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CD82 Regulation of Hematopoietic Stem/ Progenitor Cell - Niche Adhesion

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CD82 REGULATION OF HEMATOPOIETIC STEM/PROGENITOR CELL – NICHE ADHESION

BY

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THESIS

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DEDICATION

I dedicate this work to my wonderful husband, Matthew Roll. I cannot even begin to express how grateful I am for your unwavering support and immense patience. Two years ago, we began a journey with many unknowns. As I navigated through the unknowns of graduate school and life in a new city, and you worked to overcome the unknowns of starting a business, we also grappled with the resounding unknowns of a new and long-distance relationship. A relationship that quickly became a lifelong commitment and partnership. Despite the inherently difficult nature of our situation, I cannot thank you enough for allowing me this time to experience incredible academic and personal growth and to pursue my independence, while never failing to be there to encourage me along the way. I have learned a great deal during my time in graduate school, but I truly believe that I have learned the most from you, always the teacher. You have taught me much about life and love and the importance of having goals. You have taught me the value of openness and spontaneity and reminded me that the unknown is not so scary. You constantly challenge and inspire me to do things I never thought I could do. I admire your generosity, selflessness, and humility. You have kept me sane and relieved my anxieties through several thousand hours spent talking on the phone, and I will never forget the endurance, strength, and willpower you garnered to drive back and forth over 1,000 miles round trip on your visits (thank goodness for the Prius!). And although we have spent much of our time apart in a geographical sense, no one has ever felt more like home to me. Thus, I look forward to completing this chapter in my life and beginning a new one with you, my husband and best friend.

I would also like to dedicate this thesis to my family. To my mother, Maureen Sullivan Cotter (Mho), I hardly have the words to express my gratitude for all that you have done for me over the last 24 years. Let me start by saying thank you for your infinite love, unrelenting encouragement, and tireless belief in me. Thank you for allowing me to discover my own path and individuality and for playing the most important role in my early years as a student and scientist. To my father, Barry Cotter, thank you for your constant strength and support and for always providing valuable advice on navigating the real world. It is by your example that I have learned the values of hard work and sacrifice. To my brother, James Cotter, thank you for your irreplaceable friendship over the years. To my sister, Colleen Cotter, thank you for reminding me to laugh and not take life so seriously. To my grandmother, Dolores Sullivan, I owe you special thanks for taking me into your home with open arms and putting up with me during my graduate studies. I know it wasn't always easy for you, and I cannot thank you enough for your true generosity.

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ABSTRACT

The spatial organization and dynamics of proteins and lipids within the cell membrane is important for the regulation of cell signaling, adhesion, and cell communication. Within the bone marrow niche, communication between hematopoietic stem/progenitor cells (HSPCs) and niche cells is essential for regulating their proliferation, differentiation, and survival. Our previous work has ascertained that HSPCs utilize a polarized domain on the plasma membrane that serves as the contact site with osteoblasts, which are important members of the bone marrow niche. Using human primary CD34+ stem/progenitor cells and the progenitor-like KG1a cell line, we found this domain to be enriched in the specific tetraspanin proteins, CD63, CD81, and CD82. Tetraspanins are multi-spanning membrane proteins that act as scaffolds for the organization of membrane domains important for regulating adhesion and signaling. CD82 is of particular interest, as it is highly expressed on HSPCs and downregulated during HSPC differentiation. Our characterization of CD82 function using CD82-blocking antibodies revealed a significant decrease in adhesion of HSPCs to niche cells as well as in the *in vivo* homing and engraftment capabilities of these cells. To determine the molecular mechanisms of CD82's role in adhesion, we have generated CD82

overexpression and knockdown cell lines using the KG1a background. Our data indicate that the level of CD82 expression positively correlates with the extent of adhesion to fibronectin and osteoblasts but has no effect on binding to collagen I or laminin. The increase in adhesion we observed with CD82 overexpression was inhibited by the VLA-4-specific peptide, LDV, indicating a potential role for the VLA-4 (α4β1) integrin. Investigations into potential CD82-mediated mechanisms of VLA-4 regulation have revealed that CD82 regulates both the expression and avidity of VLA-4 but does not regulate its affinity. Taken together, the VLA-4 expression and avidity changes could account for the observed adhesion changes with differing CD82 expression levels. Finally, assessment of CD82 palmitoylation using KG1a CD82 palmitoylation mutant cells revealed that palmitoylation may be required for the CD82-induced changes in adhesion.

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List of Abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Ang-1	angiopoietin
APC	antigen presenting cell
BCA	bicinchoninic acid
BME	β-mercaptoethanol
BMP	bone morphogenic protein
BMP-2	bone morphogenic protein 2
BMP-7	bone morphogenic protein 7
BMPR1a	BMP receptor 1a
BSA	bovine serum albumin
CAR cell	CXCL12-abundant reticular cell
CCG	cysteine-cysteine-glycine
$CD82^{-/-}$	CD82 knockout mice
cDNA	complementary DNA
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CS-1	connecting segment-1
CXCL12	CXC-chemokine ligand 12
DHHC	aspartic acid-histidine-histidine-cysteine
DMSO	dimethyl sulfoxide
dSTORM	direct stochastic optical reconstruction microscopy
EC1	small extracellular loop
EC2	large extracellular loop
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FRET	Förster resonance energy transfer
G-CSF	granulocyte colony-stimulating factor
G418	Geneticin
GM-CSF	granulocyte/macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HBSS	Hank's buffered salt solution
HeNe	helium/neon
HIF	hypoxia-inducible factor
HIF-1	hypoxia-inducible factor 1
HIF-2	hypoxia-inducible factor 2
HRP	horseradish peroxidase
HSPC	hematopoietic stem/progenitor cell
HUVEC	human umbilical vein endothelial cell

IC	intracellular loop
ICAM	intercellular adhesion molecule
ICAM-1	intercellular adhesion molecule 1
ICAM-2	intercellular adhesion molecule 2
IgSF CAM	immunoglobulin superfamily cell adhesion molecule
IL-1	interleukin 1
IL-6	interleukin 6
IL-7	interleukin 7
KAI1	Kangai-1, also referred to as CD82
K_d	equilibrium dissociation constant
k_{off}	dissocation rate constant
LDV	leucine-aspartic acid-valine
LFA-1	lymphocyte function-associated antigen 1, also referred to as αLβ2
LIC	leukemia-initiating cell
LIF	leukemia inhibitory factor
M-CSF	macrophage colony-stimulating factor
mAb	monoclonal antibody
MARCKS	myristolated alanine-rich C-kinase substrate
MCF	mean channel fluorescence
MHC II	major histocompatibility complex class II
miRNA	microRNA
PAB	1X PBS containing 0.1% sodium azide and 2% BSA
pAb	polyclonal antibody
PAT	protein acyltransferase
PBS	phosphate-buffered saline
PBST	1X PBS containing 0.2% Tween-20
PE	phosphatidyl ethanolamine
PenStrep	penicillin and streptomycin
PFA	paraformaldehyde
PI	propidium iodide
PI4K	phosphatidylinositol 4-kinase
PKC	protein kinase C
PKCα	protein kinase C-α
PKCβ2	protein kinase C-β2
PKCε	protein kinase C-ε
PKCζ	protein kinase C-ζ
PPR	PTH-related peptide receptor
PSF	point spread function
PTH	parathyroid hormone
PVDF	polyvinylidene difluoride
RIPA	radioimmunoprecipitation assay
RT-qPCR	reverse transcription quantitative polymerase chain reaction
RTK	receptor tyrosine kinase
SCF	stem cell factor
SCID	severe combined immunodeficiency
TCR	T cell receptor

TEM	tetraspanin-enriched microdomain
TGFβ1	transforming growth factor beta 1
THPO	thrombopoietin
TIRF	total internal reflection fluorescence
VCAM	vascular cell adhesion molecule
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4, also referred to as α4β1
VLA-5	very late antigen-5, also referred to as α5β1
YXXφ	tyrosine-X-X-phi

CHAPTER 1: INTRODUCTION

1.1 Overview

Cells receive signals or cues from their surrounding environment and respond in ways to optimize survival, maintain quiescence, promote proliferation and differentiation, (or self-renewal in the case of stem cells), and regulate many other essential processes. To do this, cells have established signal transduction mechanisms that allow the transfer of extracellular signals through the plasma membrane into the cell cytoplasm and nucleus, thus promoting information flow from outside to inside (or vice versa). The cell can then respond by regulating gene expression and protein levels, altering molecular localization and dynamics, and adjusting enzymatic activity to balance cellular processes and maintain homeostasis (Alberts, 2002). Different cell types may respond differently to environmental cues, and in the case of stem cells, the microenvironment or "niche" in which the cell originates and resides determines the cell's future cellular and differentiation programs (Alberts, 2002; Schofield, 1978). In this way, environmental conditions are crucial for maintaining or encouraging particular cell behaviors and functions. However, our understanding of the signal transduction mechanisms, especially the spatiotemporal aspects of the molecular interactions involved in such cell-niche communication is not complete. In particular, further studies are needed to better characterize the spatial organization and clustering dynamics of membrane proteins and cell surface receptors and to understand how such membrane organization can serve as protein interaction platforms and regulate complex signaling systems (Vereb et al., 2003).

The work presented in this thesis will provide insights into some of these issues with a focus on the molecular mechanisms involved in regulating the interactions

between hematopoietic stem/progenitor cells (HSPCs) and their bone marrow niche microenvironment. The studies discussed here provide evidence for the functional role of a membrane tetraspanin protein found on the HSPC surface, known as CD82, in regulating HSPC-niche adhesion. The thesis will begin with background information on the CD82 scaffold protein, membrane organization, and potential CD82-interacting proteins, with a focus on integrins. The succeeding sections will outline the initial experiments demonstrating CD82's involvement in bone marrow niche interactions and provide literary support for the stated hypothesis. This will be followed by a detailed description of the methods and techniques used to assess the mechanisms utilized by CD82 at the molecular level. Herein, the attention will be on investigating the involvement of integrins, especially α4β1 (also referred to as very late antigen-4, or VLA-4), and membrane organization, including trafficking and protein clustering at the single molecule level. The data and insight gained from these investigations will serve to illustrate, albeit only in part, the CD82 story in hematopoietic stem/progenitor cells. Finally, this thesis will close with an overview of results, important conclusions, and the significance of this work and will again draw upon the literature and available resources in proposing new and exciting future directions.

1.2 Background

1.2.1 Key Words

HSPC, Bone Marrow, Niche, Osteoblast, Fibronectin, Adhesion, Integrin, α4β1 (VLA-4), Tetraspanin, CD82

1.2.2 Hematopoietic Stem/Progenitor Cells (HSPCs)

Hematopoietic stem/progenitor cells (HSPCs) are defined by their ability to undergo self-renewal, their potential to differentiate into any one of the distinct hematopoietic/blood cell lineages that make up the entire hematopoietic system, and their capacity to proliferate and replenish several million blood cells throughout a lifetime (Szilvassy, 2003). Self-renewal is an important cellular process, in which a steady pool of stem cells is persistently maintained to ensure the continuation of hematopoiesis. In this process, a parent HSPC can divide and preserve at least one of its daughter cells in the undifferentiated, stem-like state (Renström et al., 2010). While HSPCs are predominantly found in the quiescent, G0 phase of the cell cycle with a slow cell cycling/turnover rate, they are also multipotent, possessing the ability to differentiate when necessary into one of the eight major cell lineages of the hematopoietic system, including red blood cells, or erythrocytes, and white blood cells, encompassing platelets/megakaryocytes, B lymphocytes, T lymphocytes, monocytes/macrophages, neutrophils/granulocytes, basophils/mast cells, and eosinophils (Renström et al., 2010; Szilvassy, 2003). These different cell types offer a wide range of essential functions to support overall health. They help carry nutrients and oxygen throughout the body, regulate vasodilation and blood pressure to promote normal blood flow, control immune homeostasis, digest and remove damaged cells and tissue, support wound healing, and fight off infections (Alberts, 2002; Kawamoto et al., 2010; Renström et al., 2010; Wan et al., 2008). In addition, an abundant proliferation capacity allows the estimated 50 million HSPCs in the human body to produce up to 10^{13} new blood cells each, thus ensuring the successful replenishment of mature blood cells throughout a lifetime (Szilvassy, 2003). These HSPC

characteristics ultimately confer a healthy homeostatic balance to the hematopoietic system by allowing the continual regeneration of the stem cell pool and by affording the ability to regulate proliferation and differentiation in response to the body's needs.

With regards to maintaining the various cell lineage populations, hematopoietic stem/progenitor cells can be thought of as the founding regulators of a healthy hematopoietic system. However, in order for HSPCs to know which cellular program to follow, they themselves must receive regulatory cues. Regulatory influences typically arise from the cell's surrounding environment, such that the cell adapts its response to meet changing environmental conditions. HSPCs reside primarily within the bone marrow, and this surrounding microenvironment, or niche, provides the external signals to optimize survival, promote quiescence, self-renewal, proliferation, or differentiation, and modulate other important processes. Thus, the physical and communicative interactions between HSPCs and their bone marrow niche are essential for maintaining HSPC functions (Mercier et al., 2011; Renström et al., 2010).

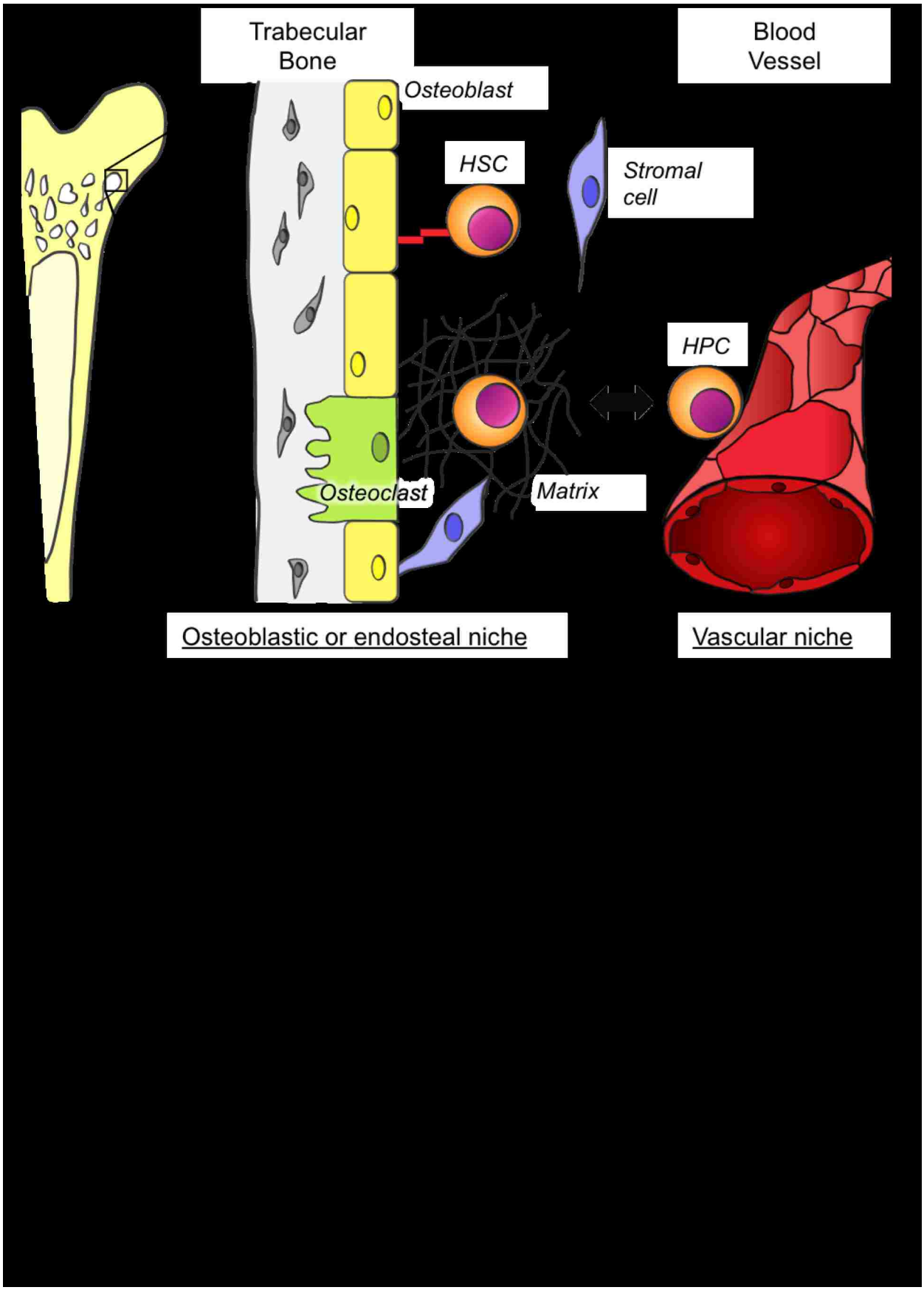
1.2.3 Bone Marrow Niche Interactions

The bone marrow is the spongy network of fatty and vascular connective tissue within the hollow cavities of bones. About half of adult bone marrow consists of fatty yellow marrow, or medulla ossium flava, which is found predominantly in the main medullary cavity within the lengthy middle segment, or diaphysis, of long bones. This marrow region houses blood vessels, adipocytes, and other stromal cells not directly involved in hematopoiesis, but it can be converted to red marrow during cases of severe blood loss. Red marrow, or medulla ossium rubra, on the other hand, is found in flat and short bones, the pelvis, vertebrae, sternum, ribs, scapulae, cranium, and the articulating,

epiphyseal ends of long bones. It is the source of hematopoiesis and contains red vascular connective tissue and blood vessels, which serve as a conduit for transporting newly produced red and white blood cells into the systemic circulation. The epiphyseal ends of bones contain a lattice network of thin, branching bone spicules, also known as cancellous bone or trabeculae, which increase the tissue surface area, contain the red marrow, and support the surrounding vasculature (Bianco and Riminucci, 1998; Clayman, 1989; Purton and Scadden, 2008). Lining the bone marrow cavity and the spaces between the trabeculae is the endosteum, a thin layer of cells consisting of bone- and matrix-producing osteoblasts and bone-degrading osteoclasts. Osteoblasts and other stromal cells found throughout the bone marrow environment, namely reticular cells and fibroblasts, produce extracellular matrix (ECM) components, including collagen, osteopontin, fibronectin, and laminin (Long et al., 1992; Scadden, 2006). Furthermore, the bone marrow contains adipocytes, macrophages, and sinusoidal endothelial cells that line the blood vessels and allow the passage of new blood cells into circulation (Purton and Scadden, 2008).

The cells, stromal tissues, and matrix components comprising the bone marrow form specific microenvironments or "niches" in which hematopoietic stem/progenitor cells thrive and function. Ray Schofield first postulated this niche hypothesis in 1978, in which "the stem cell is seen in association with other cells which determine its behavior" (Schofield, 1978). Since then, both conventional and intravital microscopy techniques have shown that early, primitive hematopoietic cells primarily localize within trabecular bone at or near the endosteal surface and closely interact with osteoblasts of varying differentiation stages as well as with matrix components, while more mature,

differentiated progenitors are seen more centrally located within the marrow cavity away from osteoblasts. The osteoblastic environment is often referred to as the "endosteal niche" (Calvi et al., 2003; Mercier et al., 2011; Taichman et al., 2000; ter Huurne et al., 2010; Zhang et al., 2003a). HSPCs have also been shown to associate with perivascular reticular cells (Sugiyama et al., 2006), mesenchymal progenitors (Méndez-Ferrer et al., 2010), and endothelial cells of the sinusoid blood vessels (Kiel et al., 2005). The latter form the "vascular niche." The relationship between these specialized niches is not very well understood, but they each play a part in regulating HSPC function and behavior (Kiel and Morrison, 2008; Mercier et al., 2011; ter Huurne et al., 2010). A schematic of the bone marrow niche can be seen in Figure 1.1.


Trabecular Bone
Blood Vessel
Osteoblast
HSC
Stromal cell
HPC
Osteoclast
Matrix
Osteoblastic or endosteal niche
Vascular niche

To determine the importance of these different niches on HSPC function, both *in vitro* and *in vivo* studies have been performed. In particular, co-culture experiments, genetic manipulation studies, and drug treatment applications have been used to assess or modify specific components of the niche (Mercier et al., 2011; ter Huurne et al., 2010). Because of the close proximity of HSPCs to osteoblasts, osteoblasts are thought to be essential regulators of HSPCs. In one study, co-culture of HSPCs with osteoblasts was shown to improve overall HSPC maintenance, thus enhancing *ex vivo* self-renewal (Taichman et al., 2000). In several *in vivo* studies, osteoblast-specific expression of a constitutively active parathyroid hormone (PTH) or PTH-related peptide receptor (PPR) was used to increase the number of osteoblasts in the bone marrow and effectively look at the effect on HSPCs (Calvi et al., 2003; Calvi et al., 2001). In addition, inhibition of bone morphogenic protein (BMP) signaling through a BMP receptor 1a (BMPR1a) mutation has been used to increase osteoblast numbers (Zhang et al., 2003a). In each case, increasing osteoblastic activity led to the expansion of HSPCs within the bone marrow and improved homing and retention of donor HSPCs upon transplantation (Mercier et al., 2011; ter Huurne et al., 2010; Wu et al., 2009). On the other hand, targeted ablation of osteoblasts through thymidine kinase-responsive ganciclovir treatment led to a loss of bone marrow hematopoiesis and overall cellularity as well as an eventual decline in hematopoietic stem cells. Ablation of osteoblasts was also found to cause a shift in hematopoiesis to extramedullary organs, such as the liver, spleen, and even peripheral blood (Mercier et al., 2011; ter Huurne et al., 2010; Visnjic et al., 2004; Wu et al., 2009). These findings strongly suggest the involvement of osteoblasts in the regulation of HSPC

function, pool size, and maintenance in the bone marrow niche (Mercier et al., 2011; ter Huurne et al., 2010).

Hematopoietic stem/progenitor cells are also known to associate with endothelial cells of the sinusoidal blood vessels within the bone marrow, and more recently, studies have begun to show strong evidence for the important role these cells have in regulating HSPC function. Co-culture of HSPCs with primary endothelial cells derived from hematopoietic, vascular sources as well as certain non-hematopoietic organs has been found to promote HSPC expansion and function *ex vivo* (Li et al., 2004). In addition, human HSPCs maintain the ability to repopulate human bone fragments implanted in severe combined immunodeficiency (SCID) mice when expanded in the presence of human brain endothelial cells. Non-human primate HSPCs expanded in this manner were also able to successfully home to and engraft in the bone marrow of baboons following myeloablative, total body irradiation (Brandt et al., 1999). Montfort and colleagues (2002) showed that transplantation of whole adult blood vessels could restore hematopoiesis in lethally irradiated mice. Further studies using isolated microvascular endothelial cells in transplant also demonstrated restoration of hematopoiesis and rescue of "true" HSPCs following bone marrow lethal irradiation (Li et al., 2010). Overall, the importance of endothelial cells in HSPC homing and engraftment and maintenance of hematopoiesis is beginning to be realized.

As mentioned briefly above, the bone marrow niche provides a complex network of external signals or cues to regulate HSPC function and behavior, including but not limited to the cellular processes of proliferation, differentiation, and self-renewal (Renström et al., 2010; ter Huurne et al., 2010). These signals can be received through

direct physical cell-cell contact, indirect paracrine signaling involving secreted cytokines or growth factors, autocrine or self-signaling through regulatory feedback loops, or by interactions with components of the ECM (Scadden, 2006; Sneddon and Werb, 2007). Niche cells have been shown to express certain ligands and adhesion molecules as well as secrete a number of cytokines, chemokines, and growth factors that have been implicated in promoting HSPC localization and interaction with the niche, maintaining HSPC behavior and function, and supporting proper hematopoiesis. Several studies have shown that osteoblasts are critical components of the niche. They are thought to be very important regulators of HSPC maintenance, particularly the properties of quiescence and self-renewal, by secreting interleukins 1, 6, and 7 (IL-1, IL-6, IL-7), leukemia inhibitory factor (LIF), and stem cell factor (SCF) (Mercier et al., 2011; ter Huurne et al., 2010). Interestingly, IL-6 is thought to generate a positive feedback loop such that the presence of HSPCs near osteoblasts allows for increased production of IL-6 by osteoblasts to further enhance HSPC maintenance (Taichman et al., 1997). Osteoblasts are also believed to regulate the localization and possible homing of HSPCs to the bone marrow by secretion of CXC-chemokine ligand 12 (CXCL12) (Mercier et al., 2011; Sugiyama et al., 2006). On the other hand, they may also be involved in the mobilization or release of HSPCs into the peripheral blood through secretion of granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and GM-CSF (Winkler and Lévesque, 2006). In addition, they express Notch (Calvi et al., 2003) and WNT ligands (Fleming et al., 2008) to inhibit HSPC differentiation, as well as angiopoietin (Ang-1) (Arai et al., 2004) and thrombopoietin (THPO) (Yoshihara et al., 2007) to positively regulate HSPC numbers and promote quiescence. Another important feature of

the niche is its hypoxic environment. The relatively low level of oxygen, or hypoxia, can lead to the activation of hypoxia-inducible factors (HIFs), which are transcription factors that can induce production of vascular endothelial growth factor (VEGF) by osteoblasts and other niche cells. VEGF secretion is not only important for generating new blood vessels, but it can also greatly contribute to HSPC survival, which may involve a VEGF autocrine feedback loop (Gerber et al., 1999; Maes et al., 2012; Rehn et al., 2011). Furthermore, osteoblast adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), annexin II, N-cadherin, CD44, and CD164 serve to localize and maintain HSCPs in the bone marrow through adhesive interactions and may also have roles in homing (Kiel and Morrison, 2008; Lewandowski et al., 2010; Lilly et al., 2011; Mercier et al., 2011; Porter and Calvi, 2008; Renström et al., 2010; ter Huurne et al., 2010).

While much of the literature to date has focused on the generalized endosteal niche, there has been increasing interest in identifying signaling components and regulatory mechanisms that are specific to candidate niche cells of each niche, including the osteoblastic, perivascular, and vascular niches. It is also of recent interest to determine the functionally important niche source(s) for each signaling factor in regulating HSPCs (Kiel and Morrison, 2008). Mesenchymal progenitor cells, reticular cells, and particularly endothelial cells utilize many of the same signaling factors as osteoblasts listed above for communicating and interacting with HSPCs, however, a few distinctions between these niches have been identified. For instance, osteopontin, a negative regulator of proliferation, is unique to osteoblasts (Stier et al., 2005). Osteoclast degradation of bone helps to release factors embedded in the bone matrix, including

transforming growth factor beta 1 (TGFβ1), bone morphogenic proteins 2 and 7 (BMP-2 and BMP-7), and divalent calcium (Ca^{2+}) ions. Such factors serve to regulate osteoblasts and HSPC maintenance and localization (Lilly et al., 2011). Reticular cells that are localized adjacent to sinusoidal blood vessels express high levels of CXCL12 and are often referred to as CXCL12-abundant reticular (CAR) cells; these cells may be especially important for HSPC maintenance, homing, and migration (Ara et al., 2003; Sugiyama et al., 2006). In addition, Ding et al. (2012) revealed through both *in vitro* and *in vivo* studies that vascular endothelial cells and perivascular CAR and other stromal cells provide functionally important sources of SCF for HSPC maintenance, while SCF from osteoblasts is not required. Although the bone marrow niche is one of the most extensively studied stem cell niches and many of the important signaling factors and cell interaction partners have been identified, there is still much to be learned.

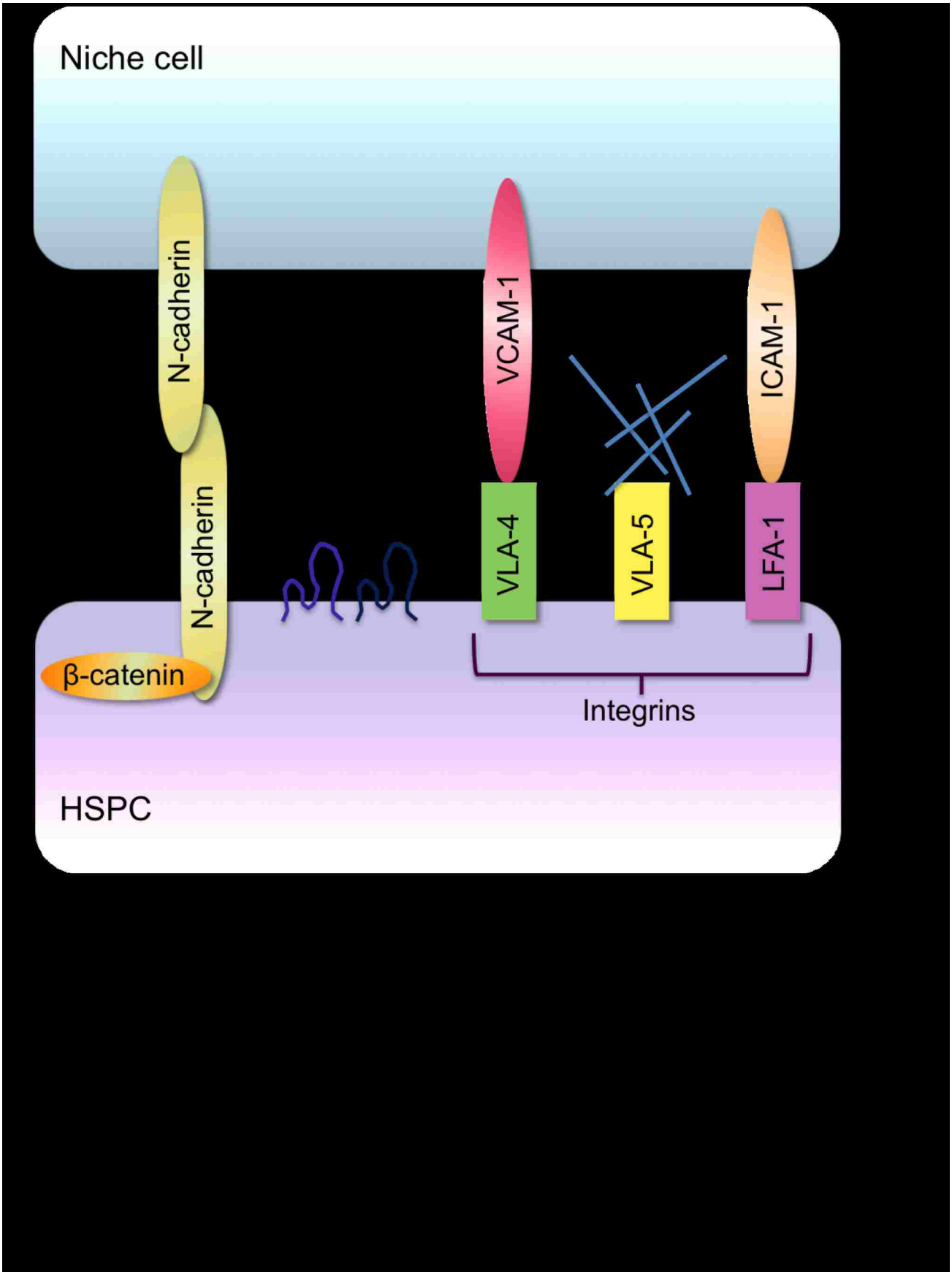
As described above, the bone marrow niche is a very complex microenvironment, providing an extensive list of secreted signaling factors suitable for contact-independent signaling. However, many interactions do rely on direct contact. The research presented here will focus predominantly on the adhesion-mediated interactions within the bone marrow niche that are important for regulating HSPC localization and behavior. Specifically, the direct cell-cell interactions between HSPCs and osteoblasts and the cell-matrix interactions involving HSPCs and ECM components will be discussed. The goal is to provide insight into the important molecules and mechanisms involved in regulating such interactions.

1.2.4 HSPC-Niche Contact Site

At the interface between hematopoietic stem/progenitor cells and niche cells are a variety of adhesion molecules that mediate direct contact and propagate intercellular signaling (Mercier et al., 2011). The expression of N-cadherin and β-catenin is thought to mediate homotypic adhesion interactions between osteoblasts and HSPCs (Kiel and Morrison, 2008; Zhang et al., 2003a). Osteoblasts also express the immunoglobulin superfamily cell adhesion molecules (IgSF CAMs), ICAM-1 and VCAM-1, which facilitate HSPC adhesion through integrins on the HSPC surface (Gillette and Lippincott-Schwartz, 2009; ter Huurne et al., 2010). Figure 1.2 illustrates HSPC-niche adhesive events involving these adhesion molecules. Contact with the surrounding bone marrow environment, namely extracellular matrix proteins produced by osteoblasts and other bone marrow stromal cells, is also important for HSPC communication, regulation, and adhesion. It has been shown that adhesive interactions between HSPCs and ECM ligands are very important for modulating the proliferation, differentiation, localization, and maintenance of HSPCs. The binding of HSPCs to the extracellular matrix is mediated primarily through integrins (Fig. 1.2) (Gu et al., 2003; Hurley et al., 1995; Lèvesque and Simmons, 1999; Long et al., 1992; Scadden, 2006). While intimate physical contact with osteoblasts and matrix components influences the behavior of HSPCs and is essential for their localization and maintenance, the molecular mechanisms orchestrating these interactions are not very well understood (Gillette et al., 2009).

To better understand the interactions between HSPCs and osteoblasts at the molecular level, previous work in our laboratory was done to characterize the organization of molecules at the HSPC-osteoblast contact site. Co-culture and live-cell

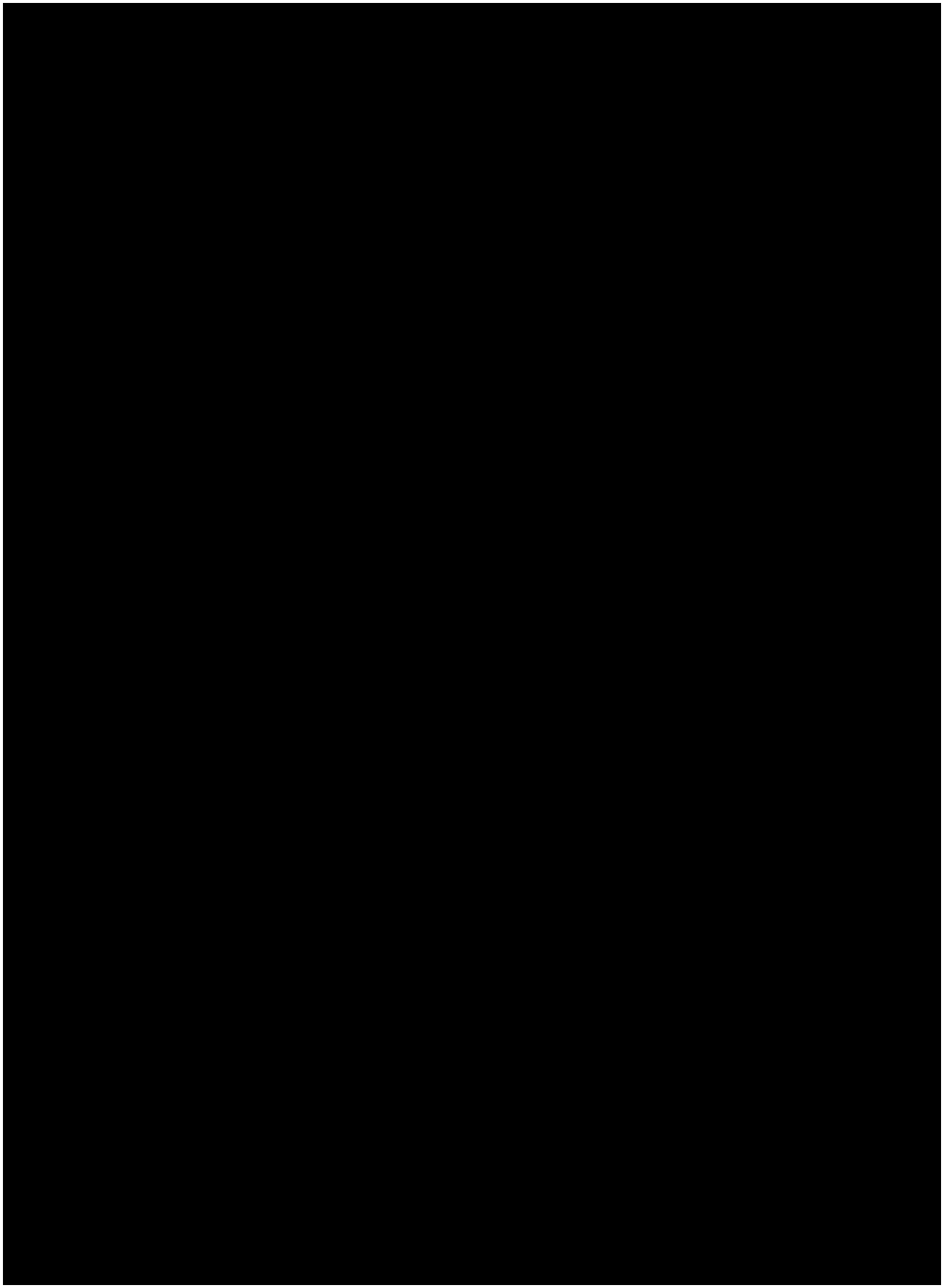
confocal fluorescence imaging techniques were utilized to assess the membrane distribution of specific molecules on the surface of HSPCs. The HSPCs were found to use a polarized domain on their plasma membrane to make contact with osteoblasts. Furthermore, this domain was found to be enriched in the stem and progenitor cell marker, prominin 1; the membrane lipid, phosphatidyl ethanolamine (PE); the integrin VLA-4; and the specific tetraspanin proteins, CD63 and CD81 (Fig. 1.2) (Gillette et al., 2009; Gillette and Lippincott-Schwartz, 2009; Larochelle et al., 2012). In addition to the tetraspanins listed, CD82 is also highly enriched within this polarized domain (Larochelle et al., 2012). Other stem/progenitor membrane surface markers, such as CD34 and CD45, were evenly distributed throughout the membrane, suggesting the polarization is specific and has an important role in mediating HSPC contact with niche cells (Gillette et al., 2009).


Niche cell
N-cadherin
N-cadherin
β-catenin
VCAM-1
ICAM-1
VLA-4
VLA-5
LFA-1
Integrins
HSPC

1.2.5 Integrins

As briefly alluded to, integrins are a type of cell surface receptor involved in mediating cell attachment, or adhesion, to other cells and extracellular matrix proteins. There are several different integrins in the integrin family, and they each have specificity for different ligands. For instance, an integrin can specifically bind one or a combination of different cell surface ligands, such as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), as well as ECM proteins, including collagens, laminins, vitronectin, and fibronectin. As transmembrane heterodimers, integrins consist of an α and a β chain, which are assembled in the endoplasmic reticulum (ER), transported to the Golgi apparatus to undergo post-translational glycosylation events, and then finally transported to the cell surface. Noncovalent association between these two chains allows a certain level of promiscuity between the different α and β subunits (Alberts, 2002; Shimaoka et al., 2002; Tiwari et al., 2011). As a result, 24 different mammalian integrin heterodimers can be formed between 18 known α subunits and 8 β subunits. Each of these possible α – β heterodimers are depicted in Figure 1.3 (Shimaoka et al., 2002). Alternative splicing of certain integrin RNAs leads to even further diversity in integrin receptors (Alberts, 2002). Each of the integrin heterodimers is found with a different prevalence in different cell types, depending on the cell's functions and needs. Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are found on many cell types and both bind collagen and laminin (Mizejewski, 1999). The $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins are also ubiquitously expressed, but bind fibronectin and laminin, respectively (Alberts, 2002). The distribution of the β_2 integrins, $\alpha_L\beta_2$ and $\alpha_M\beta_2$, is specific to lymphocytes and neutrophils as well as monocytes, respectively, and these bind the ICAM ligands, including ICAM-1 and

ICAM-2 (Kay et al., 2009; Mizejewski, 1999). Of particular interest is the $\alpha_4\beta_1$ integrin, also known as very late antigen-4 (VLA-4) or CD49d/CD29. It is largely expressed on hematopoietic cells, though it can also be found on epithelial and endothelial cells, and its ligands include fibronectin and VCAM-1 (Alberts, 2002; Humphries et al., 2006).



Each of the transmembrane α and β chains have a short cytoplasmic carboxyl tail that is only about 13-70 amino acids long, a single membrane-spanning segment of about 20 amino acids, and a very large extracellular domain consisting of approximately 700-1100 amino acids, the end of which takes on a globular head structure (Moser et al., 2009; Ross and Borg, 2001). When extended, the extracellular portion of each chain can project about 28nm from the cell membrane, and the last 5nm region of the N-terminal ends is involved in ligand binding. On the other end, the cytoplasmic tails are capable of linking the integrin to the actin cytoskeleton. The globular head of each integrin chain also contains divalent cation-binding sites, with the α chain having three or four divalent cation-binding sites and the β chain having one such site. Due to the proximal nature of the cation and ligand-binding sites, integrin-ligand binding affinity is dependent on the presence of divalent cations (Alberts, 2002; Tiwari et al., 2011).

Regulation of the affinity of integrins is crucial to various cellular processes. Divalent calcium (Ca^{2+}) ions, magnesium (Mg^{2+}) ions, and manganese (Mn^{2+}) ions each effect integrin affinity for ligand in a different manner and may themselves have a role in altering integrin conformation from the inactive to the active state and vice versa. As such, Ca^{2+} serves to inhibit ligand binding, Mn^{2+} activates ligand binding, and Mg^{2+} also supports ligand binding, albeit not as well. Under physiological conditions, Ca^{2+} and Mg^{2+} are found at relatively high concentrations in the mM range, while Mn^{2+} is present at much lower μM concentrations. A high concentration of free Ca^{2+} within the cell secretory pathway is thought to maintain integrins in the bent, inactive conformation until delivery to the cell surface. From here, the integrin can then be primed and activated,

revealing an open, extended conformation with high affinity for ligand (Tiwari et al., 2011).

Although the activation of integrins can be regulated, the intrinsic affinity of individual integrins for their ligands is not very strong. This is likely to prevent cells from binding irreversibly, such that their motility and migration is inhibited. Having the ability to initiate, stabilize, and subsequently disengage adhesive interactions is critical to a cell's behavior. As such, when effective binding is necessary, the regulation of integrin avidity, or clustering, can help to augment the overall strength or "functional affinity" of integrin-ligand binding (Ross and Borg, 2001). Avidity changes can occur in the presence of multivalent ligands and are important for the formation of early adhesion events known as focal complexes and their stabilization into focal adhesions. Moreover, the integration of integrins with the cytoskeleton may also help to stabilize integrins in clusters and lock them into a high activation state (Alberts, 2002; Ross and Borg, 2001). As discussed below, changes in integrin avidity serve important roles in propagating intracellular signals.

When integrins bind their ligands, they participate in outside-in signaling, sending information from the external environment through the plasma membrane and into the cell. This results in a number of downstream events, including changes in cell morphology, adhesion, spreading, migration, and invasion, as well as integrin clustering. Integrins not only link the ECM to the actin cytoskeleton, they can also generate and propagate intracellular signals. Due to the clustering of integrins and the formation of focal adhesions, signaling complexes assembled near the point of adhesive contact often perpetuate very localized signals and cellular changes. Because integrins do not contain

intrinsic enzymatic activity, they often interact with other proteins that do (Alberts, 2002). One protein known as focal adhesion kinase (FAK) is essential to integrin-dependent signaling. The proteins talin and paxillin, which can directly bind integrins, also help recruit FAK to focal adhesions. FAK tyrosine phosphorylation subsequently helps recruit other signaling molecules, including Src and Rho GTPases and has downstream effects on a number of intracellular signaling pathways (Alberts, 2002; Mitra et al., 2005). Integrins can also have effects on other signaling pathways by working in conjunction with other signaling receptors. Crosstalk between integrins and a variety of conventional signaling receptors, including growth factor receptors is common. Additional integrin-associated proteins include protein kinase C (PKC), calreticulin, α-actinin, and filamin, among a whole host of others with fundamental roles in intracellular signaling (Alberts, 2002; Petit and Thiery, 2000; Ross and Borg, 2001; Zhang et al., 2001).

Despite having a short cytoplasmic tail, another important function of integrins is inside-out signaling. As such, divalent metal ions are not the only regulators of integrin affinity. The direct binding of intracellular regulatory proteins to the integrin cytoplasmic tail can also affect integrin activation and adhesion. The proteins talin and kindlin bind to integrin β chains and are thought to work synergistically to displace the α cytoplasmic tail from the β tail and separate the interlinked α and β transmembrane domains that lock the integrin in an inactive state. This leads to a conformational change and subsequent activation of the integrin. In addition, talin may be required for the clustering of integrins into focal adhesions. While talin and kindlin are the only proteins known to directly regulate affinity, many other proteins can also play a part in integrin inside-out signaling

(Moser et al., 2009). Other signaling receptors may receive external cues that when propagated internally can be intercepted by integrin cytoplasmic tails. For instance, PKC is a downstream target of many signaling pathways and can bind the β tail of integrins. Interestingly, PKC is also a known interaction partner of talin, which in turn can affect integrin affinity (Zhang et al., 2001).

With regards to signaling and adhesion in the hematopoietic system, integrins are thought to be crucial for the functions and behaviors of mature immune cells and primitive hematopoietic stem/progenitor cells. The ability to regulate adhesion and migration is particularly important in white blood cells, where integrins are involved in the processes of leukocyte rolling and extravasation, inflammation, and platelet aggregation (Alberts, 2002). Integrin-propagated signals also merge with many other signaling pathways to mediate downstream events important for regulating HSPC proliferation, differentiation, quiescence, and self-renewal. Furthermore, integrins facilitate the direct physical interactions of HSPCs with the bone marrow niche, including those involving HSPC adhesion, localization, homing, and engraftment (Ellis and Tanentzapf, 2010; Lo Celso and Scadden, 2011; Wilson and Trumpp, 2006). In particular, the β1 integrin has become known for its role in crosstalk between HSPCs and the bone marrow microenvironment (Lo Celso and Scadden, 2011). Further evidence shows that HSPCs derived from β1-deficient mice fail to home to the bone marrow following transfer (Wilson and Trumpp, 2006). The β1-containing integrins α1β1 and α5β1 (also known as very late antigen-5, or VLA-5) are known to mediate HSPC adhesion to collagen or fibronectin, respectively, and both to osteopontin; however, the

integrin α4β1 (VLA-4) has been of major interest in HSPCs and will be discussed further in the next section.

1.2.6 α4β1 (VLA-4)

One of the key integrin molecules that keeps appearing in the context of HSPC adhesion, homing, and engraftment in the bone marrow niche is very late antigen-4 (VLA-4), also known as α4β1 (Ellis and Tanentzapf, 2010; Lo Celso and Scadden, 2011). As implied, the VLA-4 integrin consists of α4 and β1 chains and its cellular distribution is largely in hematopoietic cells, including HSPCs (Kolesnikova et al., 2004). This particular integrin not only binds fibronectin, but is also the main binding partner of VCAM-1 found on stromal cells, including endothelial cells and osteoblasts, which are both important niche regulators of HSPCs. The expression of VCAM-1 on bone marrow endothelial cells has been shown to correlate with the homing capacity of HSPCs (Lo Celso and Scadden, 2011). This supports the notion that binding to VCAM-1 is important for promoting the extravasation or transition of the HSPCs through the endothelial conduit of the bone marrow sinusoid and into the bone marrow niche (Ellis and Tanentzapf, 2010; Shirvaikar et al., 2012). In addition to VCAM-1, the extracellular matrix may be critical for HSPC localization and maintenance. VLA-4 binds to the alternatively spliced connecting segment-1 (CS-1) domain of fibronectin by recognizing the leucine-aspartic acid-valine (LDV) amino acid sequence in the fibronectin protein (Hynes, 2009; Kolesnikova et al., 2004). While fibronectin helps mediate the interactions of HSPCs with stromal cells, it is interesting to note that binding to VCAM-1 occurs with greater than four times higher affinity than binding to fibronectin (Masumoto and Hemler, 1993; Mould et al., 1994).

Because VLA-4 selectively binds both fibronectin and VCAM-1, it is likely that HSPCs primarily utilize this integrin in the adhesive interactions within the bone marrow niche. In fact, expression of VLA-4 is markedly higher on resident bone marrow HSPCs than on circulating HSPCs, suggesting that VLA-4 has an important role in maintaining HSPCs within the bone marrow niche (Prosper et al., 1998; Yamaguchi et al., 1998). In further support of this, it is well known that use of VLA-4 antagonists or anti-VLA-4 antibodies to block VLA-4 binding can lead to the mobilization and collection of HSPCs in the peripheral blood (Shirvaikar et al., 2012). Separate inhibition of the β1 chain, α4 chain, or VCAM-1 with inhibitory antibodies was also shown to suppress *in vivo* hematopoiesis (Williams et al., 1991), HSPC homing (Papayannopoulou et al., 1995), and *in vitro* generation of long-term bone marrow cultures (Miyake et al., 1991), respectively. In addition, various cytokines and growth factors, including IL-3, SCF, and G-CSF can alter the expression of VLA-4 in HSPCs (Bellucci et al., 1999). These dynamic expression patterns indicate the capacity of VLA-4 to functionally regulate hematopoietic homeostasis and adhesive interactions with the niche (Imai et al., 2010). Additional evidence to support the argument that VLA-4 is of critical importance includes studies involving the generation of chimeric α4 knockout mice. These mice showed significant alterations in adult hematopoiesis (Scott et al., 2003). The α4 integrin can partner with both β1 and β7 chains, and when both β chains were deleted or inactivated simultaneously, a disruption in the numbers and distribution of HSPCs was observed (Bungartz et al., 2006). In addition to α4, the β1 chain can also associate with the α6 chain. The ligand of α6β1 is laminin, which can be found in the basement membrane of the endothelial sinusoids. Although α6β1 was thought to be important for

homing, α6 knockout mice did not have any apparent effects in the homing or engraftment of HSPCs in adult bone marrow (Qian et al., 2007). Taken together, the above evidence suggests that VLA-4 is the major player in mediating homing to and retention within the bone marrow niche (Imai et al., 2010).

One family of proteins discovered to be very important in mediating the functions of integrins, including VLA-4, is the tetraspanin family. VLA-4 has been shown to associate with a number of tetraspanin proteins, including CD9, CD81, and CD82. The important regulatory and membrane organizational functions of tetraspanins will be highlighted in the next two sections.

1.2.7 Tetraspanins

Like VLA-4, a variety of tetraspanins were also found to be enriched at the site of contact between HSPCs and osteoblasts (Gillette et al., 2009). Tetraspanins are a family of multi-spanning membrane proteins with many regulatory roles, including modulating the behavior and organization of other membrane proteins and molecules, most notably, integrins and signaling receptors. As the name implies, tetraspanins span the membrane four times and consist of four hydrophobic transmembrane domains, a small extracellular loop (EC1) of about 13-31 amino acids, a large extracellular loop (EC2) of 69-132 amino acids that extends only 4-5 nm from the cell surface, and two short cytoplasmic domains, or tails, at the amino (N)- and carboxyl (C)-termini (Hemler, 2005) Although tetraspanins can range from about 200-350 amino acids in length, they contain several evolutionarily highly conserved amino acid residues, including a cysteine-cysteine-glycine (CCG) motif and two additional cysteines that form intramolecular disulphide bonds in the large extracellular loop. EC2 also contains a constant region comprised of three α-helices, A,

B, and E, and a hypervariable region known to be involved in tetraspanin protein-protein interactions (Hemler, 2005; Kitadokoro et al., 2001; Seigneuret et al., 2001). In addition, the majority of tetraspanins contain intracellular juxtamembrane cysteine residues that can undergo the post-translational modification, palmitoylation, which involves the covalent attachment of the fatty acid, palmitic acid. Furthermore, there are often several other amino acids conserved between tetraspanins, such as polar residues within the first, third, or fourth transmembrane domains (Hemler, 2005; Yáñez-Mó et al., 2009). Many tetraspanins contain asparagine or arginine residues within the large extracellular loop with the potential to undergo another type of post-translational modification known as N-glycoslylation involving addition of a carbohydrate glycan to nitrogen (Levy and Shoham, 2005; Yunta and Lazo, 2003). However, glycosylation patterns can vary widely among tetraspanins (Maecker et al., 1997).

Tetraspanins are present in most eukaryotes but interestingly, not in yeast. With respect to mice and humans, there are at least 33 known tetraspanins. A single type of tetraspanin can be present at 30,000 to 100,000 copies per cell, and there are often several different tetraspanins expressed in nearly all cell types (Hemler, 2003). Of course, certain tetraspanins are more ubiquitously expressed, while others are more cell type-specific. For instance, CD9, CD63, and CD81 can be found on nearly all cells, and CD151 is found on almost all endothelial and fibroblastic cells. However, CD53 is limited to the lymphoid/myeloid lineage, and CD37 is found almost exclusively in T- and B-lymphocytes (Hemler, 2005; Maecker et al., 1997). While the majority of tetraspanins can be found on both the cell surface and in endosomes, certain tetraspanins such as CD63 localize predominantly to late endosomal–lysosomal compartments due to the

presence of the C-terminal tyrosine-based targeting motif, GYEVM (Charrin et al., 2009; Levy and Shoham, 2005). The extensive distribution of some tetraspanins and the specificity of others indicate the many diverse regulatory and functional roles these proteins play in cellular physiology.

Of the tetraspanins expressed on a given cell type, all have been shown through immunoprecipitation experiments to characteristically associate with other members of the tetraspanin family, forming both homophilic and heterophilic dimers, multi-mers, and larger tetraspanin complexes. These interactions occur in a lateral fashion between the tetraspanin transmembrane domains and can give rise to dynamic tetraspanin-enriched microdomains (TEMs), or tetraspanin webs, which may have distinct functions in serving as protein organization or signaling platforms (Hemler, 2005; Levy and Shoham, 2005; Tarrant et al., 2003; Yáñez-Mó et al., 2009). Palmitoylation is said to promote the organization and stabilization of tetraspanins in TEMs and may also have important functions in membrane association, influencing other tetraspanin protein-protein interactions, and trafficking (Bijlmakers and Marsh, 2003; Dunphy and Linder, 1998; Resh, 1999). Glycosylation is also thought to have important implications in correct protein folding, trafficking through the ER, and encouraging tetraspanin-based interactions (Scholz et al., 2009).

The many functions of tetraspanins are not precisely known, but they do play an integral part in membrane biology, serving as scaffolds to organize membrane proteins and regulate signaling. Tetraspanins have been shown to physically associate with a wide variety of signaling molecules and receptors, including receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR), protein phosphatases, protein kinases,

G protein-coupled receptors (GPCRs), and many others. These tetraspanin-protein interactions and the formation of tetraspanin-rich signaling domains at the membrane level affect downstream processes such as survival, proliferation, differentiation, and apoptosis initiated at the DNA level. It is also well established that tetraspanins can associate with integrins and serve important roles in adhesion and migration (Hemler, 2005; Yáñez-Mó et al., 2009). The interactions tetraspanins have with other proteins can be direct or indirect. Immunoprecipitation studies are often used to identify these protein-protein interactions, but it can be challenging to tease apart direct versus indirect interactions. This is especially true for tetraspanins as they can associate with so many different proteins, and the associations observed often depend on the harshness of the detergent used for lysis in such experiments. Despite this, Yauch et al. (1998) successfully showed using harsh detergent conditions that tetraspanin CD151 directly interacts with the α3β1 integrin, and this interaction is established early in biosynthesis, likely in the ER. This direct interaction was also verified by chemical cross-linking experiments. Other primary interaction complexes include CD9-CD9P-1 and CD81-CD19-CD21 involved in B cell signaling, CD9-EWI-2 and CD81-EWI-2 important for cell spreading and migration, and CD151-α6β1, and CD81-α4β1. Secondary interactions refer to tetraspanin-tetraspanin associations, which likely occur in the Golgi following palmitoylation. These types of interactions are not disrupted by relatively mild detergents. Still, even milder lysis conditions result in tetraspanins co-immunoprecipitating with additional molecules, which are most likely due to tertiary interactions (Charrin et al., 2003; Hemler, 2005; Levy and Shoham, 2005; Yáñez-Mó et al., 2009; Yunta and Lazo, 2003). Such interactions can involve different tetraspanins

associating with the same molecules, as would be expected in the tetraspanin webs. Several studies have shown that a number of different tetraspanins (at least CD9, CD53, CD63, CD81, and CD82) associate with β1 integrins in most cell types, including α3β1, α4β1, and α6β1 (Berditchevski, 2001; Yunta and Lazo, 2003). Furthermore, several tetraspanins are known to associate with lymphocyte molecules, including CD4, CD8, and major histocompatibility complex class II (MHC II) as well as with intracellular signaling factors such as phosphatidylinositol 4-kinase (PI4K) and PKC (Boucheix and Rubinstein, 2001; Charrin et al., 2009; Hemler, 2005) Tetraspanins can also directly bind cholesterol and other membrane lipids, such as gangliosides GM2 and GM3 and phospholipids, and this may be important for TEM function and for influencing other tetraspanin-protein associations (Charrin et al., 2003; Hemler, 2005). The existence of the above tetraspanin-protein interactions has recently been confirmed in many cases by Förster resonance energy transfer (FRET) and other techniques (Boucheix and Rubinstein, 2001; Charrin et al., 2009; Charrin et al., 2003; Hemler, 2003; Hemler, 2005; Levy and Shoham, 2005; Serru et al., 1999; Yáñez-Mó et al., 2009; Yunta and Lazo, 2003).

New insights into the effects tetraspanins have within tetraspanin microdomains and specifically the effects they have on the molecules they associate with are beginning to be realized. Many of these functions involve effects on adhesion, membrane fusion, intercellular communication, and intracellular signal transduction. Tetraspanins might mediate these cellular events through a number of mechanisms, including sorting, trafficking, clustering, and stabilizing protein interactions. Tetraspanins appear to have tremendous importance in the immune system, and as such, some of these mechanistic

attributes have been extensively studied in the immunology field. Tetraspanin-deficient mice have signaling and cell communication deficits, many of which involve alterations in immune system function (Yáñez-Mó et al., 2009). In addition, tetraspanins have been identified as prominent figures in the immune synapse for T cell activation and immune cell extravasation with functions in both leukocytes and endothelial cells. It has been shown that CD81 is capable of regulating the avidity of the integrin α4β1 (VLA-4) in leukocytes (Feigelson et al., 2003) and assembling various adhesion molecules into tetraspanin-enriched adhesion platforms in endothelial cells (Barreiro et al., 2008). Furthermore, siRNA knockdown studies of CD63 in human umbilical vein endothelial cells (HUVECs) have demonstrated that CD63 is important for regulating the expression and clustering of P-selectin on the cell surface, thus leading to leukocyte rolling effects (Doyle et al., 2011). In fact, the ability to regulate integrins and other adhesion receptors through changes in avidity, stabilization, or other means may be a general feature of many tetraspanins. Studies of CD9, for example, have shown that anti-CD9 antibodies can promote the adhesion of pre-B cells to fibroblasts in the bone marrow by regulating the binding of the VLA-4 and VLA-5 integrins to fibronectin (Maecker et al., 1997). Still other roles for tetraspanins include their involvement in tumor cell migration and invasion, which also rely on integrin regulation to some extent, as well as their participation in cell fusion, morphology, differentiation, development, and infection by pathogens (Hemler, 2003; Maecker et al., 1997; Sridhar and Miranti, 2006; Yáñez-Mó et al., 2009). Interestingly, tetraspanins are also enriched in exosomes, which are small, 50-100nm membrane-enclosed vesicles secreted from immune and other cells with potential roles in intercellular signaling and transfer of protein and RNA (Escrevente et al., 2011).

Moreover, tetraspanins appear to regulate some aspects of intracellular trafficking. For instance, CD81 has been shown to promote the egress of CD19 from the ER to the Golgi in B cells (Shoham et al., 2006), and CD63 may facilitate vesicular trafficking of proteins between endosomes and lysosomes (Charrin et al., 2009). In short, from the evidence presented, it is clear that tetraspanins have a very wide range of functions and could even be referred to generally as "molecular facilitators," helping to organize and scaffold proteins into membrane domains and signaling platforms (Maecker et al., 1997).

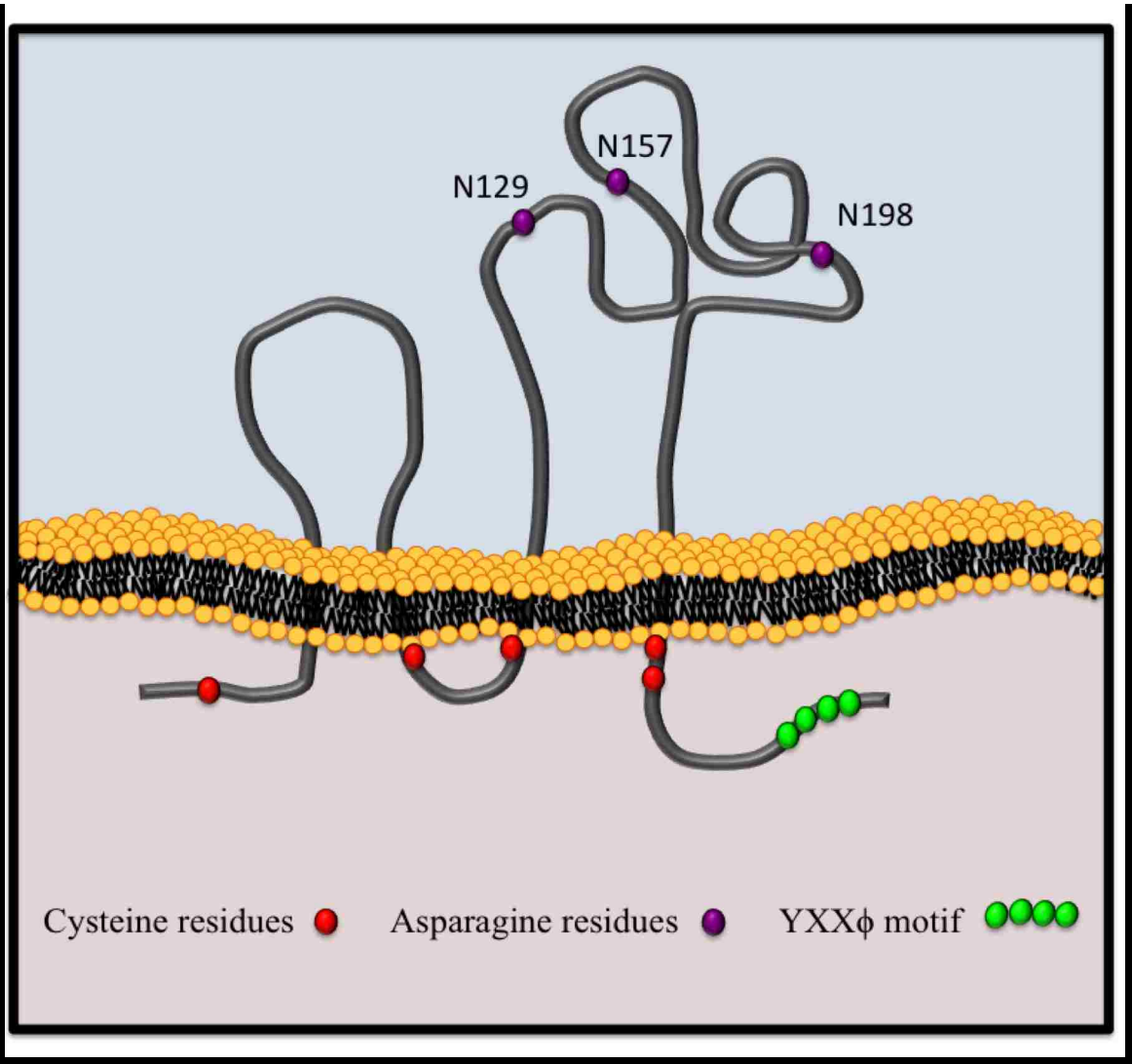
One tetraspanin that has been shown to be important in hematopoietic stem/progenitor cells is the tetraspanin CD82. CD82 is very highly expressed in bone marrow HSPCs and was one of the tetraspanins found to be enriched in the polarized domain involved in contact between HSPCs and osteoblasts. In addition, CD82 is downregulated following HSPC differentiation into mature blood cells, suggesting a possible role in hematopoiesis (Burchert et al., 1999; Larochelle et al., 2012). The discovery of CD82 is fairly recent, and there is still much to be learned about this protein, particularly in the hematopoietic cell-niche system. Therefore, the CD82 tetraspanin has been of great interest in our research.

1.2.8 CD82

1.2.8.1 General Properties of CD82

In humans, the gene encoding CD82 is found in the 11p11.2 region of chromosome 11. The CD82 protein translated from this gene is 267 amino acids in length, and like other tetraspanins, it spans the membrane four times, and contains a short intracellular loop (IC) adjoining the small and large extracellular loops that are characteristic of this family of proteins. The large EC2 loop has three potential asparagine

N-glycosylation sites at positions 129, 157, and 198 (Dong et al., 1995; Miranti, 2009). The molecular weight of CD82 can range from about 30-90 kDa, depending on its glycosylation pattern, which can vary between cell types (Zhang et al., 2010). In addition, CD82 consists of five cysteine residues at positions 5, 74, 83, 251, and 253, which can undergo palmitoylation (Zhou et al., 2004). Glycosylation, palmitoylation, and the presence of the three polar residues asparagine (N), glutamine (Q), and glutamic acid (E) found in the first, third, and fourth transmembrane domains are said to be essential for the function of CD82 (Zhang et al., 2010). A representative schematic of CD82 can be seen in Figure 1.4.


N157
N129
N198
Cysteine residues
Asparagine residues
YXXϕ motif

The distribution of CD82 expression is fairly extensive, as CD82 is found on nearly all endothelial cells, epithelial cells, fibroblasts, and immune cells (Adachi et al., 1996). It is also very highly expressed on hematopoietic stem/progenitor cells and is downregulated during the process of differentiation. Thus, its expression on granulocytes, monocytes, and other mature blood cells is comparatively lower than on HSPCs (Burchert et al., 1999; Larochelle et al., 2012). Interestingly, it does not appear to be expressed on erythrocytes (Elghetany, 2002). Like most other tetraspanins, cellular localization of CD82 is predominantly at the plasma membrane or within endosomes due to the C-terminal endosomal-sorting motif, tyrosine-X-X-phi (YXXφ), where X represents any amino acid, and φ represents a bulky hydrophobic residue (Xu et al., 2009). With regards to tissue distribution, CD82 is highly expressed in the thymus, spleen, liver, kidney, pancreas, small intestine, colon, prostate, ovary, and placenta. In contrast, the heart, brain, muscle, and testis express CD82 at significantly lower levels (Miranti, 2009).

Based on myriad studies in immune cells, CD82 has become known as a critical regulator of membrane organization, signaling, adhesion, and trafficking. It exerts its functions through associations with other tetraspanins, integrins, signaling receptors, and many other molecules and serves to organize the plasma membrane into functional microdomains. Within the immune system, CD82 has been shown to modulate T cell activation, as well as induce morphological changes by joining in complex with the T cell receptor (TCR) and acting as a costimulatory molecule. The main function of CD82 in T cells may be in regulating signaling and actin polymerization through its association with integrins (Miranti, 2009). As is characteristic of many tetraspanins, CD82 can associate

with a number of integrins in both immune and non-immune cells. These primarily include α3, α4, α5, α6, β1, and β3 (Berditchevski, 2001; Sridhar and Miranti, 2006; Yunta and Lazo, 2003). In T cells, CD82 coimmunoprecipiates with both VLA-4 and lymphocyte function-associated antigen 1 (LFA-1, also known as αLβ2) and mediates LFA-1 binding to ICAM-1 (Miranti, 2009). Furthermore, many other studies have assessed the effects of CD82 on integrin-mediated cell adhesion. In separate cases, it was found that CD82 could affect integrin binding to fibronectin and laminin (He et al., 2005; Liu et al., 2003; Sridhar and Miranti, 2006). Observed changes in adhesion-mediated signaling may also be linked to the interactions of CD82 with a variety of signaling molecules, including among others, Rho-GTPase, PKC, and the PKC substrates, talin and myristolated alanine-rich C-kinase substrate (MARCKS). Although these interactions are likely indirect, they may still be critical to proper cell behavior. Similar to its involvement in T cells, CD82 also associates with costimulatory molecules and integrins in B cells, though its general functions in B cells are less defined. An interesting role for CD82 in antigen presenting cells (APCs) is seen in its ability to interact with MHC II-peptide complexes and assist in their transport from intracellular vesicles to the cell surface (Miranti, 2009). In addition to its functions in regulating normal cellular processes under normal conditions, CD82 has also been implicated in cancer, and its significance in this context is described below.

1.2.8.2 CD82, Tumor Metastasis Suppressor

CD82, also known as Kangai-1 (KAI1), was originally identified in 1995 as a tumor metastasis suppressor in prostate cancer, where its downregulation was shown to have important implications in promoting metastatic spread (Dong et al., 1995; Hemler,

2005). Since that time, it was also found to be downregulated in several other cancers, including lung, breast, ovarian, cervical, bladder, and colon cancers, and its loss, in protein and/or mRNA expression, was shown to directly correlate with poor prognosis (Christgen et al., 2008; Jee et al., 2006; Li et al., 1999; Liu et al., 2000; Miranti, 2009; Ruseva et al., 2009; Wu et al., 2003). Interestingly, neither the reduction in CD82 nor the altered physiology observed in tumor cells involves mutation or allelic loss of CD82 (Miranti, 2009; Zhang et al., 2003b). Tetraspanins lack intrinsic activity; therefore, their involvement in cancer, like other cellular processes, is believed to be through the association, regulation, and membrane organization of other proteins (Ruseva et al., 2009; Sridhar and Miranti, 2006).

In the highly invasive PC3 prostate cancer cell line, which lacks CD82, as well as in other metastatic cancer cell lines with little or no CD82 expression, the re-expression of exogenous CD82 led to a decrease in *in vitro* cell migration and invasion. However, the mechanisms for how CD82 inhibits cell motility and invasiveness are not completely understood (Hemler, 2005; Miranti, 2009; Sridhar and Miranti, 2006). Several studies have shown that tetraspanins, including CD82, affect cell adhesion and migration by interacting with a number of signaling molecules involved in these processes (Ruseva et al., 2009). For instance, CD82 has been known to associate with EWI-2 and EWI-F, molecules known to suppress migration. In other studies, CD82 diminished EGFR signaling by limiting receptor dimerization and promoting receptor internalization upon ligand binding, thus leading to downstream inhibition of migration and invasion. This suppression of EGFR is also thought to depend on the interaction of CD82 and protein kinase C-α (PKCα) and the subsequent degradation of EGFR following internalization.

(Hemler, 2005; Malik et al., 2009; Miranti, 2009; Odintsova et al., 2000; Odintsova et al., 2003). Although CD82 appears to exert its effects on many proteins and molecules, perhaps the most compelling research into CD82's involvement in adhesion and migration in cancer involves its interactions with integrins. Across various cancers and cell lines, CD82 seems to interact with several different integrins and integrin-linked partner molecules and can modulate integrin adhesion and signaling. For example, CD82 is thought to link PKC to integrins, resulting in integrin signaling changes and adhesion and migration effects (Malik et al., 2009; Zhang et al., 2001). In addition, CD82 might help to suppress signaling by cMet and Src kinases, thereby inhibiting integrin-mediated migration and invasion (Sridhar and Miranti, 2006). On the other hand, one study by Lee et al. (2011) demonstrated that CD82 overexpression in human prostate cancer cell lines led to increased migration and decreased adhesion to fibronectin. Based on the evidence presented, the correlation to metastasis would then be that CD82 ultimately affects integrin involvement in migration and invasion (Hemler, 2005; Miranti, 2009). While the CD82-integrin relationship is not well established, many studies suggest that perhaps the loss of CD82 in cancer leads to reduced integrin-mediated adhesion, allowing cancer cells to disengage from the primary tumor and migrate to new locations. It has also been postulated that the presence of CD82 enhances integrin-mediated adhesion, leading to cell immobilization and a reduction in metastasis, thus making claim for its role as a metastasis suppressor (Malik et al., 2009).

1.2.8.3 CD82 in Hematological Malignancies and HSPCs

In addition to epithelial-based cancers, CD82 may also play an important part in hematological malignancies, which are cancers involving the blood, bone marrow, and

lymphatic system. These cancers can be myelogenous or lymphocytic in nature and include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), lymphoma, myeloma, and others. As the name implies, myeloid leukemias primarily affect the development of myeloid cells such as monocytes and granulocytes, whereas the lymphocytic types affect the development of lymphocytes. Leukemic cells are thought to be organized in a cellular hierarchy that ultimately mirrors the developmental hierarchy stemming from normal hematopoietic stem cells. Only a small subset of these cancer cells, termed leukemia-initiating cells (LICs), has the capacity to propagate and sustain disease. Further, these cells have the proliferation, differentiation, and self-renewal properties characteristic of leukemic stem cells (Bonnet and Dick, 1997; Greaves, 2010; Larochelle et al., 2012). While CD82 is downregulated in other cancer types, it was found to be overexpressed in immature CD34+ hematopoietic blast (or progenitor-like) cells taken from patients with acute myeloid leukemia (AML) and in leukemic cells taken from patients with chronic myeloid leukemia (CML) or chronic lymphocytic leukemia (CLL) (Burchert et al., 1999; Larochelle et al., 2012). Whether the overexpression of CD82 illustrates the return of hematopoietic cells to a more primitive, undifferentiated stage or is the result of aberrant expression, the importance of elevated CD82 in these leukemias is not clearly known. It is known, however, that adhesion molecules mediate interactions between HSPCs and their bone marrow niche, and their altered expression in leukemias has also been shown to affect patient outcome. Once again, it would appear that the association of CD82 with adhesion molecules could be a potential avenue for disease progression. The VLA-4 integrin, which can associate with CD82, is readily

expressed on the HSPC surface, and its loss has been associated with severe hematological defects (Arroyo et al., 1996; Hirsch et al., 1996). Furthermore, VLA-4 has been implicated in CD34+ progenitor cell adhesion, migration, and invasion. Overall, CD82 upregulation in leukemia may be a key factor in regulating improper cell maturation, adhesion, and homing, leading to aberrant hematopoiesis and deficiencies in immune function (Burchert et al., 1999).

1.2.8.4 CD82 in HSPC Adhesion and Homing

From the evidence presented, it is clear that CD82 is a tetraspanin protein with a very broad set of functions. However, it appears to be very important in immune cells and hematopoietic stem/progenitor cells, playing a crucial part in the homeostasis of hematopoiesis through membrane organization and regulation of adhesion. Not only is CD82 very highly expressed on HSPCs, but it is also downregulated during the process of differentiation (Burchert et al., 1999; Larochelle et al., 2012), correlating with the release of mature blood cells into circulation. This, in conjunction with the fact that CD82 can associate with and regulate adhesion molecules, especially integrins, suggests that CD82 may be required for adhesive and communicative interactions of HSPCs with the bone marrow niche. As mentioned earlier, characterization of the HSPC-niche interaction site revealed that HSPCs utilize a polarized membrane domain enriched in prominin 1, cholesterol and other lipids, VLA-4, and the tetraspanins, CD63 and CD81 as the point of contact with osteoblasts *in vitro* (Gillette et al., 2009; Gillette and Lippincott-Schwartz, 2009; Larochelle et al., 2012). In addition, the tetraspanin CD82 is also highly enriched within this polarized contact site (Larochelle et al., 2012). The observation of this CD82-

enriched polarized domain led to a critical investigation into the function of CD82 in HSPC-niche interactions (Larochelle et al., 2012).

To look more closely at the functional importance of CD82 in these polarized domains, CD82-function blocking antibodies were used to inhibit CD82 function on normal CD34+ cells. Treatment with CD82-blocking antibodies led to a 2.5 fold decrease in adhesion to osteoblasts, and a nearly 2-fold decrease in *in vivo* homing ability compared to cells treated with isotype control antibodies. It was noted that these observations were not the result of polarity effects or expression changes but rather were likely the result of antibody blocking an epitope on CD82 important for its ability to interact with proteins involved in adhesion and homing. Therefore, a polarized domain enriched in functional CD82 is essential for the proper homing and adhesive interactions of HSPCs within the bone marrow niche microenvironment (Larochelle et al., 2012).

The results of the aforementioned studies, the supporting literature regarding the importance of CD82 in membrane organization, integrin-mediated adhesion, and regulation of other cellular process, in addition to the fact that CD82 is highly expressed in hematopoietic stem/progenitor cells, serve to illustrate a potential role for CD82 in regulating HSPC interactions with the bone marrow niche. Thus my hypothesis is centered on the involvement of CD82 in HSPCs, which I put forward in the next section, along with the specific aims that will address this hypothesis.

1.3 Hypothesis

1.3.1 Hypothesis and Aims

The focus of this thesis lies on the tetraspanin membrane protein, CD82, and the investigations into its roles in regulating interactions between hematopoietic

stem/progenitor cells and the bone marrow niche microenvironment in the context of adhesion, protein interaction, and membrane organization. My hypothesis is CD82 expression regulates HSPC adhesion to components of the bone marrow niche.

Aim 1 will determine the effect of CD82 expression on HSPC adhesion to components of the bone marrow niche. In testing this hypothesis using CD82-overexpression and -knockout systems in progenitor-like KG1a cells, it became clear that CD82 had an effect on integrin-mediated adhesion. This effect also seemed to be specific to the VLA-4 integrin. This led me to my next aim, which encompasses CD82 regulatory effects on VLA-4.

Aim 2 will determine the mechanisms for how CD82 regulates VLA-4 integrin-mediated adhesion. For this aim, we set out to determine the effects of CD82 on VLA-4 with regards to 1) expression, 2) affinity, and 3) avidity. These assessments relied on a number of biochemical approaches as well as the novel super resolution imaging technique known as dSTORM.

In addition to looking at the relationship between CD82 and VLA-4, it was also of interest to assess the importance of CD82 in homphilic and heterophilic interactions with other tetraspanins as a result of its palmitoylation state. Thus, Aim 3 will determine the effect of CD82 oligomerization on HSPC adhesion. For this aim, a palmitoylation mutant form of CD82, in which cysteines were mutated to serines, was generated and expressed in the KG1a cell line.

A detailed description of the materials and methods enlisted to test my hypothesis and address each of my specific aims is provided in Chapter 2. Chapter 3 then presents the data and results of these studies in manuscript format. Finally, this thesis culminates

in Chapter 4 with a thorough discussion section, which highlights the important conclusions, focuses on the significance and impact of this research, and introduces intriguing ideas for future directions.

CHAPTER 2: MATERIALS AND METHODS

The following is a detailed and comprehensive description of the materials and methods utilized in the experiments presented in Chapter 3.

2.1 Materials and Methods

2.1.1 Cell Culture

KG1a human hematopoietic myeloid progenitor cells (ATCC CCL-246.1, Manassas, VA) and SaOS-2 human osteosarcoma (ATCC HTB-85, Manassas, VA) cells were cultured in RPMI 1640, 1X Medium (Mediatech, Manassas, VA.), supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 100 Units/mL penicillin and 100 μg/mL streptomycin (PenStrep; Invitrogen, Carlsbad, CA). Cells were incubated at 37°C, 5% humidity, and 5% CO_2. Suspended KG1a cells were maintained at a concentration between 2.0×10^5 and 1.0×10^6 cells/mL, as recommended by ATCC. Adherent SaOS-2 cells were maintained at 70% confluency, and treated with trypsin (Invitrogen, Carlsbad, CA) when detaching or passaging.

2.1.2 CD82 Overexpression and Knockdown Vector Constructs

To create the N-terminus mCherry-tagged CD82 plasmid, CD82 was subcloned from the YFP-CD82 construct (Addgene) into the mCherry-C1 Vector (Invitrogen) using the XhoI and SacII restriction sites. The YFP-Palm$^-$-CD82 (CD82 palmitoylation mutant) construct was a generous gift from D. Derse (NIH). To crease the mCherry version of the construct, the PALM$^-$-CD82 insert was PCR amplified with the following primers (Forward: 5'-CTCGAGCGATGGGCTCAGCC-3' and Reverse: 5'-CCGCGGAAGCTTTCAGTACTTGGG-3'). The PCR product was cleaned and digested with the XhoI and SacII restriction enzymes and then inserted into the mCherry-C1

vector. The CD82 shRNA plasmid (Santa Cruz Biotechnology, Santa Cruz, CA) consisted of a pool of three to five plasmids encoding 19-25 nucleotides (plus hairpin). CD82-targeted siRNAs consisting of pools of three 20-25 nucleotide siRNA sequences and the scrambled control siRNA were also purchased from Santa Cruz Biotechnology.

2.1.3 Nucleofection

KG1a cells were transfected with the appropriate vector constructs using the Lonza CLB-Transfection Kit and Amaxa Nucleofector device (Lonza, Walkersville, MD) according to the manufacturer's instructions (program V-001, 2 μg plasmid DNA or 1 μg siRNA duplex). One million cells were usually transfected. Successful transfection was selected for with 500 μg/mL Geneticin® (G418; Invitrogen, Carlsbad, CA), and stable cell lines expressing mCherry, mCherry-CD82, and mCherry-Palm$^-$-CD82 constructs were generated. Stably expressing cells were isolated via fluorescence-activated cell sorting (FACS; UNM Core Facilities), and cells were continually maintained under G418 selection throughout the course of experiments.

2.1.4 Proliferation Assays

KG1a mCherry, mCherry-CD82, and mCherry-Palm$^-$-CD82 cells were plated at 20,000 cells/well in a Greiner CELLSTAR® 96-well plate (Sigma-Aldrich, St. Louis, MO). Cell proliferation, as determined by relative cell number or viability, was assessed over 5 days using CellTiter 96© AQueous One Solution (Promega, Madison, WI) according to the manufacturer's instructions. Following a 1.5 hr incubation time at 37°C, absorbance was measured at 490 nm using the iMark^{TM} microplate absorbance reader (Model 168-1135; Bio-Rad, Hercules, CA).

2.1.5 Adhesion Assays

2.1.5.1 Adhesion

Microplate wells of a 96-well plate were coated with either fibronectin (10 μg/mL in phosphate-buffered saline (PBS); Millipore, Billerica, MA), collagen I (10 μg/mL in PBS; Sigma-Aldrich, St. Louis, MO), laminin (10 μg/mL in PBS; BD Biosciences, Franklin Lakes, NJ), SaOS-2 cells (plated at 50,000 cells/well to create an osteoblastic monolayer), or 10% FBS as a control. Cells were labeled for 20 min with 2 μM calcein AM fluorescent dye (Invitrogen, Carlsbad, CA) in Hank's buffered salt solution (HBSS). After washing twice with HBSS, the cells were plated at 100,000 cells/well and incubated at 37°C for 2 hrs to promote adhesion. Non-adherent cells were removed and extent of adhesion was measured in fluorescence units using a fluorescence plate reader (BioTek Instruments, Winooski, VT) with excitation wavelength of 488 nm. Fluorescence data were then normalized to the mean fluorescence obtained for control cells.

2.1.5.2 VLA-4-Specific Adhesion

KG1a mCherry and mCherry-CD82 cells were treated with either dimethyl sulfoxide (DMSO) or blocked with the monovalent peptide LDV (1 μM), which was a generous gift from Drs. Larry Sklar and Tione Buranda (UNM). Again, the cells were labeled with calcein AM, plated at 100,000 cells/well on a fibronectin-coated 96-well plate, incubated for 2 hrs, and washed. Extent of adhesion was measured as previously described.

2.1.6 Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA), containing a protease inhibitor cocktail (1:100) and protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). For immunoblotting, 25 μg protein samples were prepared with 5X protein loading buffer under nonreducing

conditions and boiled at 90°C for 5 min. Samples were then run through SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PVDF), blocked with 5% non-fat milk in 1X PBS containing 0.2% Tween-20 (PBST) for 1 hr and incubated overnight with the appropriate primary antibodies diluted in PBST. Rabbit anti-human CD82 polyclonal antibody (pAb; 1:1000) was from Abcam (Cambridge, MA), rabbit anti-RFP pAb (1:1000) was a generous gift from M. Hedge (NIH), rabbit anti-human α4 monoclonal antibody (mAb; 1:1000) was from Novus Biologicals (Littleton, CO), rabbit anti-β1 pAb (1:500) was from Cell Signaling (Danvers, MA). Protein loading control antibodies included rabbit anti-calnexin mAb (1:1000) from Cell Signaling (Danvers, MA), and mouse anti-β-actin mAb (1:6500) from Sigma-Aldrich (St. Louis, MO). Blots were washed 3 times in PBST for 20 min and incubated in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hr. Goat anti-mouse-HRP and anti-rabbit-HRP secondary antibodies (1:1000) were from Jackson ImmunoResearch Laboratories (West Grove, PA). HRP conjugate enzymes were stimulated with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Blots were imaged using the ChemiDoc™ XRS Imager (Bio-Rad, Hercules, CA).

2.1.7 Flow Cytometry

2.1.7.1 Surface Expression

One million stably expressing cells or 2.0×10^5 transiently transfected cells were used per expression assay, and all washing and labeling was done on ice. Transfected KG1a cells were washed once in cold staining buffer containing 2% bovine serum albumin (BSA) and 0.1% sodium azide in PBS (PAB). Cells were then centrifuged at 800g, 8°C for 5 min, resuspended in 100 μL staining buffer and incubated for 30 min in the dark with the

appropriate directly conjugated primary antibody or isotype control at 5 μL per million cells. Mouse anti-human CD82-Alexa 647 mAb and mouse anti-human β1-Alexa 647 mAb were from BioLegend (San Diego, CA), and mouse anti-human α4-Alexa 488 mAb was from R&D Systems (Minneapolis, MN). Following antibody labeling, cells were centrifuged at 300g, 8°C for 5 min and washed twice in 1mL cold PAB. Dead cells were then labeled with 1 μL propidium iodide (PI) on ice for 5 min in the dark, followed by one wash. Cells were kept on ice in the dark until data collection, which was performed on the BD Accuri C6 Flow Cytometer using the appropriate wavelength filter setting.

2.1.7.2 Affinity Binding Assays

Two million KG1a mCherry and mCherry-CD82 cells at 2.5×10^5 cells/mL in media were treated with either 0.1% DMSO or blocked with LDV (1 μM) and incubated for 30 min at 37°C. LDV-FITC at increasing concentrations (0nM, 0.25nM, 0.75nM, 2.5nM, 7.5nM, 25nM, 75nM, and 250nM) was then added in duplicate to eppendorf tubes containing 400 μL blocked or non-blocked cells, and the cells were incubated for an additional 30 min at 37°C with gentle shaking. Following centrifugation and resuspension in 200 μL media, blocked and non-blocked cells were assessed by flow cytometry using the FL1 filter setting to assess levels of specific ligand-integrin binding, as measured by mean fluorescence minus baseline (blocked). LDV-FITC concentration was plotted against mean channel fluorescence. The dissociation constant, K_d, was determined from the nonlinear fit using the built-in one site – specific binding (hyperbola) model in Prism.

2.1.7.3 Affinity Dissociation "Off-Rate" Assays

Two million KG1a mCherry and mCherry-CD82 cells were treated with either DMSO or blocked with LDV in a volume of 800 μL media. A saturating LDV-FITC concentration

of 75 nM was then added to 200 µL blocked or non-blocked cells in triplicate. Samples were continuously stirred with a 5×2 mm magnetic stir bar, and real-time flow cytometry was used to assess the dissociation kinetics or "off-rate" of LDV-FITC upon addition of a saturating, competitive concentration of unlabeled LDV (1 µM), added 1 min after starting the measurements. The mean fluorescence readings were collected over a 6-min time period and were baseline-corrected and normalized to 1. The dissociation rate constant, k_{off}, was determined from the nonlinear fit using the dissociation – one phase exponential decay model in Prism.

2.1.8 Fluorescence Microscopy

KG1a mCherry, mCherry-CD82, and mCherry-Palm$^-$-CD82 cells were plated in 8-well chamber slides (Nunc, Rochester, NY), and imaged by laser scanning confocal microscopy using a Zeiss Axiovert 100M inverted microscope (LSM 510) equipped with a 63X 1.2 N.A. oil immersion objective. The helium/neon (HeNe) laser was used to excite mCherry fluorphores at a wavelength of 543 nm, and fluorescence emission was collected by an electron multiplying CCD camera using the appropriate emission filter set. Image analysis was performed using the Zeiss LSM 510 software or Image J (NIH, Bethesda, MD).

2.1.9 Super Resolution Microscopy (dSTORM)

2.1.9.1 Fixation and Immunostaining

KG1a mCherry and mCherry-CD82 cells were plated on fibronectin-coated 8-well chamber slides or 25mm coverslips overnight. Non-adherent cells were removed, and attached cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature followed by a 1X PBS wash. Cells were then blocked for 1 hour in 1X PBS

with 10mg/mL BSA (block). Cells were stained for either α4 or β1 integrin at room temperature by incubating with the appropriate primary or directly conjugated antibody for 1 hour. Primary mouse anti-human VLA-4 (α4) mAb (1:500) was from Millipore (Billerica, MA), and directly conjugated mouse anti-human β1-Alexa 647 mAb was from BioLegend (San Diego, CA). Cells labeled with β1-Alexa 647 were gently washed twice with block, while cells labeled with primary α4 were washed twice, incubated with secondary anti-mouse-Alexa 647 Ab (1:500; Dianova, Hamburg, Germany), and then washed twice. After labeling, cells were fixed again with 4% PFA for 10 min at room temperature followed by a wash with block. Cells were maintained in 1X PBS until imaging.

2.1.9.2 Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

Prior to imaging, the 1X PBS buffer was removed, and cells were placed under reducing conditions with a dSTORM cocktail consisting of 20% glucose, 40 μg/mL catalase, 500 μg/mL oxidase, and β-mercaptoethanol (BME; 1:1000) in 1X PBS. All chemicals were from Sigma-Aldrich (St. Louis, MO). Cells were then imaged with an Olympus IX71 inverted microscope (Olympus America, Center Valley, PA) equipped with a 150X 1.45 N.A. TIRF oil immersion objective. A 633nm diode laser was used to excite Alexa 647 fluorphores, and fluorescence emission was collected by an Andor iXon 897 electron multiplying CCD camera (Andor Technology PLC, Belfast, Northern Ireland) using the appropriate emission filter set.

2.1.9.3 Image Processing

All image processing was performed using MatLab (The MathWorks, Inc., Natick, MA) in conjunction with the image-processing library, DIPImage (Delft University of

Technology). For descriptions of specific analysis routines see Huang et al. (2011) and Veatch et al. (2012).

CHAPTER 3: CD82 REGULATION OF HEMATOPOIETIC STEM/PROGENITOR CELL – NICHE ADHESION

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3.1 Abstract

The spatial organization and dynamics of proteins and lipids within the cell membrane is important for the regulation of cell signaling, adhesion, and cell communication. Within the bone marrow niche, communication between hematopoietic stem/progenitor cells (HSPCs) and niche cells is essential for regulating their proliferation, differentiation, and survival. Our previous work has ascertained that HSPCs utilize a polarized domain on the plasma membrane that serves as the contact site with osteoblasts, which are important members of the bone marrow niche. Using primary CD34+ cells and the progenitor-like KG1a cell line, we found this domain to be enriched in the specific tetraspanin proteins, CD63, CD81, and CD82. Tetraspanins are multi-spanning membrane proteins that act as scaffolds for the organization of membrane domains important for regulating adhesion and signaling. CD82 is of particular interest, as it is highly expressed on HSPCs and downregulated during HSPC differentiation. Our characterization of CD82 function using CD82-blocking antibodies revealed a significant decrease in adhesion of HSPCs to niche cells as well as in the *in vivo* homing and engraftment capabilities of these cells. To determine the molecular mechanisms of CD82's role in adhesion, we have generated CD82 overexpression and knockdown cell lines using the KG1a background. Our data indicate that the level of CD82 expression positively correlates with the extent of adhesion to fibronectin and osteoblasts but has no effect on binding to collagen I or laminin. The observed increase in adhesion we observed with CD82 overexpression was inhibited by the VLA-4-specific peptide, LDV, indicating a potential role for the VLA-4 ($\alpha4\beta1$) integrin. Investigations into potential CD82-mediated mechanisms of VLA-4 regulation have revealed that that CD82 regulates

both the expression and avidity of VLA-4 but does not regulate its affinity. Taken together, the VLA-4 expression and avidity changes could account for the observed adhesion changes with differing CD82 expression levels. Finally, assessment of CD82 palmitoylation using KG1a CD82 palmitoylation mutant cells revealed that palmitoylation may be required for the CD82-induced changes in adhesion.

3.2 Introduction

Cells receive signals or cues from their surrounding environment and respond in ways to optimize survival, maintain quiescence, promote proliferation and differentiation, and regulate many other essential processes. As such, cells have established diverse mechanisms to control the exchange of signaling molecules required for cell-cell communication. Long- or short-range paracrine signaling events involve the secretion of cytokines, growth factors, and hormones for the purpose of contact-independent communication; however, many cell interactions depend on direct physical contact. Stem cells, in particular, rely on intimate physical interactions with their surrounding microenvironment or "niche" for the regulation and maintenance of proper stem cell function (Schofield, 1978). In the case of hematopoietic stem/progenitor cells (HSPCs), which reside in the bone marrow niche, direct contact with surrounding niche cells is essential for regulating HSPC proliferation, multipotentiation, and self-renewal (Renström et al., 2010; ter Huurne et al., 2010; Zhang and Li, 2008).

The bone marrow niche is a complex microenvironment consisting of a number of different cellular and extracellular matrix (ECM) components. Endothelial cells, adipocytes, reticular cells, and osteoblasts are all known to be important regulators of HSPCs (Fuchs et al., 2004). In addition, the stromal cells, namely osteoblasts and

fibroblasts, are responsible for generating bone marrow ECM proteins, including collagen, osteopontin, fibronectin, and laminin (Long et al., 1992; Scadden, 2006). HSPCs can engage in direct contact with niche cells and ECM through adhesive events involving N-cadherin/β-catenin interactions (Kiel and Morrison, 2008; Zhang et al., 2003a) and integrin-mediated adhesion (Gillette and Lippincott-Schwartz, 2009; Gu et al., 2003; ter Huurne et al., 2010). These interactions are also thought to be important for the homing of HSPCs to the niche and their long-term engraftment (Calvi et al., 2003; Kiel and Morrison, 2008; Zhang et al., 2003a).

While intimate physical contact with osteoblastic, endothelial, and matrix constituents of the bone marrow niche influences the behavior of HSPCs and is essential for their localization and maintenance, the molecular mechanisms orchestrating these interactions are not very well understood (Gillette et al., 2009). Previous work from our laboratory was done to characterize the membrane interface at the HSPC-niche contact site. HSPCs were found to use a polarized membrane domain enriched in prominin 1, the α4β1 integrin, also referred to as very late antigen-4 (VLA-4), and the specific tetraspanin proteins, CD63, CD81 (Gillette et al., 2009; Larochelle et al., 2012), and CD82 (Larochelle et al., 2012) to make contact with osteoblasts.

Tetraspanins are a large family of multi-spanning membrane proteins with many regulatory roles in signaling and adhesion. They promote the organization of various other membrane proteins and molecules, most notably signaling receptors and integrins, into tetraspanin-enriched microdomains (TEMs). These domains can then serve as signaling platforms to recruit adaptor molecules, thereby modulating downstream signaling and altering cell function. Tetraspanins have been shown to effect cell adhesion

and migration, membrane fusion, intercellular communication, and intracellular signaling (Charrin et al., 2009; Hemler, 2005; Larochelle et al., 2012; Yáñez-Mó et al., 2009). CD82 is one such tetraspanin with the capacity to regulate these and other cellular events through a number of mechanisms, including sorting, trafficking, clustering, and stabilizing protein interactions (Hemler, 2005; Yáñez-Mó et al., 2009).

CD82, also known as KAI1, has implications in cancer, as its expression levels correlate with suppression of tumor cell migration and invasion in the context of metastasis (Ruseva et al., 2009). Interestingly, CD82 appears to exert most of its effects on adhesion and migration through its association with integrins (Hemler, 2005; Malik et al., 2009; Miranti, 2009; Ruseva et al., 2009). CD82 is also found in many immune cells (Tarrant et al., 2003), and its high expression in HSPCs and subsequent downregulation during differentiation suggests an important role in hematopoiesis. In addition, previous work from our laboratory has shown that CD82 function-blocking antibodies can significantly decrease HSPC adhesion to osteoblasts as well as the *in vivo* homing and engraftment capabilities of these cells in mice (Larochelle et al., 2012). These observations support a potential role for CD82 in regulating HSPC adhesive interactions with the bone marrow niche.

In this study, we set out to determine the molecular mechanisms underlying CD82's involvement in adhesion to components of the bone marrow niche. Through the use of CD82 overexpression and knockdown in progenitor-like KG1a cells, we found that CD82 expression positively correlates with adhesion *in vitro*, and its effects appear to be mediated through the integrin, VLA-4. To better understand how CD82 could be

impacting the function of VLA-4, we will assess potential mechanisms by which CD82 could be regulating VLA-4 function and adhesion.

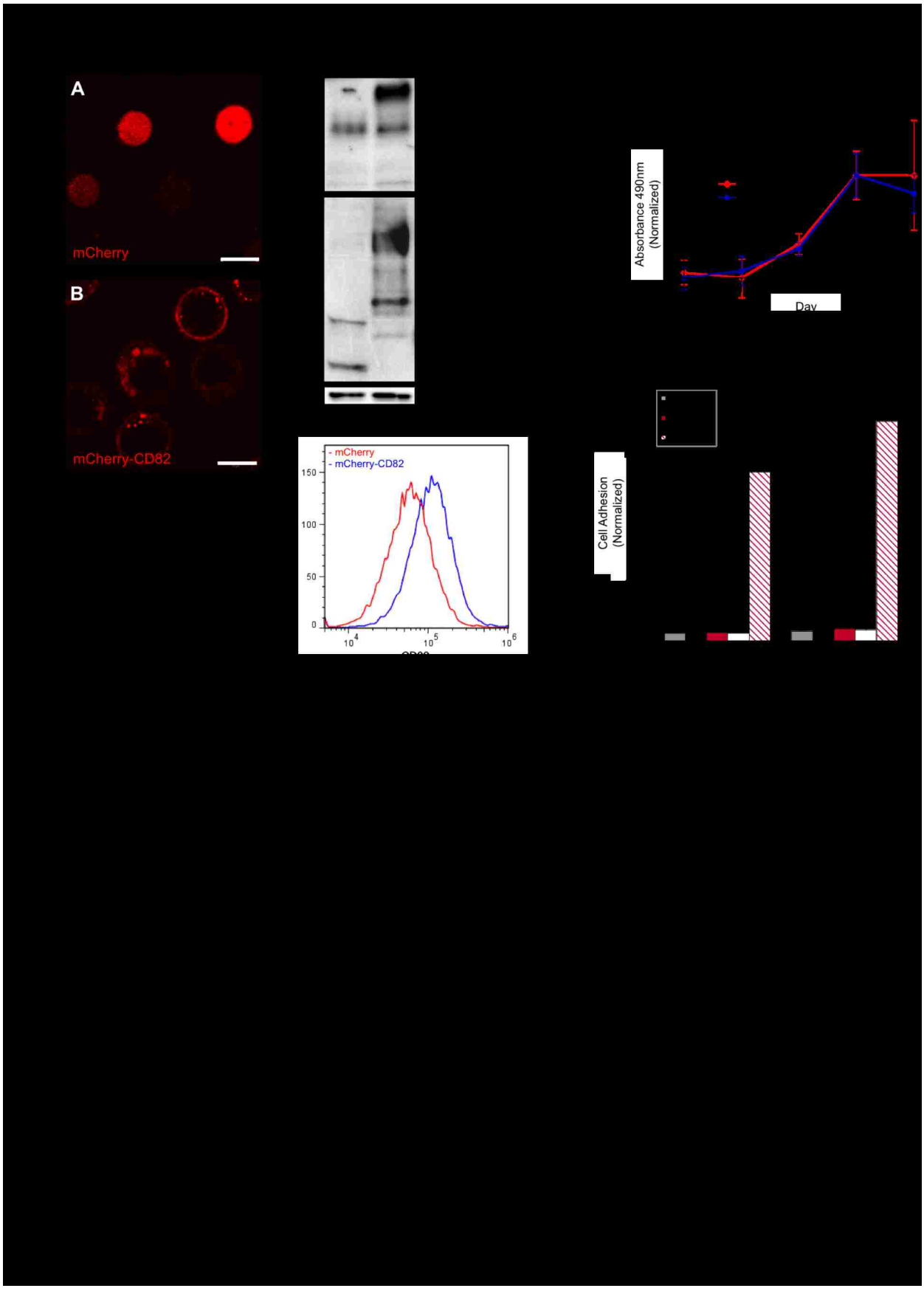
3.3 Results

3.3.1 CD82 overexpression increases KG1a cell adhesion

Since we previously showed that CD82 function-blocking antibodies can significantly decrease adhesion of HSPCs to osteoblasts as well as decrease the *in vivo* homing and engraftment capabilities of these cells in mice (Larochelle et al., 2012), we wanted to analyze the molecular mechanisms of CD82's involvement in HSPC interactions with the bone marrow niche. To do this, we first overexpressed CD82 in the human acute myelogenous leukemia progenitor-like cell line, KG1a. CD82 was genomically tagged with the mCherry fluorescent protein, and stable KG1a transfectants overexpressing mCherry-CD82 were generated through selective G418 treatment. As a control, KG1a cells were transfected with the mCherry vector alone. Using fluorescence microscopy, we detected both cell surface and endosomal mCherry-CD82 (Fig. 3.1, B), which is consistent with the endogenous localization of CD82 (Xu et al., 2009). In contrast, the mCherry control was detected throughout the cytoplasm (Fig. 3.1, A). Western blot analysis identified both the endogenous and the exogenous forms of CD82 (50 kDa and 75 kDa, respectively) and indicated a greater than 2-fold increase in CD82 expression in the mCherry-CD82 cells (Fig. 3.1, C). In addition, we quantified the surface expression of CD82 by flow cytometry (Fig. 3.1, D). Mean fluorescence readings revealed at least a 2-fold increase in CD82 surface expression on the CD82-overexpressing cells.

To further characterize the mCherry-CD82 cells, we evaluated whether CD82 expression had an effect on cell proliferation. KG1a mCherry and mCherry-CD82 cells were plated at a starting concentration of 20,000 cells/well and maintained in culture for a total of 5 days. Relative cell number was measured every 24 hours using the colorimetric CellTiter 96® AQueous One Solution proliferation assay (Fig. 3.1, E). These data indicate no difference in proliferation between control and CD82-overexpressing cells.

We next set out to examine more closely the involvement of CD82 in adhesion to components of the bone marrow niche *in vitro*. In order to quantifiably evaluate changes in cell adhesion, we performed a fluorescence adhesion assay. Briefly, calcein-labeled KG1a mCherry or mCherry-CD82 cells were plated on FBS, fibronectin, collagen I, laminin, or an osteoblastic monolayer and allowed to adhere. After washing off the non-adherent cells, the extent of adhesion was measured in mean fluorescence units and normalized to mCherry control cells. Both mCherry and mCherry-CD82 cells displayed a significant increase in adhesion. However, the CD82 overexpressing cells demonstrated an even greater increase in cell adhesion, which suggests that CD82 can modulate cell adhesion.


A
mCherry
B
mCherry-CD82
- mCherry
- mCherry-CD82
150
100
50
0
10^4
10^5
10^6
Absorbance 490nm (Normalized)
Day
Cell Adhesion (Normalized)

3.3.2 CD82 expression regulates cell adhesion to VLA-4-specific ligands

To further evaluate potential changes in cell adhesion mediated by CD82 expression, we also generated transient and stable CD82 knockdown cells in the KG1a cell line by using both siRNAs and shRNAs, respectively. As a control, KG1a cells were transfected with a scrambled, non-targeting siRNA. Knockdown of CD82 was verified by western blot analysis (Fig. 3.2, A) and flow cytometry (Fig. 3.2, B). The siRNA knockdown cells had about a 5-fold decrease in CD82 surface expression at 48 hours post-transfection, and the stable shRNA knockdown cells showed a subsequent 5-fold reduction in CD82 surface levels.

Next, using these control siRNA, CD82 siRNA, and CD82 shRNA cells, we assessed how the loss of CD82 expression affected cell adhesion, specifically to fibronectin and osteoblasts. In these experiments, we detected a significant decrease in adhesion to fibronectin as well as a decrease in adhesion to osteoblasts between the control and transient CD82 siRNA knockdown cells (Fig. 3.2, C). Stable knockdown with shRNAs led to an even greater decrease in adhesion to fibronectin and osteoblasts with respect to control (Fig. 3.2, C). In combination, these data suggest that the extent of cell adhesion correlates with the expression levels of CD82.

The specific increase in adhesion to fibronectin and osteoblasts observed in the CD82-overexpressing cells and the decrease in adhesion to these substrates in the knockdown cells suggests that CD82 potentially interacts with or has some effect on the α4β1 integrin, also referred to as VLA-4. Both fibronectin and VCAM-1 on osteoblasts are ligands specific to VLA-4. To look more closely at the molecular involvement of CD82 with VLA-4, we utilized the monovalent peptide, LDV, derived from the leucine-

aspartic acid-valine sequence found in fibronectin (Chigaev et al., 2001). By specifically binding the VLA-4 integrin, LDV can block its interaction with fibronectin. As such, we evaluated whether this peptide could interfere with the CD82-induced cell adhesion. Calcein-labeled KG1a mCherry and mCherry-CD82 cells were either treated with DMSO or blocked with 1μM of LDV and then plated on fibronectin to assess the effects of LDV block on adhesion. Treatment with LDV resulted in reduced adhesion to fibronectin in both the control and CD82-overexpressing cells; however, the decreased adhesion was more pronounced in the overexpressing cells (Fig. 3.2, D). These data implicate, at least in part, the involvement of the VLA-4 integrin in the increased cell adhesion observed with CD82 overexpression.

Control siRNA
CD82 siRNA
CD82 shRNA

Cell Adhesion
(Normalized)

- Control siRNA
- CD82 siRNA
- CD82 shRNA
250
200
150
100
50
0
10^2
10^3
10^4
10^5
10^6
CD82

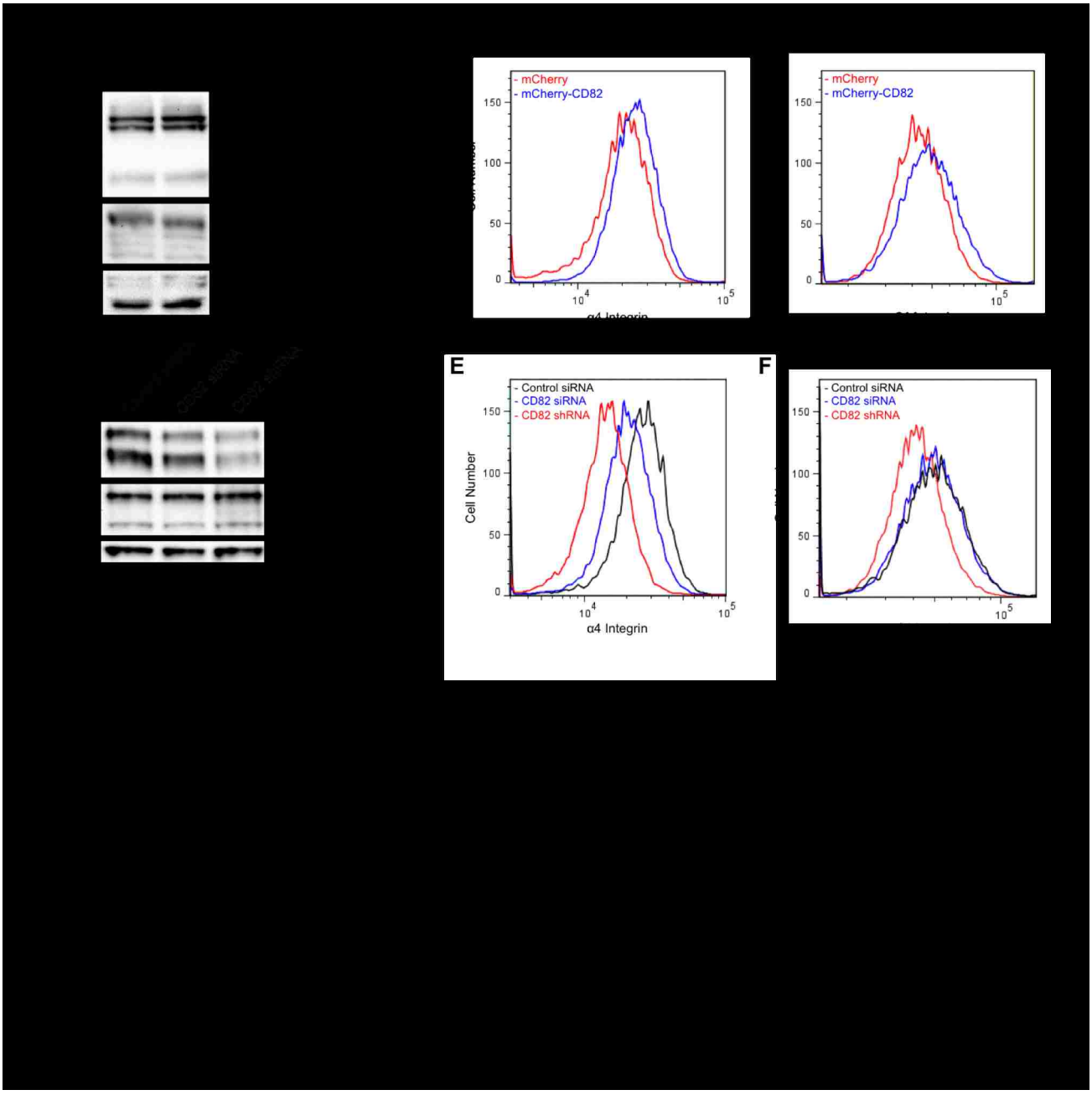
Cell Adhesion to Fibronectin
(Normalized)

3.3.3 CD82 regulates VLA-4 (α4β1) integrin expression

To determine how CD82 could be regulating adhesion through the VLA-4 integrin, we first wanted to assess whether CD82 could alter VLA-4 expression. Western blot analysis was performed to look at total expression levels of the α4 and β1 integrin chains in the control and overexpressing cells (Fig. 3.3, A). Bands representing the mature and precursor α4 chains at 150 kDa and 140 kDa, respectively, indicate no change in total α4 levels with CD82 overexpression. In addition, bands identifying the mature and precursor β1 chains at 130 kDa and 115 kDa show no change in total β1 expression. Flow cytometry was also used to measure surface expression levels of both α4 and β1 (Fig. 3.3, B and C). Although there is no change in total expression levels, histograms show a slight right shift, or increase, in surface levels of α4 and β1 in the CD82-overexpressing cells compared to control. Therefore, these data suggest that CD82 overexpression leads to a modest increase in the surface expression of both α4 and β1.

Total α4 and β1 expression levels were also determined by western blot analysis in the control, transient CD82 knockdown, and stable CD82 knockdown cells (Fig. 3.3, D). Results indicate a decrease in total α4 expression between the control and CD82 siRNA cells and an even greater decrease in the shRNA cells. However, a decrease in total β1 expression was not observed. Flow cytometry was used to assess surface expression of both α4 and β1 (Fig. 3.3, E and F). Transient CD82 siRNA cells show a decrease in α4 surface expression from control, and stable CD82 shRNA cells show an even more pronounced decrease. Interestingly again, there is no change in β1 expression on the surface between the control siRNA and CD82 siRNA cells; however, there is a decrease in β1 surface expression in the shRNA cells. Like the adhesion data presented

above, expression of α4 and β1 appears to correlate with the differing levels of CD82 expression observed between the control, CD82 siRNA, and CD82 shRNA cells (Fig. 3.1, E and F).

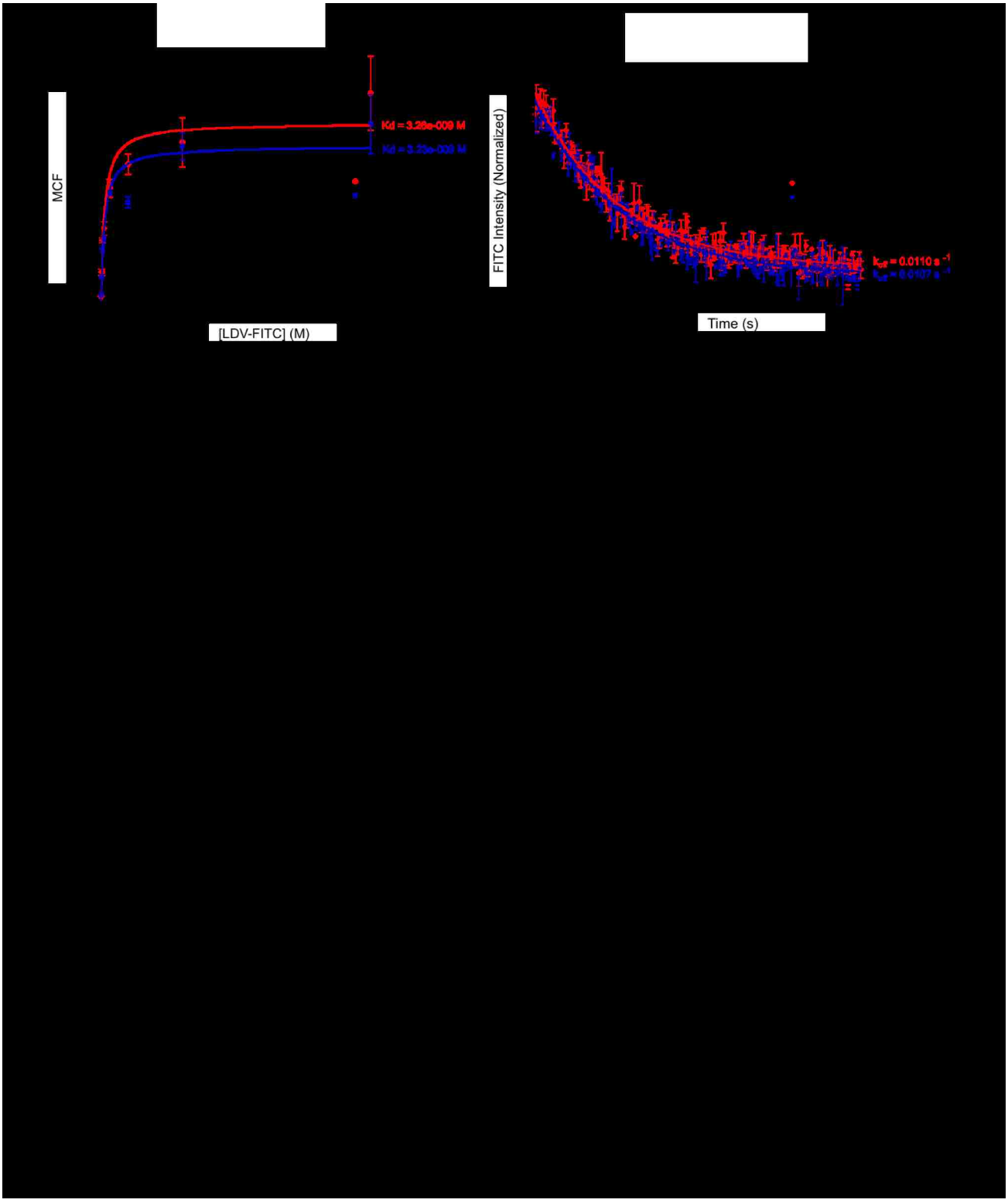

- mCherry
- mCherry-CD82
150
100
50
0
10^4
10^5
- mCherry
- mCherry-CD82
150
100
50
0
10^5
E
- Control siRNA
- CD82 siRNA
- CD82 shRNA
Cell Number
150
100
50
0
10^4
10^5
α4 Integrin
F
- Control siRNA
- CD82 siRNA
- CD82 shRNA
150
100
50
0
10^5

3.3.4 CD82 does not regulate VLA-4 integrin affinity

While CD82 appears to have an effect on VLA4 expression, we also wanted to determine whether CD82 could alter the affinity or activation of VLA-4. As a protein known to associate with integrins, we wanted to know if CD82 could interact with VLA-4 in such a way as to change its conformation and lead to its activation or increased affinity for ligand. To assess VLA-4 affinity, we performed affinity assays in which we looked at binding and dissociation of the VLA-4-specific ligand, LDV. For the affinity binding assays, the KG1a mCherry and mCherry-CD82 cells were either treated with DMSO or blocked with a saturating concentration of LDV and then incubated with increasing concentrations of fluorescently labeled LDV, LDV-FITC (0nM – 250nM). The LDV block provided a means to measure baseline, non-specific binding. Blocked and non-blocked cells were then analyzed by flow cytometry to assess levels of specific ligand-integrin binding, as measured by mean fluorescence minus baseline. Figure 3.4, A shows the FITC mean channel fluorescence (MCF) with respect to concentration of labeled LDV-FITC, and the dissociation constant, K_d, was determined from the nonlinear fit. The K_d values for the mCherry and mCherry-CD82 cells were nearly identical; 3.26×10^{-9} M and 3.23×10^{-9} M, respectively. These data indicate that CD82 is not regulating VLA4 integrin affinity.

To confirm these results, we also examined the dissociation of LDV-FITC in affinity assay "off-rate" experiments. The cells were again treated with either DMSO or blocked with LDV, but they were then incubated with a saturating LDV-FITC concentration. Real-time flow cytometry was used to analyze the dissociation kinetics of LDV-FITC upon addition of a saturating, competitive concentration of unlabeled LDV.

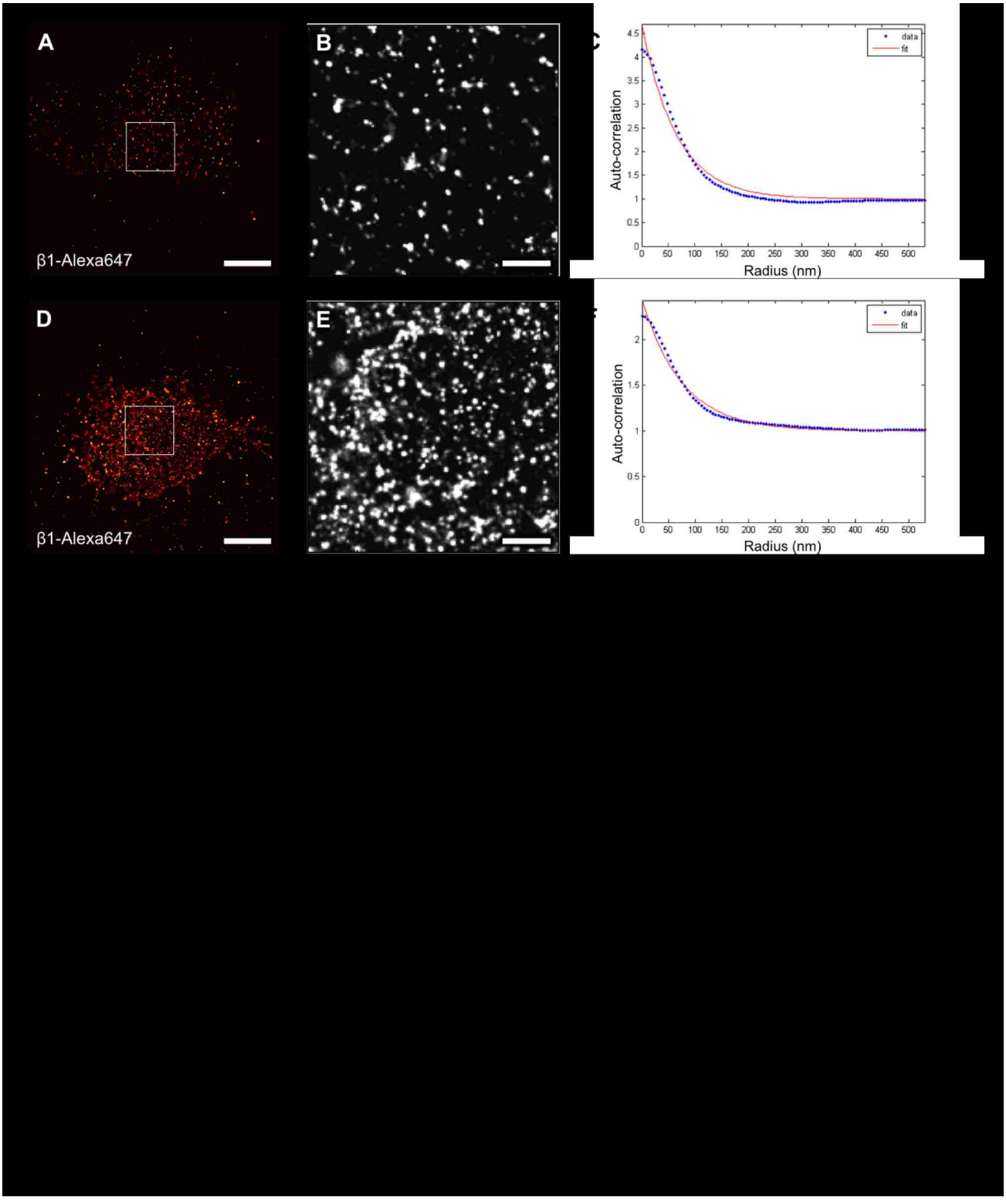
Figure 3.4, B shows the normalized mean fluorescence readings of LDV-FITC over time, and the dissociation rate constant, k_{off}, was determined from the nonlinear fit. The k_{off} values for mCherry and mCherry-CD82 were again nearly identical at 0.0110 s^{-1} and 0.0107 s^{-1}, respectively. These data indicate no difference in dissociation kinetics between control and CD82-overexpressing cells, thus confirming that CD82 is not affecting integrin affinity.


MCF
Kd = 3.28e-009 M
[LDV-FITC] (M)
FITC Intensity (Normalized)
Time (s)

3.3.5 CD82 regulates VLA-4 integrin avidity

As a membrane scaffold protein, CD82 has the potential to organize the proteins and molecules it associates with into clusters on the cell surface. Therefore, we next wanted to investigate whether CD82-mediated expression changes in VLA-4 are accompanied by changes in the clustering, or avidity, of VLA-4. To evaluate avidity, we utilized the innovative super resolution imaging technique, direct stochastical optical reconstruction microscopy (dSTORM), to look for distribution and clustering changes of the β1 chain of VLA-4 between KG1a mCherry control and CD82-overexpressing cells plated on fibronectin. In these experiments, cells were fixed and labeled with a directly conjugated β1-Alexa 647 antibody. The dSTORM technique takes advantage of the ability of Alexa647 fluorophores to transition between the "bright" and "dark" energy states without photobleaching, or in other words to blink, when placed under reducing conditions. By collecting a series of images of this blinking in TIRF, each fluorophore representing a single molecule can be localized based on its point spread function (PSF) and fit to generate a reconstructed image, which contains spatial information regarding the molecular organization of the cell surface. Figure 3.5 shows the reconstructed super resolution fluorescence localization images of (A) a representative mCherry control cell and (D) a representative mCherry-CD82 overexpressing cell. A 1000×1000 pixel region of each cell type was selected for analysis (Fig. 3.5, B and E). These magnified images show the localization of single β1 molecules on the cell surface. By applying the auto-correlation function to these regions in the mCherry control (Fig. 3.5, C) and CD82-overexpressing cells (Fig. 3.5, F), localized single molecule centers can be fit by the equation $g_{meas}(r) = \exp\{-r^2/4\sigma^2\}/\{4\pi\sigma^2\rho\}+g(r>0)*g_{psf}(r)$ for 30 nm < r < 500 nm

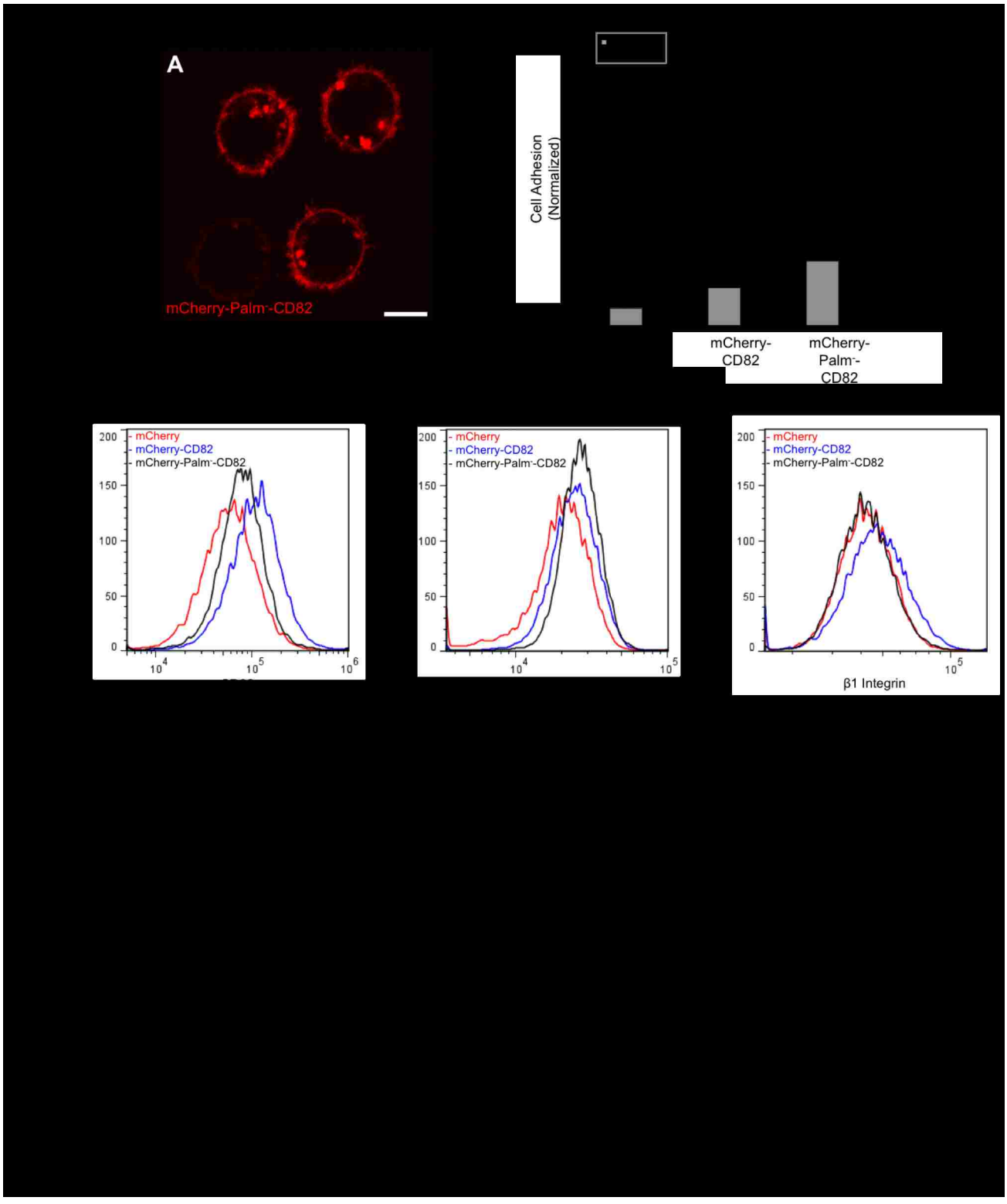
assuming an exponential form of $g(r>0)*g_{psf}(r) = 1+A\exp\{-r/\zeta\}$, where σ represents the PSF radius, ρ indicates the surface density of labeled molecules, A is the amplitude, and ζ gives the aggregate or cluster size (Veatch et al., 2012). For the mCherry control cells, extracted fit parameters were found to be: σ = 4.82 nm, ρ = 110.47 nm^{-2}, A = 3.70, and ζ = 64.75 nm. For the mCherry-CD82 cells, extracted fit parameters were: σ = 4.17 nm, ρ = 122.25 nm^{-2}, A = 1.43, and ζ = 74.86 nm. Here, the parameter ζ indicates β1 cluster size. As such, the difference in ζ values, with 64.75 nm for the mCherry control cells and 74.86 nm for the CD82-overexpressing cells, suggests that CD82 overexpression leads to an increase in the clustering of the β1 integrin chain on the cell surface.


A
β1-Alexa647
B
C
data
fit
Auto-correlation
Radius (nm)
D
β1-Alexa647
E
data
fit
Auto-correlation
Radius (nm)

3.3.6 CD82 palmitoylation can regulate VLA-4 adhesion

We next wanted to investigate the importance of CD82 palmitoylation in CD82-mediated adhesion. Palmitoylation is said to promote the organization and stabilization of tetraspanins in TEMs and may serve to influence CD82 oligomerization, membrane association, trafficking, and CD82 interactions with other proteins. For this purpose, we generated CD82 palmitoylation mutants, in which normally palmitoylated cysteines at positions 5, 74, 83, 251, and 253 were mutated to serines. The CD82 palmitoylation mutant DNA was genomically tagged to mCherry and transfected into KG1a cells. Selection with G418 led to the creation of the stable KG1a mCherry- Palm$^-$-CD82 cell line. Expression of the mCherry- Palm$^-$-CD82 vector was confirmed by fluorescence microscopy (Fig. 3.6, A), and its cell surface and endosomal localization was found to be consistent with the endogenous localization of CD82 (data not shown) and the exogenous localization of mCherry-CD82 in the overexpressing cells (Fig 3.1, B). We then quantified the surface expression of CD82 by flow cytometry (Fig. 3.6, C). Mean fluorescence readings revealed about a 1.5-fold increase in CD82 surface expression on the CD82 palmitoylation mutant cells, with levels directly between the mCherry control and CD82-overexpressing cells. These cells were also used in adhesion assays to assess the effects of palmitoylation on adhesion, specifically to fibronectin. The data revealed an increase in adhesion to fibronectin compared to mCherry control; however, this increase was not found to be significant as it was for the CD82-overexpressing cells (Fig 3.6, B). Interestingly, the extent of adhesion again appears to correlate with the level of CD82 detected on the surface of these cell lines. Next, flow cytometry was used to determine surface expression of both α4 and β1 integrins (Fig. 3.6, D and E). Our results show a

slight increase in surface expression of α4 in the mCherry- Palm$^-$-CD82 cells that is comparable to, if only marginally higher than the CD82-overexpressing cells. On the other hand, the modest increase in surface β1 observed in the mCherry-CD82 cells was not seen in the CD82 palmitoylation mutants. Taken together, these data suggest that the palmitoylation state of CD82 does not affect the surface expression of α4 but may be required for enhanced stabilization of β1 on the surface.


A
mCherry-Palm-CD82
Cell Adhesion (Normalized)
mCherry-CD82
mCherry-Palm-CD82
mCherry
mCherry-CD82
mCherry-Palm-CD82
β1 Integrin

3.4 Discussion

As in many stem cell-niche systems, the interactions between hematopoietic stem/progenitor cells and their bone marrow niche are crucial for regulating the function and behavior of HSPCs, especially their proliferation, differentiation, and maintenance of stemness via self-renewal (Renström et al., 2010; Schofield, 1978; ter Huurne et al., 2010; Zhang and Li, 2008). Many of these interactions involve direct physical contact between HSPCs and the cellular and matrix structures within the bone marrow, including osteoblasts and stromal cell-derived extracellular matrix proteins (Kiel and Morrison, 2008; Zhang et al., 2003a). The attachment of HSPCs is typically mediated through cell adhesion molecules, particularly integrins (Gillette and Lippincott-Schwartz, 2009; Gu et al., 2003; ter Huurne et al., 2010); however, the regulatory mechanisms for such adhesive events among HSPCs have not been fully elucidated (Gillette et al., 2009). CD82 is a tetraspanin protein highly expressed on HSPCs whose functionality lies predominantly in its ability to associate with, organize, and modulate the activity of other membrane proteins, including integrins and integrin-linked proteins. CD82 not only has important implications in adhesion and migration of immune and cancer cells (Hemler, 2005; Miranti, 2009; Ruseva et al., 2009), but it has also been linked to HSPC mobilization and release from the bone marrow niche as well HSPC homing to and engraftment within the niche (Larochelle et al., 2012). The role of CD82 in each of these events is likely a result of its influence on integrins (Hemler, 2005; Malik et al., 2009; Miranti, 2009; Ruseva et al., 2009). In this study, we utilized CD82 overexpression and knockdown systems as well as CD82 palmitoylation mutants to examine the effect of CD82 expression and oligomerization on HSPC adhesion to components of the bone marrow niche.

The results of our investigation indicate that CD82 plays an important role in regulating the adhesion of progenitor-like KG1a cells to components of the bone marrow niche *in vitro*. First, cells overexpressing the mCherry-CD82 vector exhibited at least a 2-fold increase in adhesion to fibronectin as well as an increase in adhesion to osteoblasts compared to control mCherry cells. Interestingly, neither control nor CD82-overexpressing cells were able to adhere to collagen I or laminin. Second, transient knockdown of CD82 reduced CD82 surface expression 5-fold and resulted in a significant decrease in adhesion to fibronectin as well as a decrease in adhesion to osteoblasts compared to control siRNA cells. Stable knockdown reduced surface CD82 an additional 5-fold and led to an even greater decrease in adhesion to fibronectin and osteoblasts. These data strongly implicate CD82 in the regulation of adhesion and indicate that the level of CD82 expression positively correlates with the extent of adhesion, such that highly expressing cells have a greater adhesion capacity than poorly expressing cells. Furthermore, the effects of CD82 expression on adhesion were specific to fibronectin and osteoblasts, which provide ligands for the α4β1 integrin, or VLA-4. Therefore, we hypothesized that VLA-4 was the most likely mediator of this adhesion. VLA-4 specifically binds the LDV sequence in fibronectin and a homologous sequence in the osteoblastic ligand, VCAM-1. Thus, to better analyze the relationship between CD82 and VLA-4 in adhesion, we utilized the monovalent LDV peptide to specifically block VLA-4 binding to fibronectin. Our results demonstrated a significant decrease in adhesion to fibronectin in the CD82-overexpressing cells, providing further support that CD82-induced adhesion effects are mediated through VLA-4. As CD82 serves as a molecular scaffold and exerts its functions through interacting with, regulating, and

organizing membrane proteins, we then assessed whether CD82 could be altering the expression, affinity, or avidity of VLA-4 in KG1a cells.

By looking at VLA-4 expression, we determined that CD82 could functionally regulate the expression levels of the α4 and β1 chains. While we saw no differences in total VLA-4 expression in the CD82-overexpressing cells, we did observe a modest increase in both α4 and β1 chains on the surface of these cells, which could account, at least in part, for the increase in CD82-induced adhesion to fibronectin and osteoblasts. Like many other tetraspanins, CD82 has been implicated in the control of maturation, trafficking, and internalization of integrins and other proteins (Berditchevski and Odintsova, 2007; Hu et al., 2005; Miranti, 2009; Ng et al., 1999; Zhang et al., 2003b) as well as their stabilization on the cell surface by establishing links to other proteins or tetraspanins in the tetraspanin web (Bass et al., 2005; Berditchevski and Odintsova, 2007; Hu et al., 2005; Stipp, 2010). As such, the increase in surface α4 and β1 could indicate a potential role for CD82 in α4β1 trafficking to the cell surface, slowing its internalization or recycling rate, and/or enhancing its stabilization on the surface. In the knockdown cells, we again saw no change in total expression of the β1 chain; however, there was a marked decrease in total α4 expression that appeared to correlate with the extent of CD82 knockdown and the associated decrease in adhesion. This in itself could suggest some level of CD82-mediated transcriptional or translational regulation of α4, or potentially, an increase in the degradation or turnover rate of α4 in the absence of CD82. Assessment of α4β1 surface expression showed a similar consecutive decrease in α4 associated with the level of CD82 knockdown. This is likely to be a consequence of the global decrease in total α4 or an indication that CD82 is required to traffic and/or stabilize α4 on the surface,

thereby preventing its internalization and targeting for lysosomal degradation. Relative to α4, surface expression of β1 did not change between the control and transient CD82 knockdown cells; however, there was a noticeable decrease in surface β1 in the stable knockdown cells, which correspondingly also exhibited the lowest levels of CD82. Because there is no difference in total β1 in these cells, the decrease in surface β1 could again harken back to potential CD82-mediated alterations in trafficking or stabilization of β1 on the surface. This could also suggest that it may take a more significant loss of CD82 to negatively influence the trafficking or stabilization of β1 on the surface. In addition, perhaps the lack of its α4 binding partner limits the extent of β1 trafficking, as these chains assemble in the endoplasmic reticulum (ER) and are transported to the surface together (Tiwari et al., 2011). In combination, our data indicate that changes in surface expression of α4 and β1 could be one avenue in which CD82 is regulating adhesion to fibronectin and osteoblasts. Moreover, these data implicate CD82 in the trafficking and/or stabilization of VLA-4 on the cellular surface and suggest that CD82 might have a more influential role in the regulation of α4 with less of an effect on β1. Studies to pinpoint the exact mechanism for CD82 regulation of VLA-4 expression, whether transcriptional, translational, or trafficking-based, still need to be performed.

Our assessment of VLA-4 affinity in the context of CD82 overexpression revealed no observable difference in VLA-4 activation, or affinity for ligand. When measuring the binding of a labeled form of the LDV monovalent peptide, LDV-FITC, we saw no significant differences in the binding curves between the control and overexpressing cells. The equilibrium dissociation constant, K_d, which corresponds to the concentration of ligand needed to meet half receptor occupancy, can be calculated from

the non-linear fit curve (Lineweaver and Burk, 1934). For the mCherry control and mCherry-CD82 overexpressing cells, the K_d values were nearly identical at 3.26 nM and 3.23 nM, respectively. Typically, the low affinity or resting state of VLA-4, as determined in the absence of Mn^{2+}, is indicated by a K_d of ~12 nM, while the high affinity or activated state of VLA-4, measured in the presence of high Mn^{2+}, is indicated by a K_d of ~1-2 nM (Chigaev et al., 2011). The nearly identical K_d values observed in our cells under normal culture conditions suggest that CD82 does not have an effect on VLA-4 affinity. In addition, the calculated dissociation rate constants, or k_{off} values, were also nearly identical between the control and overexpressing cells at 0.0110 s^{-1} and 0.0107 s^{-1}, respectively, further confirming that CD82 is not regulating VLA-4 affinity. These data are also consistent with other studies showing no evidence for tetraspanins in altering integrin conformation or affinity for ligand (Hemler, 2003).

Although we saw no differences in VLA-4 affinity, we did observe changes in the avidity, or clustering of VLA-4 with CD82 overexpression. Using the innovative super resolution imaging technique, dSTORM, we assessed distribution and clustering changes of the β1 chain of VLA-4 on the surface of mCherry control and CD82-overexpressing cells plated on fibronectin. We were able to localize and fit β1 integrins expressed on the cell surface down to the single molecule level. Auto-correlation analysis was used to analyze the aggregation or clustering of β1 molecules. From the auto-correlation equation, the ζ value can be calculated, which gives an indication of cluster size. Between the mCherry control and mCherry-CD82 overexpressing cells, the ζ values were calculated to be 64.75 nm and 74.86 nm, respectively. This indicates that CD82 overexpression leads to an increase in the cluster size of β1 on the cell surface, which in

turn suggests that CD82 is involved in the regulation of β1 avidity. While we have yet to complete dSTORM experiments to assess α4 avidity, we do expect a similar trend, partly because there was a similar increase in α4 and β1 surface expression between these cells suggesting that they are trafficked and regulated together. This would not be surprising, as the ability to regulate integrins and other adhesion receptors through changes in avidity, stabilization, or other means may be a general feature of many tetraspanins. In our studies, we believe that the increase in avidity of β1 (and possibly α4) in conjunction with the increase in surface expression of VLA-4 largely account for the increase in adhesion we observed with the CD82-overexpressing cells. Therefore, we can conclude that regulation of VLA-4 expression and avidity are the primary mechanisms by which CD82 can regulate HSPC adhesion to components of the bone marrow niche.

The post-translational modification involving the addition of palmitate to cysteine residues, otherwise known as palmitoylation, is thought to be essential to normal CD82 function. Palmitoylation can promote the lateral association of CD82 into clusters, which can influence its role in TEMs and effect the interactions, trafficking, and regulation of other proteins (Berditchevski, 2001; Hemler, 2005; Levy and Shoham, 2005; Tarrant et al., 2003). Cells expressing a mutant form of CD82 tagged to mCherry and lacking the intrinsic ability to undergo palmitoylation were used in adhesion and expression assays. These cells, which exhibited about a 1.5-fold increase in CD82 surface expression compared to mCherry control, also showed a correspondingly modest increase in adhesion to fibronectin, again higher than control but lower than cells with a 2-fold overexpression of non-mutant CD82. Assessment of VLA-4 surface expression in the palmitoylation mutant cells revealed an increase in surface α4 similar to that seen in the

CD82-overexpressing cells, but no change in surface β1 from control. As a whole, these data suggest that the palmitoylation state of CD82 may not only affect the trafficking and/or stabilization of CD82 on the surface, but may also be required for the enhanced stabilization of β1 on the surface. It may also be possible that a certain threshold level of CD82 on the surface is needed to maintain β1 there. On the other hand, our results appear to indicate that the increased expression of CD82, regardless of its palmitoylation state, can lead to increased surface expression of α4. While it is possible that the increase in surface α4 may still be a result of CD82-mediated trafficking and/or stabilization, α4 could also be getting to the surface by other means, particularly if inhibition of CD82 palmitoylation has an effect on other tetraspanins or proteins within the cell. Furthermore, perhaps α4 prefers to associate with a different β chain in these cells, most likely β7, and together they are transported more readily to the surface. Whatever the reason for the increase in surface α4 in the palmitoylation mutants, the lack of a corresponding increase in surface β1 could account, at least in part, for the observation of only a modest increase in adhesion to fibronectin compared to the CD82-overexpressing cells. This may be because β1 is the limiting factor in the assembly of VLA-4. Another possible reason for observing only a modest increase in adhesion is that palmitoylation might be involved in regulating the VLA-4 avidity changes that we observed with CD82 overexpression. Further studies to assess total VLA-4 expression in these cells as well as the expression levels of other potential binding partners for the separate integrin chains need to be done.

Our findings provide insight into the cellular and molecular mechanisms that regulate HSPC interactions with the bone marrow niche. The data strongly indicate that CD82 plays a role in mediating adhesion *in vitro* to components of the bone marrow

niche by modulating the expression and avidity of VLA-4. We would next like to assess the importance of CD82 expression in an *in vivo* setting. The clinical relevance of such research lies in the possibility that CD82 may be a key mediator of integrin function, thereby altering HSPC homing to and adhesion and engraftment within the bone marrow niche, as well HSPC mobilization and release from the niche into the peripheral blood. While HSPC transplantation is the predominant clinical therapy for the treatment of hematological malignancies, including leukemia, lymphoma, and myeloma, it is currently challenging to collect an adequate number of functional HSPCs and to expand them *ex vivo* such that they maintain the ability to successfully home to and engraft within the bone marrow of transplant patients. Therefore, understanding how to target or manipulate CD82 in such a way to alter its expression could provide a means to regulate HSPC release from, as well as homing and adhesion to, the bone marrow niche. Further studies into the downstream events initiated by CD82 as well as identification of other key players involved in regulating HSPC adhesion will improve our understanding of these complex HSPC-niche interactions and result in important innovations in the field of HSPC transplantation.

CHAPTER 4: DISCUSSION AND SIGNIFICANCE

4.1 Summary

The concept of a specific microenvironment, or "niche," in which stem cells are housed and regulated, was first proposed by Ray Schofield in 1978. Like most stem cell-niche systems, the interactions between hematopoietic stem/progenitor cells and their bone marrow niche are critical for regulating the function and behavior of HSPCs, especially their proliferation, differentiation, and maintenance of stemness via self-renewal. Without the niche, maintaining the proper homeostasic balance required for normal hematopoiesis would not be possible (Renström et al., 2010; Schofield, 1978; ter Huurne et al., 2010; Zhang and Li, 2008). While HSPC-niche interactions can involve long- or short-range paracrine signaling through the release of cytokines, growth factors, and hormones, the focus of this thesis has been on the direct physical contact or adhesive interactions between HSPCs and the cellular and matrix components of the bone marrow niche (Kiel and Morrison, 2008; Zhang et al., 2003a). HSPC adhesion is typically facilitated through adhesion molecules, including N-cadherin/β-catenin interactions, and most notably, integrins (Gillette and Lippincott-Schwartz, 2009; Gu et al., 2003; ter Huurne et al., 2010). The molecular mechanisms involved in regulating HSPC integrin-mediated adhesion, including the spatiotemporal aspects of this process are not very well understood. To gain insight, our laboratory previously characterized the molecules found at the HSPC-osteoblast contact site and found that HSPCs use a polarized membrane domain enriched in the integrin, VLA-4, and the specific tetraspanin proteins, CD63, CD81 (Gillette et al., 2009; Larochelle et al., 2012), and CD82 (Larochelle et al., 2012). In particular, CD82 has become of major interest to our research, as it is highly expressed

on HSPCs and has been linked to HSPC mobilization and release from the bone marrow niche as well HSPC homing to and adhesion and engraftment within the niche. CD82 functionality relies on its ability to associate with, organize, and modulate the activity of other membrane proteins, including signaling receptors, adaptor proteins, and especially integrins. In addition, previous work from our laboratory has shown that CD82 function-blocking antibodies can significantly decrease HSPC adhesion to osteoblasts as well as the *in vivo* homing and engraftment capabilities of these cells in mice (Larochelle et al., 2012). These observations support a potential role for CD82 in regulating HSPC adhesive interactions with the bone marrow niche.

The goal of this thesis has been to shed light on the molecular mechanisms underlying CD82's involvement in adhesion to components of the bone marrow niche. Through the generation of CD82 overexpression and knockdown cell lines as well as CD82 palmitoylation mutants, we have discovered that CD82 expression positively correlates with adhesion *in vitro* and that adhesion may be at least somewhat dependent on CD82's palmitoylation state. Interestingly, the effects of CD82 on adhesion appear to be mediated through the integrin, VLA-4. Furthermore, in looking at the molecular involvement of CD82 with VLA-4, we demonstrate that CD82 can regulate the expression and avidity of this integrin.

4.2 Significance of Results

4.2.1 CD82 Expression and Adhesion

In the context of cancer, CD82 expression levels have been shown to correlate with suppression of tumor cell migration, invasion, and metastasis (Ruseva et al., 2009). CD82 is thought to participate in the above processes by regulating adhesion, most likely

through its associative and scaffolding interactions with integrins (Hemler, 2005; Malik et al., 2009; Miranti, 2009; Ruseva et al., 2009). While the CD82-integrin relationship is not well established, the loss of CD82 in cancer may result in reduced integrin-mediated adhesion, allowing cancer cells to disengage from the primary tumor and migrate to new locations. On the other side, the presence of CD82 may serve to promote integrin-mediated adhesion, leading to cell immobilization and a reduction in metastasis (Malik et al., 2009). Much of the early research on CD82 centered on its involvement in cancer; however, CD82 expression does not only have functional implications in regulating the adhesive events of tumor cells. CD82 expression in immune cells is also critical to integrin signaling and adhesion (Miranti, 2009; Tarrant et al., 2003). Furthermore, its high expression in HSPCs and subsequent downregulation during differentiation also suggest that CD82 may be an important regulator of hematopoiesis by mediating membrane organization and adhesion to the niche. As such, we previously showed that blocking CD82 function with antibodies resulted in decreased HSPC adhesion to osteoblasts and an inhibition in their capacity to home to and engraft within the bone marrow niche *in vivo*, an outcome which may have important implications in HSPC transplantation (Larochelle et al., 2012). It is currently a challenge to expand HSPCs that have been isolated from a healthy donor in such a way that they can maintain their ability to successfully home to and engraft within the bone marrow of a transplant patient. Thus, in order to spur innovative improvements in HSPC transplantation, we need to better understand the mechanistic workings of HSPC adhesive interactions with the niche. In doing so, we have begun our investigations by more thoroughly assessing the importance of CD82 expression in HSPC adhesion.

From the data presented in this thesis, we can conclude that CD82 does indeed play an important role in regulating the adhesion of HSPCs to cellular and matrix components of the bone marrow niche. By utilizing *in vitro* CD82 overexpression and knockdown systems, we found that CD82 expression positively correlates with the extent of progenitor-like KG1a cell adhesion to fibronectin and osteoblasts but does not affect adhesion to collagen I or laminin. Because fibronectin and osteoblasts provide ligands specific to the α4β1 integrin, or VLA-4, we then hypothesized that the CD82-induced adhesion effects were most likely mediated through VLA-4. As VLA-4 specifically binds the LDV sequence in fibronectin and a homologous sequence in the osteoblastic ligand, VCAM-1, we were able to use the monovalent LDV peptide to specifically block VLA-4 binding to fibronectin. From these data, we can conclude that VLA-4 is involved, at least in part, in mediating the effects of CD82 on adhesion. CD82 is known to serve as a molecular scaffold, and it exerts its functions primarily through interacting with, regulating, and organizing membrane proteins. Therefore, we then speculated that CD82 could be altering the expression, affinity, or avidity of VLA-4 in KG1a cells.

4.2.2 CD82 Regulation of VLA-4 Expression

We have determined that CD82 can functionally regulate VLA-4 expression, mostly by affecting surface levels of the α4 and β1 chains. In addition, the levels of α4 and β1 generally appear to correlate with the extent of CD82 expression; however, CD82 seems to have a greater effect on α4 than on β1. Like many other tetraspanins, CD82 has been implicated in the control of maturation, trafficking, and internalization of integrins and other proteins (Berditchevski and Odintsova, 2007; Hu et al., 2005; Miranti, 2009; Ng et al., 1999; Zhang et al., 2003b) as well as their stabilization on the cell surface by

establishing links to other proteins or tetraspanins in the tetraspanin web (Bass et al., 2005; Berditchevski and Odintsova, 2007; Hu et al., 2005; Stipp, 2010). Thus, we can postulate that CD82 may be increasing or decreasing VLA-4 surface expression by altering VLA-4 trafficking to the cell surface, adjusting its internalization or recycling rate, and/or mediating its stabilization on the surface. While we did not typically observe CD82-mediated changes in total integrin expression, we did observe a decrease in total α4 expression in the CD82 knockdown cells. We can assume that this might be occurring through a number of mechanisms. For one, it is possible that CD82 is involved in regulating the transcription of α4 by activating downstream transcription factor(s) that can bind the designated promoter region to promote α4 transcription. Alternatively, CD82 may be regulating the translation of α4 by allowing its message to be translated or by promoting its biosynthesis in the ER and maturation to the Golgi. In either case, the absence or downregulation of CD82 would lead to a decrease in α4. It could also be possible that CD82 expression prevents the degradation or slows the turnover rate of α4, perhaps by stabilizing it on the surface and thereby preventing its internalization and targeting for lysosomal degradation. Whatever the mechanism, CD82-mediated regulation of VLA-4 expression may account, at least in part, for the increase or decrease in adhesion we observed for CD82 overexpression or knockdown, respectively.

The effects of CD82 on VLA-4 expression may also carry over into a more clinical and *in vivo* system. As such, the ability of CD82 to alter VLA-4 expression levels may be critical for the regulation of HSPC homing to, adhesion, and engraftment within the bone marrow niche, as well as HSPC mobilization and release from the niche into the peripheral blood. In fact, the expression of VLA-4 is markedly higher on resident bone

marrow HSPCs than on circulating HSPCs, suggesting that VLA-4 has an important role in maintaining HSPCs within the niche (Prosper et al., 1998; Yamaguchi et al., 1998). In addition, it is well known that use of VLA-4 antagonists or anti-VLA-4 antibodies to block VLA-4 binding can lead to the mobilization and collection of HSPCs in the peripheral blood (Shirvaikar et al., 2012). Separate inhibition of the α4 or β1 chain with inhibitory antibodies was also shown to suppress HSPC homing (Papayannopoulou et al., 1995) and *in vitro* generation of long-term bone marrow cultures (Miyake et al., 1991) or *in vivo* hematopoiesis (Williams et al., 1991), respectively. Furthermore, various cytokines and growth factors, including IL-3, SCF, and G-CSF can alter the expression of VLA-4 in HSPCs (Bellucci et al., 1999). These dynamic expression patterns indicate the capacity of VLA-4 to functionally regulate adhesive interactions with the niche (Imai et al., 2010). Taken together, the above evidence suggests that not only could VLA-4 be the major player in mediating retention within the bone marrow niche, but also CD82 is likely the key regulator of the fluctuations in VLA-4 expression involved in promoting HSPC release into circulation and homing back to the niche. Therefore, understanding how to target or manipulate CD82 in such a way to alter its expression or ability to regulate VLA-4 expression could provide a means to overcome some of the difficulties of HSPC transplantation.

4.2.3 CD82 Regulation of VLA-4 Avidity, Not Affinity

While we did observe CD82-mediated changes in VLA-4 expression, we were unable to detect any differences in VLA-4 activation, or affinity for ligand between our mCherry control and CD82-overexpressing cells. Regulation of integrin affinity involves altering the conformation of integrins from the inactive to the active state and vice versa.

As such, integrins in the bent, inactive, or low affinity conformation can be primed and activated, revealing an open, extended conformation with high affinity for ligand (Tiwari et al., 2011). From our assessments of VLA-4 affinity, we saw no significant differences in LDV-FITC binding or dissociation between the control and overexpressing cells. The calculated equilibrium dissociation constants, or K_d values, were nearly identical at 3.26 nM and 3.23 nM, respectively. K_d values of 3 nM are approaching the high affinity state of VLA-4 (high affinity: K_d ~12 nM; low affinity: K_d ~1-2 nM), thus it is not surprising that we saw no difference in VLA-4 affinity. In addition, the calculated dissociation rate constants, or k_{off} values, were nearly identical between the control and overexpressing cells at 0.0110 s^{-1} and 0.0107 s^{-1}, respectively, further confirming that CD82 is not regulating VLA-4 affinity. These data are also consistent with other studies showing no evidence for tetraspanins in altering integrin conformation or affinity for ligand (Hemler, 2003).

Although we saw no differences in VLA-4 affinity, we did observe changes in the avidity, or clustering of VLA-4 with CD82 overexpression. Using the innovative super resolution imaging technique, dSTORM, we assessed distribution and clustering changes of the β1 chain of VLA-4 on the surface of mCherry control and CD82-overexpressing cells plated on fibronectin. We were able to localize and fit β1 integrins expressed on the cell surface down to the single molecule level. Auto-correlation analysis was used to assess the aggregation or clustering of β1 molecules. Between the mCherry control and mCherry-CD82 overexpressing cells, the ζ values, which indicate cluster size, were calculated to be 64.75 nm and 74.86 nm, respectively. This indicates that CD82 overexpression leads to an increase in the cluster size of β1 on the cell surface, which in

turn suggests that CD82 is involved in the regulation of β1 avidity. While we have yet to complete dSTORM experiments to assess α4 avidity, we do expect a similar trend, partly because there was a similar increase in α4 and β1 surface expression between these cells suggesting that they are trafficked and regulated together. We believe that the increase in avidity of β1 (and possibly α4) in conjunction with the increase in surface expression of VLA-4 largely account for the increase in adhesion we observed with the CD82-overexpressing cells. Therefore, we can conclude that regulation of VLA-4 expression and avidity are the primary mechanisms by which CD82 can regulate HSPC adhesion to components of the bone marrow niche.

The regulation of integrin avidity, or clustering, can help to augment the overall strength or "functional affinity" of integrin-ligand binding (Ross and Borg, 2001). In fact, the ability to regulate integrins and other adhesion receptors through changes in avidity, stabilization, or other means may be a general feature of many tetraspanins. For instance, it has been shown that CD81 is capable of regulating VLA-4 avidity in leukocytes (Feigelson et al., 2003) and assembling various adhesion molecules into tetraspanin-enriched adhesion platforms in endothelial cells (Barreiro et al., 2008). Furthermore, siRNA knockdown studies of CD63 in human umbilical vein endothelial cells (HUVECs) have demonstrated that CD63 is important for regulating the expression and clustering of P-selectin on the cell surface, thus leading to leukocyte rolling effects (Doyle et al., 2011). Here we show for the first time that CD82 can strengthen the overall adhesive interactions of VLA-4 with its ligands, fibronectin and VCAM-1, by altering not only its expression patterns but also its avidity on the HSPC surface. Taken together, our data

provide additional scientific rationale for the interest in targeting and manipulating CD82 in HSPCs with the end goal being to generate improvements in HSPC transplantation.

4.2.4 CD82 Palmitoylation and Adhesion

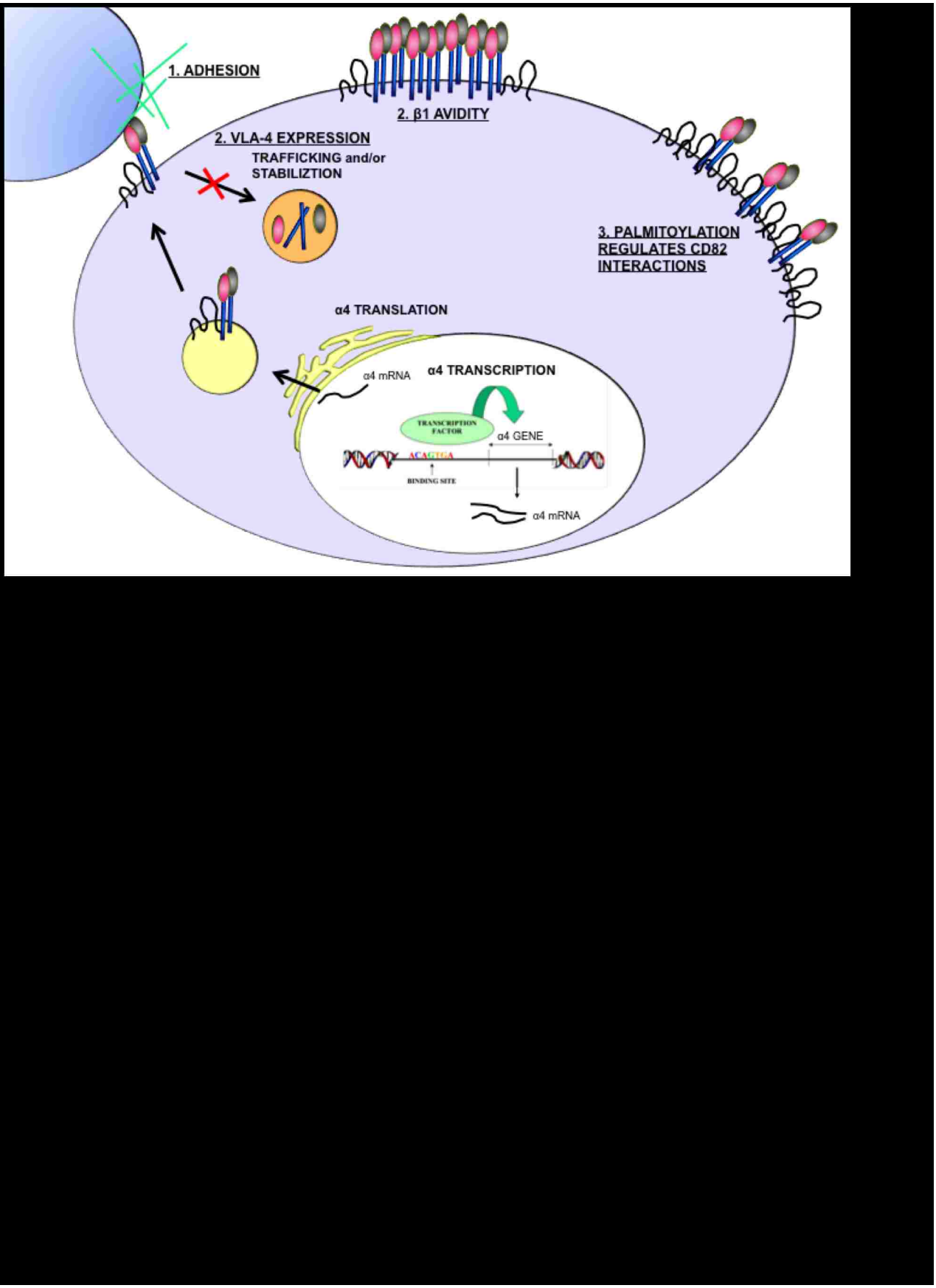
As a tetraspanin, CD82 characteristically associates with other members of the tetraspanin family, forming both homophilic and heterophilic dimers, multi-mers, and larger tetraspanin complexes. These interactions occur in a lateral fashion between the tetraspanin transmembrane domains and can give rise to dynamic tetraspanin-enriched microdomains (TEMs), or tetraspanin webs (Hemler, 2005; Levy and Shoham, 2005; Tarrant et al., 2003; Yáñez-Mó et al., 2009). These TEMs generally also include nontetraspanin members such as signaling receptors, adaptor proteins, and notably, integrins. The involvement of CD82 in TEMs may affect the organization of TEMs and subsequently the functions of receptor and cell adhesion molecules in these domains (Berditchevski, 2001; Hemler, 2003; Maecker et al., 1997; Tarrant et al., 2003). Palmitoylation is said to promote the organization and stabilization of tetraspanins in TEMs and may also have important functions in membrane association, influencing other tetraspanin protein-protein interactions, and trafficking (Bijlmakers and Marsh, 2003; Dunphy and Linder, 1998; Resh, 1999). Because of the observed effects of CD82 expression on VLA-4-mediated adhesion to components of the bone marrow niche, we wanted to know whether CD82 palmitoylation was required for regulating this adhesion. In addition, because of the ability of CD82 to increase VLA-4 avidity, we set out to determine whether the clustering or oligomerization of CD82 itself, as a result of its palmitoylation state, could impact the interactions of CD82 with VLA-4 in such a way to regulate its adhesion.

From our studies using CD82 palmitoylation mutant cells, we have determined that the palmitoylation state of CD82 may not only affect the trafficking to and/or stabilization of CD82 on the surface, but may also be required, at least in part, for the CD82-induced increase in adhesion. In addition, increased CD82 surface expression, regardless of its palmitoylation state, was found to correlate with an increase in α4 surface expression much like in the CD82-overexpressing cells. On the other hand, the lack of an increase in surface β1 suggests that CD82 palmitoylation may be required for enhanced trafficking to and/or stabilization of β1 on the surface. It is important to note, however, that α4 may prefer to associate with a different β chain in these cells, most likely β7, and together they may be transported more readily to the surface. Whatever the reason for the increase in surface α4 in the palmitoylation mutants, the lack of a corresponding increase in surface β1 could account, at least in part, for the observation of only a modest increase in adhesion to fibronectin compared to the CD82-overexpressing cells. This may be because β1 is the limiting factor in the assembly of VLA-4. Therefore, without palmitoylation, CD82 may not be able to properly associate with VLA-4, or at least β1, in such a way to promote VLA-4 trafficking and/or stabilization on the surface. Another possible reason for detecting only a modest increase in adhesion is that palmitoylation might be involved in regulating the VLA-4 avidity changes that we observed with CD82 overexpression. Because there is still endogenous wild-type CD82, and we see a slight increase in surface CD82 in these palmitoylation mutants, these cells might still be able to achieve some level of increased VLA-4 clustering, thus leading to the modest increase in adhesion. However, without palmitoylation, CD82 may not be

able to fully promote the proper organization of TEMs, which in turn may inhibit the clustering of VLA-4 that is necessary for adhesion strengthening.

4.3 Overall Significance

Our findings provide insight into the cellular and molecular mechanisms that regulate HSPC interactions with the bone marrow niche. The data strongly indicate that CD82 plays a role in mediating adhesion *in vitro* to components of the bone marrow niche by modulating the expression and avidity of VLA-4. Based on our results, we propose a working model for CD82 regulation of VLA-4 adhesion (Fig. 4.1). We would next like to assess the importance of CD82 expression in an *in vivo* setting. The clinical relevance of such research lies in the possibility that CD82 may be a key mediator of integrin function, thereby altering HSPC homing to and adhesion and engraftment within the bone marrow niche, as well HSPC mobilization and release from the niche into the peripheral blood. While HSPC transplantation is the predominant clinical therapy for the treatment of hematological malignancies, including leukemia, lymphoma, and myeloma, it is currently challenging to collect an adequate number of functional HSPCs and to expand them *ex vivo* such that they maintain the ability to successfully home to and engraft within the bone marrow of transplant patients. Therefore, understanding how to target or manipulate CD82 in such a way to alter its expression could provide a means to regulate HSPC release from, as well as homing and adhesion to, the bone marrow niche. Further studies into the downstream events initiated by CD82 as well as identification of other key players involved in regulating HSPC adhesion will only serve to improve our understanding of these complex HSPC-niche interactions and could result in important innovations in the field of HSPC transplantation.


1. ADHESION
2. β1 AVIDITY
2. VLA-4 EXPRESSION
TRAFFICKING and/or
STABILIZTION
3. PALMITOYLATION
REGULATES CD82
INTERACTIONS
α4 TRANSLATION
α4 mRNA
α4 TRANSCRIPTION
TRANSCRIPTION FACTOR
α4 GENE
ACAGTGA
BINDING SITE
α4 mRNA

4.4 Future Directions

In light of the data presented in this thesis, many new and exciting ideas for directions in which to take this project have developed. For one, we hope to further evaluate the importance of CD82 expression in an *in vivo* setting by taking full advantage of the newly available resource of CD82 knockout mice ($CD82^{-/-}$), which have been generously offered to us by Dr. Cindy K. Miranti and her lab at the Van Andel Research Institute in Grand Rapids, Michigan. We plan to use these mice to isolate CD82-deficient HSPCs for use in adhesion and expression assays as well as *in vivo* homing and engraftment studies. It may also be worthwhile to assess other tetraspanins in these knockout cells to look for any compensatory or redundant functions, especially since the tetraspanins CD63 and CD81 can also specifically associate with VLA-4 (Mannion et al., 1996). In addition to having complete knockout of CD82, the advantage of using these cells is that they are primary cells isolated directly from mouse bone marrow. While cell lines are typically much easier to work with and can be maintained in culture for long-term experimentation, primary cells have more clinical relevance than their cell line counterparts. Not only are cell lines often derived from tumors, but they also adapt to growth in culture, despite attempts to maintain physiologic conditions. This can lead to genetic and phenotypic drift and altered cellular functionality. As such, primary cells are often more ideal as they are more representative of cells *in vivo* (Pan et al., 2009).

Altering CD82 expression in our KG1a cells has provided important information regarding the regulation of HSPC-niche interactions. However, it would be of great benefit to know what regulates CD82 expression in HSPCs. In addition to the complex signaling network within the niche, an important feature of the niche is its hypoxic

environment. The relatively low level of oxygen, or hypoxia, can lead to the activation of hypoxia-inducible factors (HIFs), including HIF-1 and HIF-2. These transcription factors can then regulate certain subsets of genes in response to hypoxia (Eliasson et al., 2010; Nagao and Oka, 2011; Rehn et al., 2011). In one study, it was discovered that HIF-2 can upregulate the expression of CD82 in human umbilical vein endothelial cells (HUVECs) under hypoxic conditions. Because hypoxia is thought to promote the maintenance of HSPCs in the more quiescent state, a property that might involve CD82-mediated contact with osteoblasts, we would like to assess the potential HIF-1 and HIF-2 regulation of CD82 in HSPCs. To do this, we could generate cells overexpressing HIF-1 and HIF-2 and then analyze their effects on CD82 expression in normoxic and hypoxic conditions. In addition, we could place our other cell lines, including the mCherry control, CD82-overexpressing cells, and CD82 knockdown cells in normoxic and hypoxic environments to compare HIF-1 and HIF-2 levels and look for any changes in CD82 expression. Finally, it may also be interesting to use these cells, especially the HIF-overexpressing cells, in adhesion assays and VLA-4 expression assays.

To complement the adhesion data for our mCherry control and CD82-overexpressing cells, which showed that adhesion to fibronectin could be blocked with the LDV monovalent peptide, we plan to repeat the adhesion assays using both α4- and β1-blocking antibodies. The specific monoclonal antibodies, PS/2 (Miyake et al., 1991; Papayannopoulou et al., 1995), HP1/2 and HP2/1 (Abraham et al., 1994; Huo et al., 2000), have been reported to block α4 function and ability to bind its ligands. Likewise, the specific monoclonal antibodies JB1A (Akimov and Belkin, 2001), P4C10 (Rinaldi et al., 1997), and mAb13, known to block the function of all β1 integrins (Lee et al., 1995)

can block β1 adhesive binding. For these VLA-4 function-blocking adhesion assays, it would be worthwhile to assess α4 and β1 block separately and in combination to determine if there is a synergistic or additive effect of the α4 and β1 chains in mediating CD82-induced adhesion. This could also serve to provide further information about whether VLA-4 truly is the predominant integrin involved in the effects on adhesion, or whether α4 or β1 separately have different levels of involvement by interacting with different partners in this process. We would also like to utilize the α4- and β1-activating antibodies, BU49 (activating potential observed in our lab) and TS2/16 (Lee et al., 1995), respectively, as positive adhesion controls for fully activated integrins. Alternatively, adhesion in the presence of Ca^{2+} and absence of Mn^{2+} and vice versa could be included as negative and positive controls for the integrin low and high activation states, respectively (Tiwari et al., 2011). Furthermore, because we have mainly focused on adhesion to fibronectin, we could look more closely at the CD82-induced increase in adhesion to osteoblasts, which we believe is mediated through VLA-4 binding to VCAM-1. Because VLA-4 binding to VCAM-1 is thought to occur with greater than four times higher affinity than binding to fibronectin (Masumoto and Hemler, 1993; Mould et al., 1994), it would be of benefit to assess VLA-4/VCAM-1 interactions in HSPCs. As such, we could use anti-VCAM-1 antibodies to block cell binding. Together with anti-VLA-4 antibodies, this could potentially further support our claim that VLA-4 is involved in CD82-mediated adhesion.

Because we saw a decrease in the total expression of α4 in our CD82 knockdown cells, we would like to determine whether this decrease is due to transcriptional or translational regulation of α4 or the result of increased α4 degradation. As direct

translational regulation is not very common, we would first focus on transcriptional regulation or protein degradation. To assess whether CD82 has the potential to regulate α4 transcription and/or mRNA turnover, we could perform real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) to measure levels of α4 mRNA transcript in the knockdown cells versus the siRNA control and KG1a parental cells. This process works by first using the reverse transcriptase enzyme to reverse transcribe mRNA strand into its complementary DNA (cDNA). The cDNA is then amplified using PCR with primers specific to the gene of interest, in this case α4. As the cDNA is progressively amplified over a number of PCR cycles, there is a measurable increase in fluorescence emitted by a dye such as ethidium bromide or SYBR green, which intercalates into the DNA, and this is proportional to the amount of cDNA amplified. Detection of PCR products in this assay serves as a way to quantify the original amount of mRNA transcript underlying the cDNA content (Nolan et al., 2006). Transcription may not be the only way α4 is regulated. To measure protein degradation or turnover, we could utilize techniques involving labeled metabolic tracers, such as stable isotope-labeled amino acids. In this procedure, cells can first be grown in medium containing a given stable isotope-labeled amino acid such as [$^{2}H_{10}$] leucine until all the proteins are labeled, and then the medium can be switched to one containing unlabeled leucine. The cells could be sampled every few hours over the course of a day, and then proteins would be resolved in conjunction with 2-D gel electrophoresis or immunoprecipitation. After isolating the α4 protein, it would then be subjected to mass spectrometry. Over the course of time, as the proteins become unlabeled, there is a shift in the mass spectrum of leucine-containing peptides from “heavy,” or stable isotope-labeled, to “light,” or unlabeled. The

loss in labeled peptides over time would allow us to compare the degradation rate of α4 between the CD82 knockdown cells and control (Beynon, 2005).

Although our focus has been on the VLA-4 integrin, there are other integrins on the HSPC surface that could mediate adhesive binding to fibronectin and/or VCAM-1. For instance, the integrin α5β1, or VLA-5, binds the arginine-glycine-aspartic acid (RGD) motif in fibronectin (Stipp, 2010), while α9β1 binds VCAM-1 (Schreiber et al., 2009). In addition, α4β7 can bind both the LDV sequence in fibronectin and VCAM-1 (Humphries et al., 2006). The integrin VLA-5 is involved in HSPC homing to and engraftment within the bone marrow niche (Carstanjen et al., 2005). Integrin α9β1 has only recently been discovered on the HSPC surface with important functions in promoting HSPC adhesion to osteoblasts as well as inhibiting HSPC proliferation and differentiation (Schreiber et al., 2009). The α4β7 integrin has also been implicated in HSPC homing to and release from the bone marrow (Tada et al., 2008), and some studies suggest that both α4β1 and α4β7 contribute equally to HSPC homing (Katayama et al., 2004). Furthermore, because CD82 appears to have a greater effect on the α4 chain than on β1 in the α4β1 integrin, perhaps CD82 regulation of α4 also extends to its other binding partner, β7. Considering the potential involvement of the aforementioned integrins in HSPCs, we will begin by assessing the total and surface expression of each of these integrin chains in our mCherry control and CD82-overexpressing cells. Of these, α9β1 and α4β7 may be the most promising. However, while α9β1 may indeed be involved in HSPC adhesive interactions with the niche, our preliminary expression data obtained through both flow cytometry and immunofluorescence using an α9β1 specific antibody indicate that there is very little expression of α9β1 on our progenitor-like KG1a

parental and stably transfected cell lines. It might still be worthwhile to utilize a different α9 antibody in repeating these studies. Overall, if we find that CD82 could be regulating the expression of these other integrins, we would then consider examining CD82 effects on affinity and avidity as well.

With regards to avidity, we also plan to improve and expand upon the super resolution imaging technique, dSTORM, such that we can successfully perform two-color, simultaneous labeling of α4 and β1 in our mCherry control and CD82-overexpressing cells. Thus far, we have only been able to label with antibodies conjugated to Alexa Fluor 647, as we have had little success with the Alexa 488 dyes. We have yet to find a way to induce a consistent and effective transition of the 488 dye between the dark and light energy states, which is required to obtain fluorophore localization. If this does not work, another option is to utilize Cy3-conjugated antibodies, which still excite and emit at wavelengths distinct enough from Alexa 647 to use in two-color labeling but have recently shown more promise for dSTORM than 488. In addition to improving our labeling and technique, we also need to develop better tools and algorithms for analyzing our fit images. Arriving at this point will allow us to obtain data for both α4 and β1 together and more critically evaluate α4β1 distribution or clustering changes between these cells at the single molecule level.

The CD82 palmitoylation mutant cells have provided some very interesting information; however, there is still much to be obtained from studying this cell line. We still need to complete adhesion assays to assess adhesion of these mCherry-Palm$^-$-CD82 cells to osteoblasts. While we see a slight increase in adhesion to fibronectin, it would be beneficial to know whether this carried over to osteoblasts in order to instill more

confidence in our data. We would also like to assess the total expression of CD82, α4, and β1 in these cells, as we have not yet successfully developed the blots to compare expression between mCherry control and mCherry-CD82 overexpressing cells. Analysis of affinity using flow cytometric methods and avidity using our dSTORM technique could prove interesting, especially since palmitoylation is thought to be important for clustering-type interactions between tetraspanins and other proteins (Hemler, 2005). Palmitoylation is known to occur through the enzymatic action of thiol-directed protein acyltransferases (PATs). A family of PATs containing the aspartic acid-histidine-histidine-cysteine (DHHC) motif, also known as the DHHC protein family, is involved in the palmitoylation of various substrates, including tetraspanins. Among the DHHCs, DHHC2 has been shown to be the most efficient in stimulating palmitoylation of tetraspanins CD9 and CD151. (Miranti, 2009; Sharma et al., 2008). While DHHC2 has not been specifically implicated in CD82 palmitoylation, it is very likely to be the predominant enzyme involved in this process (Sharma et al., 2008). As far as I know, DHHC2 mutation or knockdown to inhibit palmitoylation has only been assessed for CD9 and CD151 (Miranti, 2009; Sharma et al., 2008). Through [^{3}H] palmitate labeling and subsequent CD82 immunoprecipitation (Sharma et al., 2008), we could assess the effects of DHHC2 expression on CD82 palmitoylation and then utilize cells with altered CD82 palmitoylation in other experiments. The advantage of mutating or knocking down DHHC2 over transfecting with a palmitoylation mutant version of CD82 is that all CD82 molecules in the cell would potentially lack palmitoylation. However, DHHC2 mutation or knockdown could also lead to global effects in palmitoylation of various tetraspanins, though this could be interesting to study as well.

In conjunction with the obvious CD82 palmitoylation defects of the mCherry-Palm$^-$-CD82 cells, we have made some unusual observations about these cells when grown in culture. They not only appear to grow at a slightly slower rate than the parental KG1a, mCherry control, or mCherry-CD82 overexpressing cells, but they also seem to have potential differences in metabolism or breakdown of energy sources provided by nutrients in the culture medium. The latter observation is based on noticeable color differences in the liquid medium, as these cells tend to rapidly turn the color from a more reddish-pink to a golden yellow, which could be a result of certain metabolic by-products. Thus, it could be fruitful to perform proliferation or cell cycle analysis of these cells as well as assess their metabolic output and regulation. Preliminary proliferation data (not shown) does indeed indicate a slower rate of cell division, though more trials need to be conducted, and an alternative means of confirming proliferation could be employed. As for analyzing the cell cycle of these cells in comparison to the control and overexpressing cells, we could utilize flow cytometric methods by first permeabilizing and then staining the DNA with propidium iodide (PI). We also have access to the Seahorse XF analyzer for purposes of examining the state of the mitochondria, which in extension, provides an assessment of metabolism by measuring the two major energy yielding pathways, aerobic respiration and glycolysis.

In addition to looking at the functional importance of palmitoylation, we plan to generate other CD82 mutants to look at the consequence of modifying various other structural aspects of CD82 on adhesion and regulation. For instance CD82 glycosylation involving the addition of a carbohydrate glycan to asparagine residues at positions 129, 157, and 198 in the large extracellular loop (EC2) loop (Dong et al., 1995; Miranti, 2009)

is thought to have important structural and functional implications in correct protein folding, trafficking through the ER, and encouraging CD82-based protein interactions (Scholz et al., 2009). Therefore, the generation of glycosylation mutants, in which one or more asparagine residues are mutated to glycine, could provide insight into the importance of glycosylation to CD82 function in the context of HSPC-niche communication. We have begun to generate and sequence the DNA for these mutants, and upon stable transfection, we plan to use the CD82 glycosylation mutant cell lines in each of our adhesion and expression assays as previously described. Glycosylation in the EC2 loop is one potential avenue for regulation of CD82-protein interactions; however, in most tetraspanins, EC2 contains a constant region comprised of three α-helices, A, B, and E, and importantly, a hypervariable region that is also critical for tetraspanin protein-protein interactions (Hemler, 2005; Yáñez-Mó et al., 2009). This region in CD82 has been proposed to be important for interactions with integrins (Mazurov et al., 2007). Therefore, mutations in the hypervariable region of CD82 could be useful to determine whether this region provides the mode of interaction between CD82 and other proteins in HSPCs, and specifically whether mutating this region has any effect on integrin-mediated adhesion or integrin surface expression. Other possible CD82 domains to consider include the transmembrane domains and intracellular loop (IC). Mutating the polar residues found in the first, third, and fourth transmembrane domains (asparagine (N), glutamine (Q), and glutamic acid (E), respectively) could impact the stabilization of the overall structure of CD82, as these residues are thought to be important for CD82 folding and maturation. Furthermore, transmembrane domain interactions, which are thought to be mediated by hydrogen bonds between the polar residues, can help to stabilize the

conformation of the EC2 loop. Thus, by association, mutating these residues could affect the interaction potential of the EC2 loop (Cannon and Cresswell, 2001; Hemler, 2003). Likewise, mutations in the intracellular loop could impact interactions with various cytoplasmic or submembrane proteins (Mazurov et al., 2007).

Because tetraspanins can interact with many different proteins, their association with and regulation of integrins may be occurring indirectly. One such protein that can associate with both tetraspanins and integrins is protein kinase C (PKC), and as such, tetraspanin interactions with integrins may be mediated at least partly through PKC (Malik et al., 2009; Zhang et al., 2001). The many PKC isoforms, including classical (α, β1, β2, and γ), novel (δ, ε, η, and θ), and atypical (ζ, ι, and λ), are part of a subfamily of serine-threonine kinases (Spitaler and Cantrell, 2004). Of these, PKCα, PKCβ2, PKCε, and PKCζ have been detected in HSPCs (Myklebust et al., 2000). Although the involvement of PKC with integrins is not very well understood in HSPCs, PKCα has been shown in other cells to be important in the trafficking of integrins, particularly β1, and its expression can lead to an increase in β1 on the surface (Ng et al., 1999). Thus, potential CD82 regulation of integrin trafficking in KG1a cells could be mediated through PKCα. Not only is CD82 thought to link PKC to integrins, but CD82 has also been observed to associate with the PKC substrates, talin and MARCKS, which in turn can have effects on integrin adhesion and signaling (Miranti, 2009). Therefore, overall it could be worthwhile to assess PKC levels and activation, which is indicated by its phosphorylation state, in relationship to CD82 expression levels. Preliminary data via immunofluorescence and immunoblotting suggest that the CD82 knockdown cells may have less total PKCα expression than control KG1a cells. We have yet to assay for

changes in total PKC between the mCherry control and CD82-overexpressing cells; however, it may also be interesting to assess levels of activated or phosphorylated PKC (phospho-PKC) in these cells. Ultimately, it would be interesting to use each of the CD82 mutant cells described above to look at the involvement of PKC signaling as well.

While CD82 may be important for intracellular signaling, it may also be important for intercellular signaling via the secretion of exosomes. Tetraspanins, including CD82 are enriched in exosomes, which are small, 50-100 nm membrane-enclosed vesicles secreted from immune and other cells with potential roles in intercellular signaling and transfer of protein and RNA (Escrevente et al., 2011). Interestingly, exosomes can express integrins and participate in adhesive interactions with extracellular matrix proteins and cellular ligands. As a result of their direct contact with other cells and surrounding environment, exosomes may be able to send signals from their cell of origin to receiving cells (Clayton et al., 2004). In addition, HSPCs are known to promote the release of exosomes and may even transfer microRNAs (miRNAs) through this process to post-transcriptionally regulate mRNA expression in other niche cells (Bauer et al., 2011; Bissels et al., 2012). Therefore, it may be interesting to investigate the potential role of exosomes and their CD82 and integrin expression patterns in HSPC communication with the bone marrow niche. To do this, we would first isolate exosomes from HSPCs using the ExoQuick-TC isolation kit. We could then characterize the total and surface expression patterns of tetraspanins and integrins for these exosomes and utilize them in co-culture experiments with niche cells, particularly osteoblasts. After incubating osteoblasts with the exosomes and allowing any signaling exchange to occur, we could then perform enzyme-linked immunosorbent assays

(ELISAs) on exosome-treated osteoblasts compared to osteoblasts under normal culture conditions to look for any differences in signaling factors. Some signaling factors that could be interesting to measure include IL-6, stem cell factor (SCF), CXC-chemokine ligand 12 (CXCL12), and even vascular endothelial growth factor (VEGF), as HSPCs are thought to encourage the production of VEGF by other cells (Maes et al., 2012).

In conclusion, the regulatory scaffold membrane protein, CD82, is critical for regulating integrin-mediated adhesion of hematopoietic stem/progenitor cells with the bone marrow niche. Association with and regulation of integrins and other adhesion molecules through mechanisms involving membrane organization, sorting, and trafficking appear to be a common theme among tetraspanins. Much as CD81 can regulate the avidity of VLA-4 on leukocytes (Feigelson et al., 2003) and CD63 can regulate the expression and clustering of P-selectin on HUVECs (Doyle et al., 2011), here we show for the first time that CD82 can strengthen the overall adhesion of VLA-4 by altering both its expression and avidity on the HSPC surface. By gaining insight into the complexities of HSPC interactions with the bone marrow niche, particularly the underlying mechanisms of CD82 involvement in HSPC adhesion, we provide justification for CD82 as a realistic molecular target for manipulating HSPC release from, as well as homing and adhesion to, the bone marrow niche, and we hope to identify others that could one day serve to advance the field of HSPC transplantation.

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