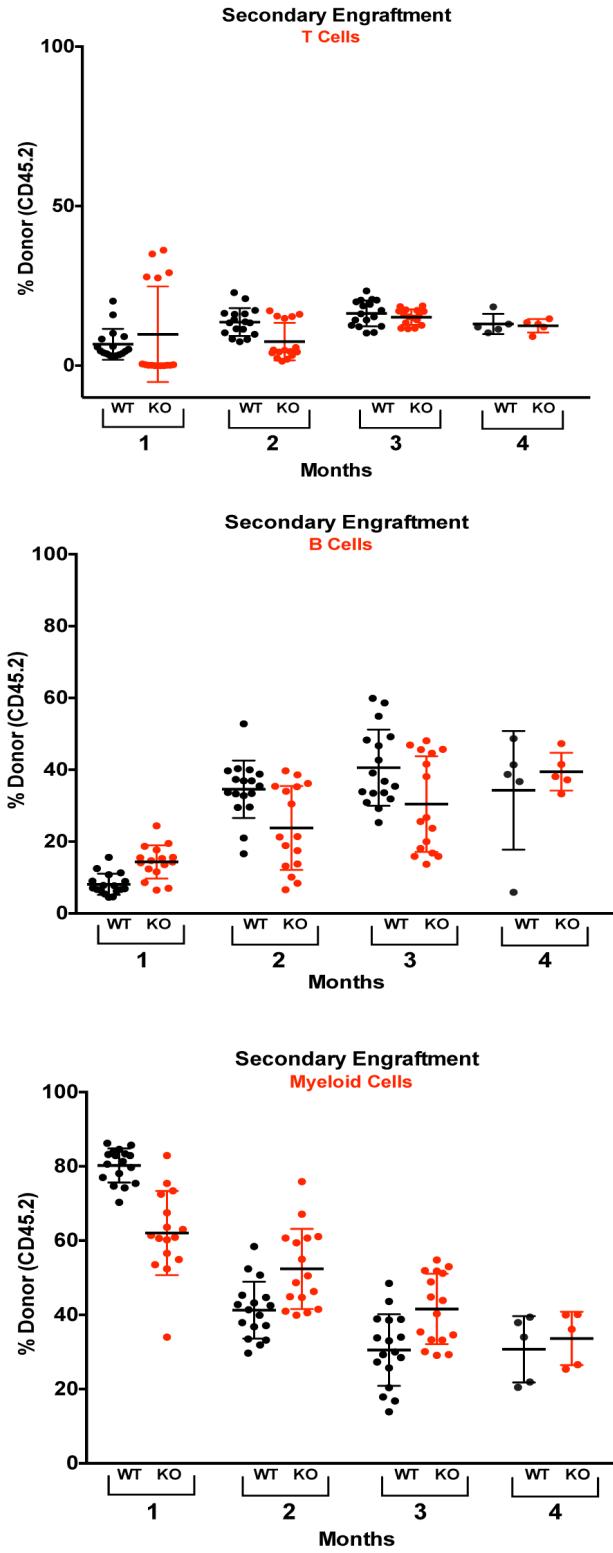
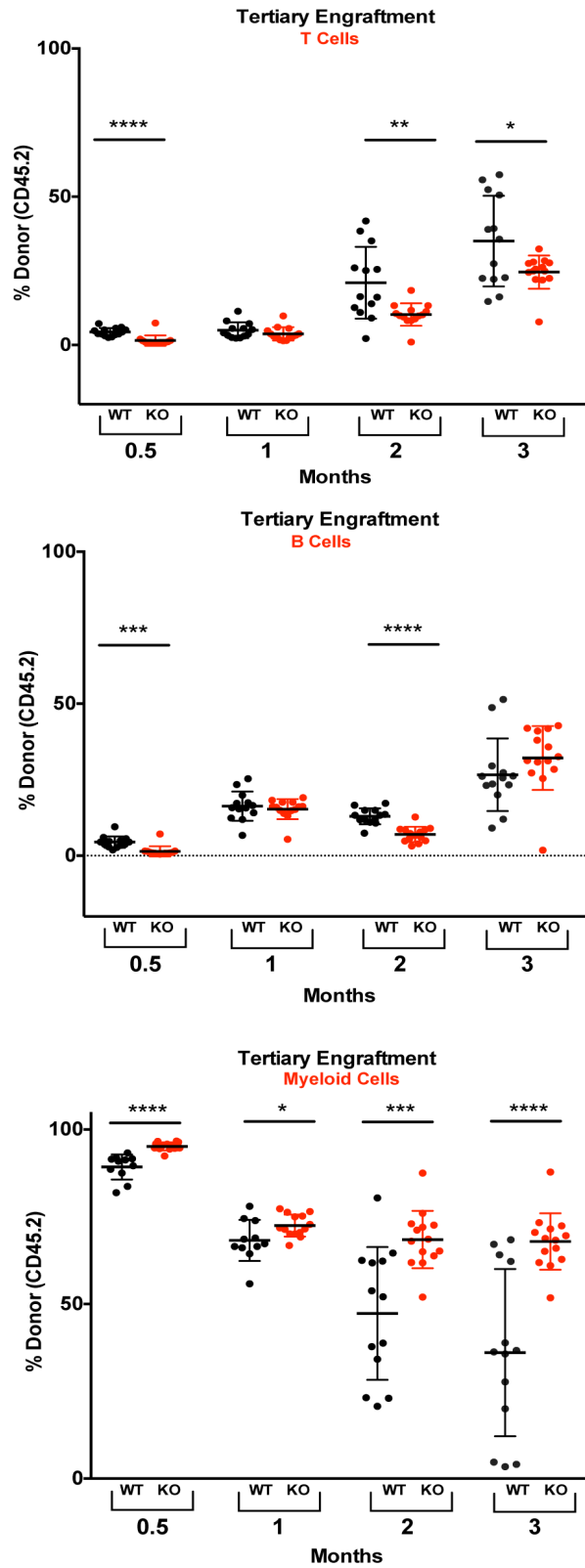


Figure S1: Serial Transplantation of WT and CD82KO HSPCs. (A) Experimental scheme for a primary engraftment experiment. (B) Experimental scheme for a serial transplantation experiment. (C-F) The percentage of donor cell repopulation of peripheral blood collected monthly from tail bleeds for primary to quaternary engraftment studies. Error bars, SEM; n= 5-15 mice per strain, Student's t-test (**p<0.01 and ****p<0.0001).



B

C

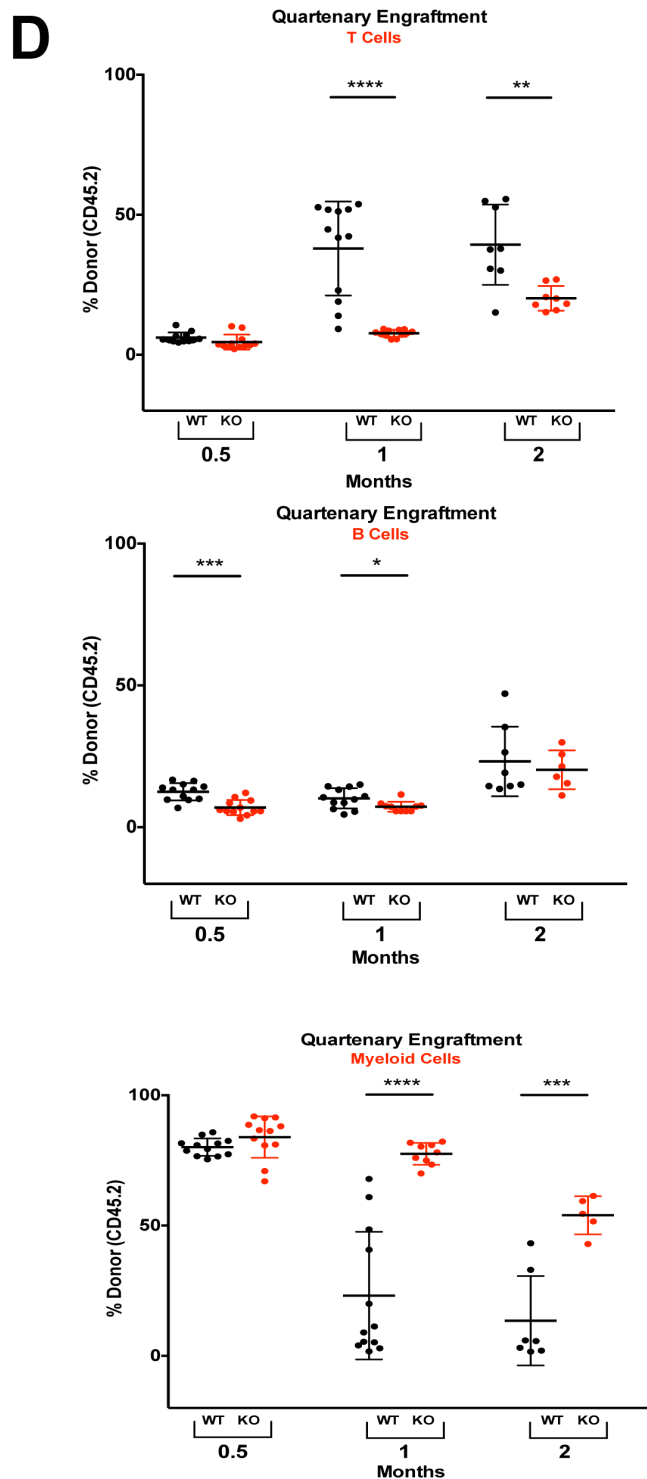


Figure S2: WT and CD82KO Serial Transplantation immune cells (A-D) Flow cytometry analysis of the percentage of donor immune cells (B cells (B220), T cells (CD3) and myeloid cells (Gr1/Mac1)) from donor population in S1. Error bars, SEM; n= 5-15 mice per strain (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001).

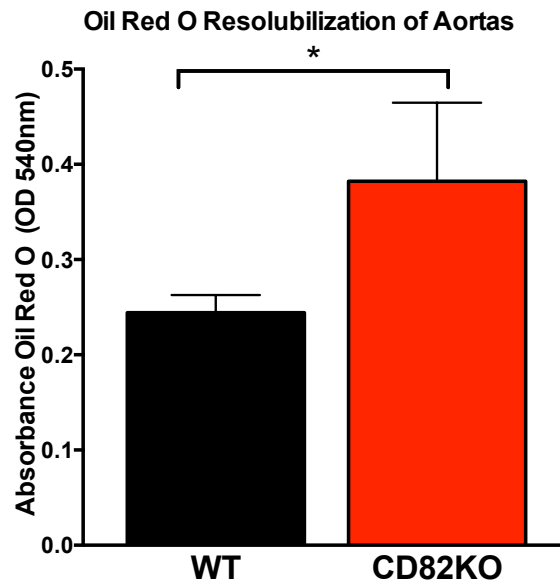


Figure S3: CD82KO mice have increased aortic lipid content. WT and CD82KO aortas stained with Oil Red O to assess lipid content. Unpaired t-test, (* $p < 0.05$).

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