Role of Podocalyxin in Hematopoiesis and Cell Migration

by

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Abstract

CD34 and its relatives, Podocalyxin and Endoglycan, comprise of a family of surface sialomucins expressed by hematopoietic stem/progenitor cells, and vascular endothelia. Recent data suggest that they serve as either pro- or anti-adhesion molecules depending on their cellular context and their post-translational modifications. We were interested in identifying Podocalyxin ligands and their cellular distribution and understanding the role of these factors in signaling, adhesion and migration. Using both a lambda phage screen assay and mass spectrometry, we identified the Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) as a selective ligand for Podocalyxin and Endoglycan but not for the closely related CD34. Furthermore, we showed that NHERF-1 is expressed by all, lineage$^-$, Sca-1^+ and c-kit$^+$ (LSK) cells, which are known to express Podocalyxin and have long-term repopulating characteristics of hematopoietic stem cells. In addition, upon IL-3 stimulation of a factor dependent cell line (FDC-P1) these proteins re-localize and co-localize in an asymmetrical pattern. By using a lentiviral based shRNA system to silence Podocalyxin and NHERF-1 proteins, we observed that migration across stromal monolayer towards a CXCL12 and SCF gradient is significantly impeded in cells that lack Podocalyxin but not NHERF-1. Following *in vitro* stimulation with a combination of CXCL12 and SCF we observed that Podocalyxin co-associates with CXCR4. Furthermore, cells lacking Podocalyxin have decreased phospho-AKT, a key signaling molecule downstream of c-kit and CXCR4 receptors. Taken together, our data supports the conclusion that Podocalyxin co-association with CXCR4 modulates downstream signaling to efficiently regulate HSC homing.

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List of Abbreviations

BAMBI	Bio-Assembly Mosaic Building and Informatics system
BSA	Bovine serum albumin
CFTR	Cystic fibrosis transductance receptor
CFU-S	Colony forming unit-spleen
CKII	Casein kinase II
c-kit	SCF receptor
CXCL12	aka. Stromal derived factor-1, SDF-1
CXCR4	CXCL12 receptor
DKD	Double Knock-down
DNA	Deoxyribonucleic acid
ECM	Extracellular cell matrix
En_{th}	Embryonic day, where *n* is the number of days
ERM	Ezrin, radixin and moesin
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDC-P1	Factor Dependent Cell-Paterson-1
GFP	Green fluorescent protein
Gr-1	Granulocyte differentiation antigen-1
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell

IL-3	Interleukin-3
KD	Knock-down
KO	Knock-out
LC –MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
LSK	Lineage-, Stem cell antigen-1+ and c-Kit+
Luc	Luciferase
M210B4	ATCC ID for a murine bone marrow derived stromal cell line
Mac-1	Macrophage differentiation antigen-1
NHERF-1	Na^+/H^+ exchanger regulatory factor-1
NHERF-2	Na^+/H^+ exchanger regulatory factor-2
$P2Y_1$	Purinergic receptor $2Y_1$
PBS	Phosphate buffered solution
PDGF-R	Platelet derived growth factor-receptor
PDZ	Post synaptic density-95, D Drosophila disc large tumor suppressor, Zona occludins-1,
PECAM	Platelet/endothelial cell adhesion molecule
PFA	Paraformaldehyde
PKC	Protein kinase C
PLCβ	Phospholipase-β
pLL3.7	Plasmid lentiviral 3.7
PMA	phorbol 12-myristate 13-acetate
pMD.G	Envelope gene
pMDLg/pRRE	HIV-1 *gag* and *pol* genes

Podo	Podocalyxin
pRSV-REV	plasmid expressing HIV-1 Rev
RNA	Ribonucleic acid
SAP	Strepavidin alkaline phosphatase
SCF	Stem cell factor, aka. Steel Factor or mast cell growth factor
Sh	Short hairpin
shRNA	Short hairpin ribonucleic acid
SS	Steady State
ST	Starved
TBS	Tris-base salt
Trp4	Tryptophan requiring 4
Trp5	Tryptophan requiring 5
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
VLA-5	Very late antigen-5
VSVG	Vesicular stomatitis virus glycoprotein
WB	Western blot
WEHI-3B	Walter and Eliza Hall Institute -3B
β2AR	Beta-2-adrenergic receptor

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Dedication

I dedicate this thesis to stem cell transplant patients worldwide,

YOU are the reason that I strive to find the answer everyday

Co-Authorship Statement

My participation in the work presented in Chapter 2:

- generated, verified and analyzed Podocalyxin and NHERF-1 binding and identification
- performed data analysis, generated figures and wrote a complete manuscript for publication.

My participation in the work presented in Chapter 3:

- generated, verified and analyzed shLuc and shPodocalyxin cells
- developed, optimized, and conducted all migration and adhesion assays and FACS analysis
- developed, optimized and conducted all confocal microscopy work
- generated data, figures and wrote a complete manuscript for publication

My participation in the work presented in Chapter 4 is a product of collaborative work with Dr. Eric Jervis from the University of Waterloo, Ontario, Canada. My participation in this work involves:

- generated, verified and analyzed shLuc and shPodocalyxin cells
- participated in data analysis
- generated figures from the data analysis and wrote a complete manuscript for publication.

My participation in the work presented in Appendix A:

- generated, verified and analyzed shLuc and shNHERF-1 cells
- developed, optimized and conducted all microscopy, FACS and cell phenotype analysis
- generated data, figures and wrote a complete brief report for publication.

Chapter 1 : General Introduction

1.1 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) have the unique ability to self-renew and differentiate into all lineages of specialized cells in the blood (Eckfeldt et al. 2005). Throughout embryogenesis, HSCs develop in the yolk sac and eventually reside in the adult bone marrow. In this work, we will be focusing on the blood formation in the mouse system.

Early studies with mouse embryos have relied on *in vitro* and *in vivo* assays to identify hematopoietic progenitors and stem cells during development. Pioneering studies by Moore and Metcalf showed that primitive erythroid, erythroid-myeloid progenitors, colony forming unit-spleen (CFU-S) and HSC appear in the yolk sac at days E7, E8, E8.5 and E11 respectively (Moore et al. 1970). In addition to the yolk sac, hematopoietic stem cell progenitors (HSCP) and stem cells were also identified in the intra-embryonic aorta-gonad-mesonephros (AGM) region at E7.5. At E10, CFU-S frequency and activity in the AGM are higher than in the yolk sac. Thereafter, the activity in the fetal liver surpasses that of the AGM (Cumano et al. 2001); and finally, cells are believed to travel to the fetal bone marrow, which eventually becomes the adult source for HSCs.

In the adult bone marrow, HSCs develop in close association to stromal cells. Stromal cells include cells of mesenchymal origin, for example: fibroblasts, osteocytes, adipocytes and vascular smooth muscle cells. Stromal cells of bone marrow origin are known to constitute a supportive microenvironment (niches) for the survival,

proliferation and differentiation of HSCs (Lemischka 1991). These niches are thought to help maintain HSCs by tethering quiescent cells close to the bone and supporting their survival via cytokines and cell-association signals (Wilson et al. 2006). In addition to the stromal monolayer, the bone marrow niche has also been defined by the osteoblastic region in the bone and has been shown as a pertinent area for HSC maintenance. The discovery of the vascular niche within the bone has also been attributed to HSC maintenance. Sinusoid endothelial cells that make up the vascular niche are believed to provide an environment that promotes proliferation and further differentiation, while the known osteoblastic niche provides a quiescent microenvironment (Kopp et al. 2005).

The most primitive HSCs are currently used as therapeutic transplants in patients with blood disorders. HSCs can be divided into two different populations based on their activity: short-term and long-term repopulating cells (Eckfeldt et al. 2005). Short-term repopulating cells are capable of differentiating and reconstituting an irradiated recipient only transiently. Short-term cells are usually referred to as multipotent progenitor or precursor cells. Long-term cells have the ability to self-renew, differentiate and sequentially reconstitute primary, secondary and tertiary irradiated hosts (Eckfeldt et al. 2005). Because of HSCs' long-term repopulating abilities, these cells are commonly used in therapies. However, it has been challenging to properly identify, isolate and expand these cells *in vitro* for clinical applications.

HSCs are usually quiescent until they are triggered to differentiate or mobilized into the circulatory system. Mobilization and differentiation takes place under two situations: a)

normal turnover and replacement of apoptotic blood cells in circulation or b) during stem cell mobilization for clinical therapy (Wilson et al. 2006). The latter is quite commonly performed in the clinics to collect sufficient numbers of HSCs for successful transplantation.

The most common technique for isolating HSCs from the bone marrow is by using markers that appear on the surface of these cells followed by fluorescence activated cell sorting (FACS). In the mouse, c-Kit and Sca-1 markers are used to identify long-term repopulating cells; lineage markers (B220: B-cells; Mac-1: macrophages; Ter119: erythrocytes; CD3: lymphocytes and Gr-1: granulocytes) are used to exclude mature and lineage committed cell populations (Spangrude et al. 1991). Thus, long-term repopulating cells are classified as having a phenotype of, **Lin**$^-$, **Sca-1**$^+$ and c-**Kit**$^+$ (LSK). To enrich for human HSCs populations for the purpose of clinical applications, CD34 is used as marker (Greaves et al. 1992).

1.2 Hematopoietic Stem Cell Homing: A Closer Look at Migration and Adhesion

One remarkable trait of HSCs is their ability to "home". Homing is a multi-step process that involves tethering, rolling, firm adhesion, trans-endothelial migration and subsequently successful lodgment of HSCs at the appropriate niche (Whetton et al. 1999; Wright et al. 2002; Nervi et al. 2006; Kaplan et al. 2007) (Figure 1.1). This process leads to the repopulation of an irradiated host due to the self-renewal and multi-lineage

differentiation capacities - an event known as engraftment. Bone marrow progenitors, which lack homing activity, fail to engraft the bone marrow and thus fail to rescue lethally irradiated recipients. A dose of lethal irradiation damages DNA in blood and bone marrow cells and thus, freeing up the niche in the bone marrow. This creates "space" for incoming cells (as a result of intravenous delivery of host cells) to establish residence for the re-population of the host with new blood lineages.

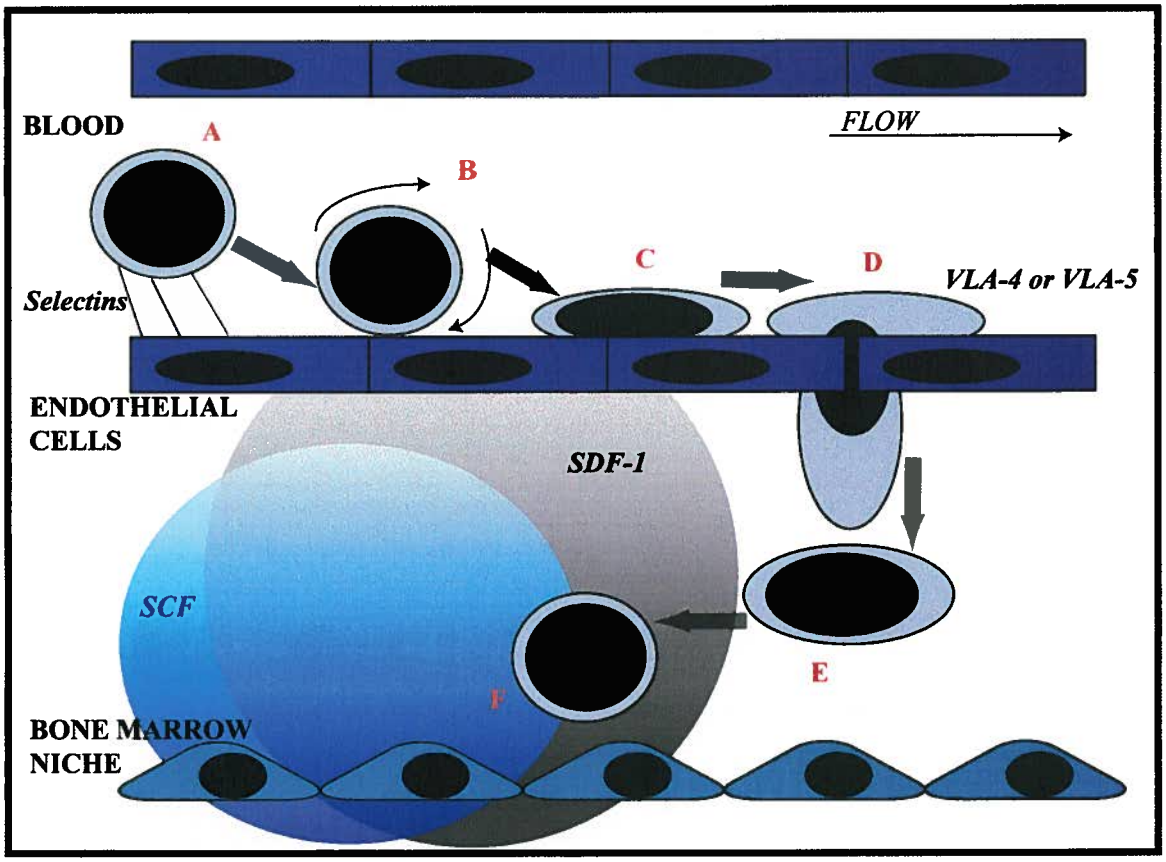


Figure 1.1 Schematic model of the HSCs homing process to the bone marrow niche.

Homing of HSCs to the stromal niche involves complex mechanisms. The main steps that are involved in successful homing and engraftment of stem cells from the blood circulation include A: tethering, B: rolling, C: firm adhesion, D: trans-endothelial migration, E: chemotaxis and finally F: lodgment.

Homing involves coordinated actions between CXCL12, previously known as stromal derived factor-1 (SDF-1), stem cell factor (SCF), and adhesion receptors such as CD44, very late antigen-4 (VLA-4) and -5 (VLA-5) (Lapidot et al. 2002; Chute 2006; Jung et al. 2006). It has been shown that a chemotactic gradient of CXCL12 significantly influence the migration of HSCs (Lapidot et al. 2002). In addition, SCF alters adhesion receptor expression (Berrios et al. 2001), which affects HSCs *adhesion* to the stroma during homing (Shimaoka et al. 2002; Driessen et al. 2003). However, successful homing is measured by efficient maintenance of populations of HSCs within the bone marrow niche.

1.3 HSCs Adhesion in the Homing Process

One of the common but pertinent mechanisms behind all the steps in HSC homing is adhesion. Adhesion molecules such as selectins and integrins mediate the interaction of cells to the blood vessel wall prior to extravasation into the proper bone marrow stromal or osteoblastic niche (Whetton et al. 1999; Hart et al. 2004; Chute 2006).

There are different classes of adhesion receptors that participate in cell-to-matrix and cell-to-cell interactions, which play a role in homing (Nilsson et al. 1996; Nilsson et al. 2004; Haylock et al. 2005; Nilsson et al. 2006). Endothelial selectins (P- and E-selectin), VLA-4 and CD44 are examples of receptors that play a role in the adhesion of marrow cells. Several groups have demonstrated that P- and E-selectins and integrins contribute equally to rolling and adhesion of marrow cells to the vessels (Frenette et al. 1998; Mazo et al. 1998). Short-term homing studies performed by infusing HSCs into P- and E-

selectin deficient, lethally irradiated recipients also showed a significant decrease in the number of donor marrow cells homing to the marrow (Frenette et al. 1998; Mazo et al. 1998). In addition, Papayannopoulou et al. (Papayannopoulou et al. 1997) demonstrated decreased bone marrow engraftment when cells were pre-incubated with an antibody to VLA-4, a key integrin in the homing process. Other groups have also shown that by blocking CD44, a cell surface sialomucin involved in cell migration decreases engraftment in mice by impeding homing mechanisms through an affect on cell-cell adhesion (Vermeulen et al. 1998).

It has also been shown that the functional activity of adhesion receptors can be affected by CXCL12 and SCF. For example, SCF affected the function of VLA-4 by activating its high affinity β1 subunit (Leavesley et al. 1994; Simmons et al. 1994; Simmons et al. 1994; Kovach et al. 1995). In addition, activation of CXCR4 by CXCL12 leads to increased affinity of integrins such as VLA-4 for their substrates (for example, VCAM-1). Thus, mediating adhesion to stromal cells during HSC homing (Hartmann et al. 2005).

1.3.1 Selectins – A Brief Introduction

Selectins are another family of molecules involved in HSC homing to the bone marrow. The selectin family of adhesion molecules contains three family members: P- (CD62P) and E-selectin (CD62E) and L-selectin (CD62L). Endothelial cells express P- and E-selectins, while mature leukocytes and hematopoietic progenitors express L-selectin.

Historically, the selectin family members are active players in leukocyte migration from circulation into injured tissues during inflammation (Vestweber et al. 1999), or during naïve lymphocyte homing to the lymph nodes (L-selectin) (Rosen 2004).

Selectins allow blood cells to recognize, target and migrate across endothelial barriers towards a gradient of inflammatory molecules or chemoattractants. They have a unique extracellular domain which is predominantly composed of an N-terminal lectin region. Furthermore, selectins have a number of epidermal growth factor (EGF)-like domains, a series of domains similar to complement binding proteins, with a transmembrane region and a short C-terminal cytoplasmic tail (Vestweber et al. 1999). All three members are conserved throughout evolution and their expression is highly tissue specific.

However, their binding ligands can be redundant (Vestweber et al. 1999). For example, all three members are able to bind P-selectin glycoprotein ligand-1 (PSGL-1) (Levesque et al. 1999; Katayama et al. 2003). This ligand was first characterized in lymphocytes and it is critical for efficient leukocyte rolling during inflammatory responses. However, P-, E- and L-selectins also bind specific ligands such as CD24 for P-selectin, which modulates B-cell activation (Simmons et al. 1997), ESL-1 for E-selectin, which mediates neutrophil adhesion (Vestweber et al. 1999), and CD34 for P-selectin, which is a common marker used for sorting human stem cells (Krause et al. 1996).

1.3.2 Integrins – A Brief Introduction

In addition to selectins, integrin molecules participate in the homing process. Integrins are adhesion molecules that are composed of an α and β subunit. These molecules mediate cell-cell and cell-matrix interactions by integrating with the cytoskeleton in response to cellular cues for cell migration. There are currently nineteen different α-subunits and eight β-subunits in vertebrates (Shimaoka et al. 2002). Together, they can form at least twenty-five different types of heterodimers with specific structural and functional roles (Figure 1.2) (Shimaoka et al. 2002).

Integrins are composed of a transmembrane and a cytoplasmic domain. Each α- and β-subunit interface contains a globular amino-terminal ligand-binding head domain (Nermut et al. 1988; Xiong et al. 2001). Integrins exist in an activated or non-activated state. In the non-activated state, integrins are bent, generating a V-shaped topology in which the binding interface is in close proximity to the membrane-proximal portions. In this orientation, integrins appear to be unfavourable for binding to the extracellular matrix or cell-surface ligands.

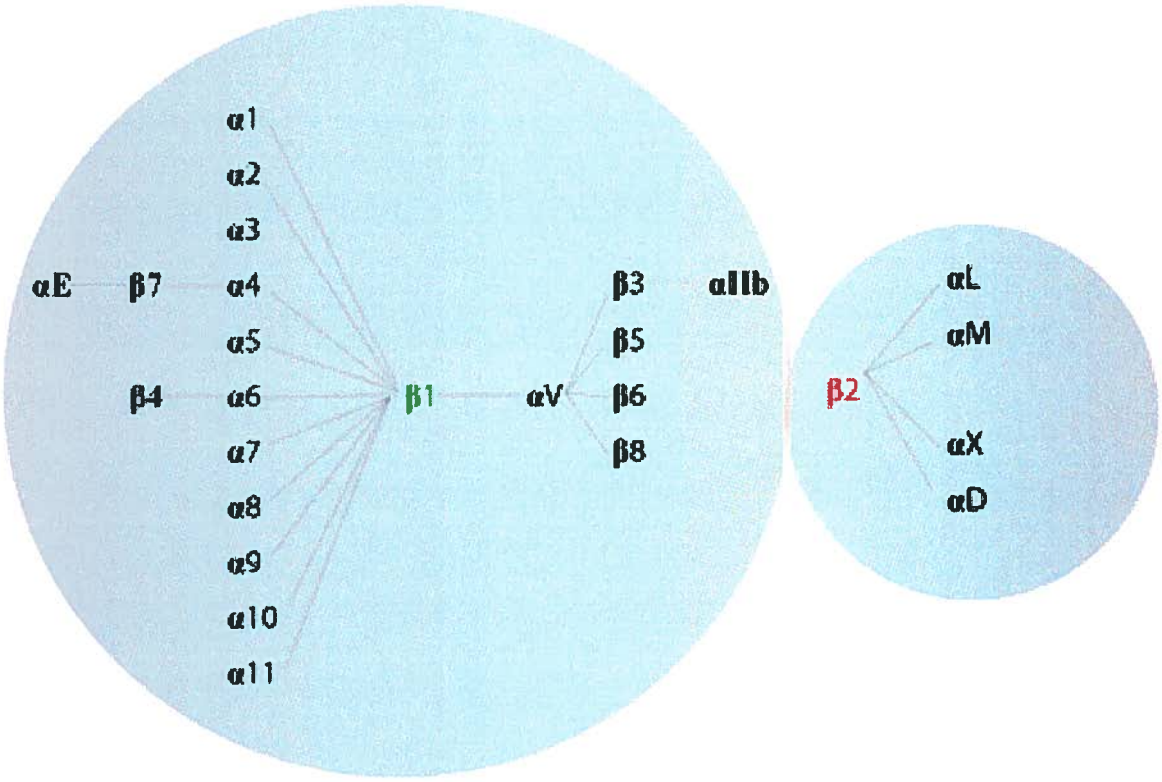
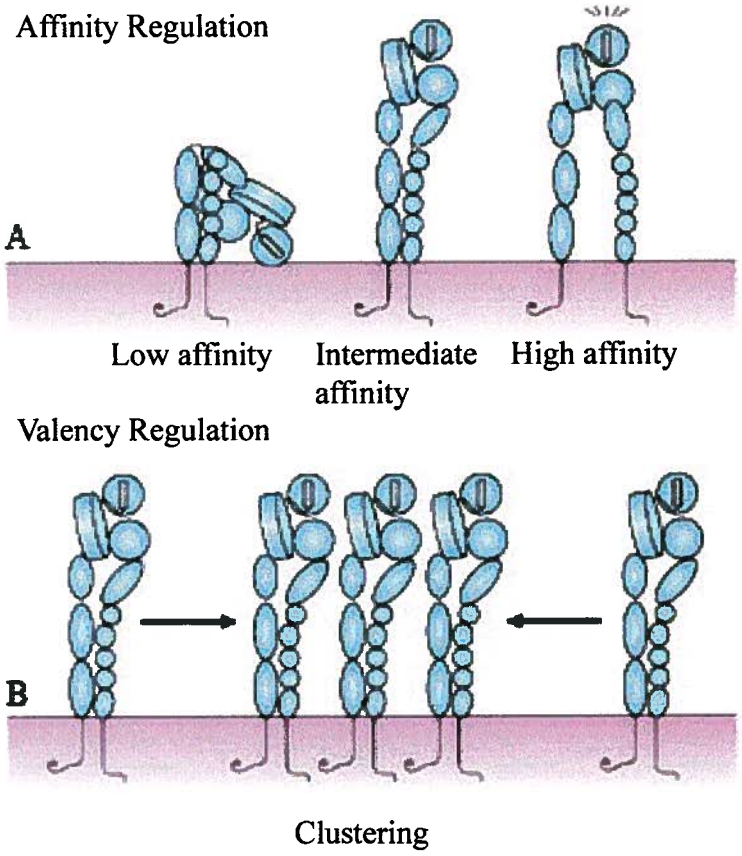


Figure 1.2 The integrin family is composed of combinations of α and β subunits.

Various associations occur as indicated by grey lines between the 18 types of integrin α-chains and the 8 types of β-chains to form at least 24 types of integrins.

Integrins are able to transduce signals by altering their affinity for extracellular ligands. Currently, three main models explain the regulation of integrin activation: a) affinity, b) valency, also known as avidity and c) a model in which affinity and valency are integrated.

The affinity regulation model is defined by the degree of attraction or specificity between a receptor and its ligand. Upon cell activation, the α and β interface rearrange and extend with a "switchblade-like" motion to bind ligand (Takagi et al. 2001; Beglova et al. 2002; Takagi et al. 2002). This change in orientation exposes integrins to the ligand and therefore modulates affinity (Figure 1.3). Subsequent binding of ligand to the exposed surface of the α and β interface causes conformational changes in the entire molecule including the cytoplasmic tail. These changes initiate intracellular signaling.



Adapted and modified from Kinashi T. Nature

Figure 1.3 Integrin regulation: Affinity versus Valency.

Two models of integrin activation are schematically presented in this figure. A) In the affinity regulation model, integrins exist in a bent, closed, "switch-blade" structure where the ligand binding site is masked between the membrane and the stalk of the integrin. Upon activation, the molecule springs up and adopts an upright position where the ligand binding region is exposed. B) In the valency regulation model integrins exists in an upright position and upon activation they re-distribute at the membrane to form a cluster which mediates multivalent interactions with ligands. (Figure adapted and modified from Kinashi T, 2005)

The valency regulation model suggests that integrin mediated adhesion is achieved by receptor clustering at the membrane surface. As valency is a difficult event to measure, clustering or patching of stained cells observed by fluorescent microscopy is often used to support the valency regulation model (Carman et al. 2003). Clustering or patching may be due to oligomerization or ligand-dependent re-distribution of integrins. Consequently, polarization and intracellular trafficking are necessary for both events to occur (Shimaoka et al. 2002). Several groups have attempted to explain the dynamic recruitment and clustering of integrins at the surface. Jacobson and colleagues hypothesized that the recruitment of leukocyte integrins into structures such as lipid rafts, mediates clustering (Jacobson et al. 1999). However, another group suggests that integrin clustering is a result of homotypic associations between the transmembrane domains of neighbouring integrins prior to switching to the "open" active state during affinity regulation (Kinashi 2005). Despite the different theories, researchers continue to explain valency regulation through the measure of adhesion strength, a method, which quantifies number of receptor-to-ligand bonds that form upon integrin activation.

Finally, an integrated model suggests that both affinity and valency are important for integrin regulation. Stewart and colleagues demonstrated that ligand-binding is necessary for the re-distribution of integrins to the zone of contact (Stewart et al. 1998). Also, it has been demonstrated that by exposing cells to potent activators such as phorbol 12-myristate 13-acetate (PMA), which is an activator of protein kinase C, enhances micro-clustering of integrins, thus increases its affinity for ligand (Lollo et al. 1993; Leitinger et al. 2002). The re-distribution of integrins into clusters at the cell membrane is commonly

visualized by using green fluorescent protein (GFP) and real time-imaging microscopy (Laukaitis et al. 2001; Plancon et al. 2001).

Regardless of which type of regulation occurs at the cell membrane, both affinity and valency regulation are dynamic processes that require a set of coordinated cell signals following integrin activation. Presently, there are two models to explain signal transmission by integrins across the plasma membrane: a) outside-in and b) inside-out signaling pathways.

Outside-in signaling is characterized by the activation of integrins following ligand, such as fibronectin, binding at the extracellular domain. Integrin activation results in propagation of a signal across the plasma membrane to intracellular mediators that influence cell adhesion, cell shape, growth and survival (Qin et al. 2004; Kinashi 2005). This type of signaling has been attributed to integrin clustering at the cell surface due to the multivalent nature of fibronectin. Clustering initiates downstream signaling pathways by enabling the recruitment of cytoplasmic mediators to the adhesion complex. Outside-in signal transduction may also be initiated by a combination of integrin affinity and activation. It is interesting to note that, integrin clustering alone may not be sufficient to reproduce a full outside-in signaling cascade (Leitinger et al. 2002).

Inside-out signaling has been shown most clearly in studies of a platelet receptor – $\alpha IIb\beta 3$ (Chen et al. 1994; O'Toole et al. 1994). Many studies on this mode of signaling have concentrated on this particular receptor mainly due to: the ease of collecting large

quantities of platelets, purification of the receptor for experiments, the existence of well defined platelet activators and the ability to directly measure changes in receptor affinity for its ligand.

Inside-out signaling is a consequence of sequential events resulting from cell stimulation. Activation of key molecules initiates a signaling cascade, causing conformational changes to the cytoplasmic region of integrins. Ultimately, this causes pertinent modifications in the extracellular ligand binding domain, leading to integrin activation. Activation increases affinity and a greater tendency for clustering (Faull et al. 1996; Miranti et al. 2002). For example, chemokines such as CXCL12 rapidly increases the avidity and thus, activation of integrins resulting in the events mentioned above (Kucia et al. 2004).

1.4 HSCs Migration in the Homing Process

Cell migration is an integrated process which involves continuous formation and disassembly of adhesion complexes. Primary HSC migration occurs during hematopoietic development in the fetus, while homing of transplanted HSCs or mobilization of activated HSCs from the niche take place during trauma or injury. There is now ample evidence to suggest that during these events HSCs migrate towards a defined niche in the bone marrow where they are kept in fine balance between quiescent and cycling states (Kaplan et al. 2007). Most bone marrow transplantations involve an intravenous delivery of freshly isolated HSCs directly into the circulation. The intricate homing

process that follows involves several different interactions including, but not limited to, binding between integrins and the extracellular matrix (ECM), a chemokine gradient and specific homing receptors present in the niche and on HSCs. As noted, VLA-4 plays a central role in homing and engraftment of transplanted HSCs. The attraction of transplanted cells to the bone marrow via specific chemoattractants, particularly CXCL12 and SCF, is important for selective homing to the niche. CXCL12 is constitutively expressed by stromal cells including osteoblasts, endothelium and fibroblasts, and is a major determinant of homing and migration in both embryonic and adult life (Lapidot et al. 2002). This chemokine efficiently binds to its cognate receptor CXCR4 expressed on HSCs for efficient activation of integrin mediated adhesion and trans-endothelial migration (Mazo et al. 1998). SCF is a cytokine expressed by the stroma cells in the niche. Because the local concentrations of CXCL12 and SCF are higher, a gradient is built to aid in attracting cells that express the corresponding receptors to the niche (Ashman 1999; Heberlein et al. 1999). Levesque et al. reported that following a short exposure to SCF, transplanted HSCs showed a significant up-regulation of pertinent homing molecules such as the indispensable CXCR4 and beta 1 integrins (Levesque et al. 1995). In parallel, efficient HSC homing is dependent on cells having the ability to regulate cell shape. Numerous migration and homing studies suggest that cells must be able to dynamically regulate their actin cytoskeleton for efficient migration and mobilization across and through the stromal layer (Holloway et al. 1999; Serrador et al. 1999; Francis et al. 2002; Fruehauf et al. 2002; Gu et al. 2003; Vicente-Manzanares et al. 2004)

1.4.1 CXCL12 and CXCR4: Roles in HSC Migration

Chemokines are small, secreted proteins that can be categorized into two main subfamilies based on conserved cysteine residues and the presence of an intervening amino acid. These two families are termed CXC or CC chemokines. Two additional classes have also been defined, lymphotactin (XC) and fractalkine (CX3C), which either lack a cysteine or have three amino acids between the first two cysteines. The receptors are designated CXCR1 to 7, CCR1 to 11, XCR1 (lymphotactin receptor) and CX3CR1 (fractalkine receptor) based on their preference for CXC or CC chemokines (Pelus et al. 2002; Kucia et al. 2004). The chemokine CXCL12 was initially identified as a pre-B-cell growth factor (as reviewed in (Chute 2006), which is constitutively secreted by marrow stromal cells. This chemokine binds to CXCR4, which is a seven transmembrane, G-protein coupled receptor. In addition, CXCR4 plays a role in the directional migration of cells, a process known as chemotaxis (Lapidot et al. 2005)

Studies have shown that CXCR4 is important in hematopoiesis and development of the immune system. Mice that lack this protein die during embryogenesis likely due to a deficiency in myelopoiesis, lymphopoiesis and abnormal development of the neuronal and cardiovascular systems (Nagasawa et al. 1996; Zou et al. 1998; Stumm et al. 2003).

CXCL12 plays a prominent role in this process by coordinating interactions between integrins, cell adhesion molecules and the cytoskeleton. Upon ligand binding, CXCR4 receptors are capable of activating pertinent signaling pathways required for efficient migration (Wysoczynski et al. 2005). Rac-1 is a GTPase belonging to the RAS

superfamily of GTP-binding proteins. Members of this family regulate a number of cellular events, particularly the control of cell growth, cytoskeletal re-organization and activation of downstream kinases (Gu et al. 2003; Guan 2004). The interaction between Rac-1 and CXCR4 may control microtubule re-organization and membrane ruffling; each event is essential to cell migration and adhesion (Kijima et al. 2002). To corroborate this finding, it has been shown that normal HSCs fail to engraft Rac-1 deficient mice. Conversely, Rac-1 deficient HSCs fail to engraft wildtype recipients. In both scenarios, HSCs remain in the circulation and thus are not detected in the bone marrow microenvironment (Gu et al. 2003). A recent study by Wysoczynki et al. showed that upon CXCL12 stimulation of human cord blood cells, Rac-1 and CXCR4 not only associate with each other, but also facilitate activation (GTP binding) of Rac-1 and thus enhancing chemotaxis.

1.4.2 SCF and c-Kit: Roles in HSC Migration

In addition to CXCL12, SCF has been implicated to modulate cell migration. SCF is also known as kit ligand, steel factor and mast cell growth factor, which binds to the receptor c-Kit (Ashman 1999). Kit was originally identified as the viral oncogene (*v-kit*) that is responsible for the Hardy-Zuckerman IV feline sarcoma virus (Besmer et al. 1986). In humans, the receptor c-Kit was first identified as a cell surface marker on acute myeloid leukemia cells. Also, c-Kit was later shown to be expressed by mast cells, (Ashman 1999), a wide range of non-hematopoietic cells (Turner et al. 1992) and breast epithelial cells (Hines et al. 1999; Ulivi et al. 2004). c-Kit is expressed by approximately 70% of

human $CD34^+$ cells in the bone marrow; this population also includes some lineage positive cells (Turner et al. 1992). In mice and humans, different isoforms of c-Kit exist as a result of alternative mRNA splicing. In the juxtamembrane region of the extracellular domain, these isoforms are characterized by the absence or presence of four amino acid sequences, GNNK (Ashman 1999).

c-Kit is known to be a receptor tyrosine kinase (RTK), which is closely related to the receptors of platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1) (Yarden et al. 1987; Qiu et al. 1988). The extracellular domain of c-Kit is divided into five immunoglobin-like regions. The first three immunoglobin-like (Ig-like) regions bind SCF to induce homodimerization of the receptor. There are currently two models for ligand-induced dimerization for c-Kit (Blechman et al. 1993; Blechman et al. 1993; Blechman et al. 1995). The first model suggests that binding of the biologically active dimeric form of SCF initiates receptor dimerization. The second model suggests that the binding of SCF to a c-Kit monomer induces conformational changes, resulting in interaction of the Ig-like domains between the two molecules of c-Kit (Blechman et al. 1993; Lev et al. 1994; Blechman et al. 1995).

SCF is a glycosylated, non-covalent homodimer, which is ubiquitously expressed by stromal cells, fibroblasts and endothelial cells. It is an important growth factor for hematopoiesis and for the generation of germ cells (Ashman 1999). SCF also exists in both soluble and membrane bound forms due to differential splicing and post-translational modifications. These two forms have very different effects on the c-Kit

kinase signaling activity (Miyazawa et al. 1995; Broudy 1997). For example, c-kit ligation by the membrane bound form of SCF mediates a long duration signal due to its ability to dimerize the receptor more frequently, rather than by activation with the soluble form alone (Broudy 1997).

As mentioned before, a feature of SCF is its capacity to synergize with other hematopoietic growth factors. This is important because HSCs respond optimally to a combination of growth factors. It has been shown that SCF activity synergizes with the activity of different factors such as IL-3, GM-CSF and CXCL12 (Gotoh et al. 1996; Mantel et al. 1996; O'Farrell et al. 1996; Christensen et al. 2004). It has been shown that activation with both SCF and CXCL12 generates a potent synergistic affect that enhances migration of LSK cells (Christensen et al. 2004). For example, SCF stimulated LSK cells show a dramatic increase in CXCR4 surface expression (Christensen et al. 2004) and thus, enhancing the cells' response to CXCL12 to mediate migration.

In addition to being a chemotactic agent, SCF promotes cell adhesion mainly by two distinct mechanisms. First, the binding of stromal membrane-bound SCF to c-Kit on HSCs may directly mediate cell attachment. Second, signaling through c-Kit strengthens β_1 integrin valency on the surface of these cells. It has been shown that the activation of the PI3-kinase pathway can mediate adhesion and migration responses through SCF by activating integrins through an inside-out signaling mechanism (Sattler et al. 1997). This adhesion response is an important step for the migration and homing process to occur.

1.5 Uropods and Leading Edges on HSCs

In addition to proper receptor localization, recruitment and efficient activation of signaling events, the migrating cell must have the ability to regulate cytoskeletal changes. For example, during leukocyte recruitment, the first requirement for a cell to initiate migration is the acquisition of a polarized morphology. Chemokines and cytokines mediate polarization by establishing two sub-cellular regions on the cell: the leading edge and the uropod (Serrador et al. 1999). In early studies, Wilkinson described the polarized morphology of leukocytes as similar to that of migrating amoeba: a leading edge followed by the cell body, and the tail or uropod trails behind (Wilkinson 1986). Situated at the rear end of a migrating cell, the uropod is a cytoplasmic projection and is not typically in contact with the matrix. CD44 and CD43 are usually concentrated in the uropod during cell migration (Dustin 2002). During the migration process, a polarization of filamentous actin (F-actin) forms at the leading edge and as a consequence generates structures such as lamellipodia and filopodia. The lamellipodia comes in contact with the matrix and propulsion is achieved by a series of contractions and retractions of the cell. The lamellipodia is comprised of cytoskeletal proteins which include microtubules and microfilaments. Microfilaments composed of F-actin regulate membrane plasticity, which includes cytoskeleton-propelled deformation and protrusion and cell motility (Sanchez-Madrid et al. 1999). The rates of actin polymerization and depolymerization are finely regulated by signaling machinery. This process also regulates the contraction and relaxation to generate a directional force that is required for cell movement.

At the leading edge, most of the actin polymerization is distributed and concentrated in a linear fashion (Sanchez-Madrid et al. 1999; Serrador et al. 1999). Many different receptors are concentrated at the leading edge including VLA-4, VLA-5 and various chemokine receptors.

Human $CD34^+$ mobilized blood stem cell populations have been shown to exhibit uropods and leading edges, similar to the ones observed in migrating leukocytes (Holloway et al. 1999; Francis et al. 2002; Fruehauf et al. 2002). These structures are augmented by exposing the cells to increasing amounts of CXCL12 (Francis et al. 2002). Chemokine induction of actin polymerization is most pronounced within the first one to two minutes after stimulation of CXCR4 (Voermans et al. 2001), which results in the re-distribution of F-actin into large polarized clusters. In migrating cells, the actin is polarized towards the side of the cell facing the CXCL12 gradient. Through a series of active actin polymerization and depolymerization events, extensions of cellular protrusions are generated (i.e. uropods). Thus, varying concentrations of CXCL12 induces a transient but substantial burst of actin polymerization for the migration process.

1.6 The CD34 Protein Family

Due to their similarities such as genomic organization, structural homology and function, we and others have collectively referred to three members: CD34, Podocalyxin and Endoglycan as the CD34 family (Sassetti et al. 1998; Doyonnas et al. 2001; Nielsen et al. 2002; Tan et al. 2006). Members of the family are expressed on different cell types including HSC. All three are transmembrane sialomucins that share several biochemical

motifs including: a heavily decorated N-linked and O-linked glycosylated mucin domain, a disulphide-bonded globular domain, a cytoplasmic tail of 70-80 amino acids with consensus phosphorylation sites for protein kinase C (PKC) and casein kinase II (CKII) and specific C-terminal amino acid PDZ (**P**ost-Synaptic-Density of 95kDa, **D**isc Large Homolog-1, **Z**ona Occuldins-1) docking site-sequences. All three proteins exist in two isoforms: full length transmembrane proteins, and alternatively spliced variants that lack most of the cytoplasmic tail (Nielsen et al. 2002) (Figure 1.4 and Figure 1.5).

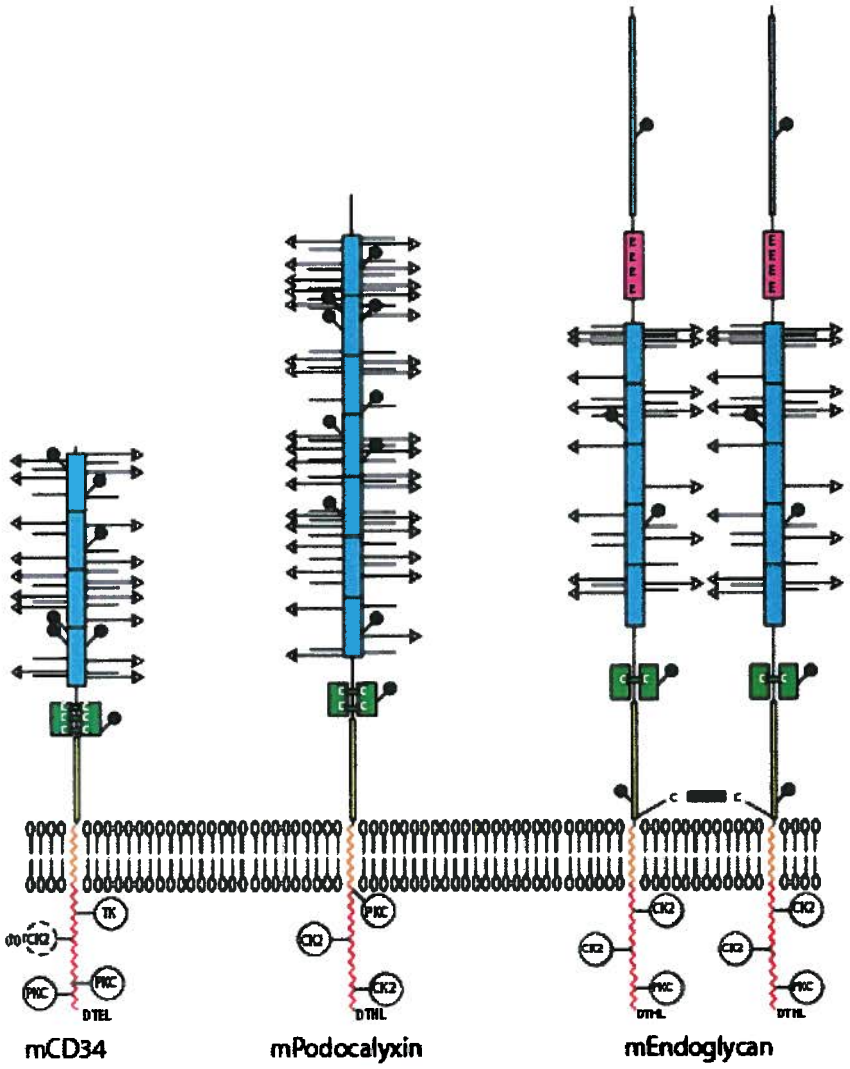


Figure 1.4 Schematic diagram to illustrate structural similarities between CD34, Podocalyxin and Endoglycan.

Schematic drawing of the three CD34 family members. Blue boxes: mucin domains; green boxes, cysteine-rich domains; yellow boxes: stalk domains; black circles: potential N-linked carbohydrates; horizontal bars: potential O-linked carbohydrates; PKC, CK2 and TK: potential phosphorylation sites; DTEL, DTHL potential PDZ-domain docking motif.

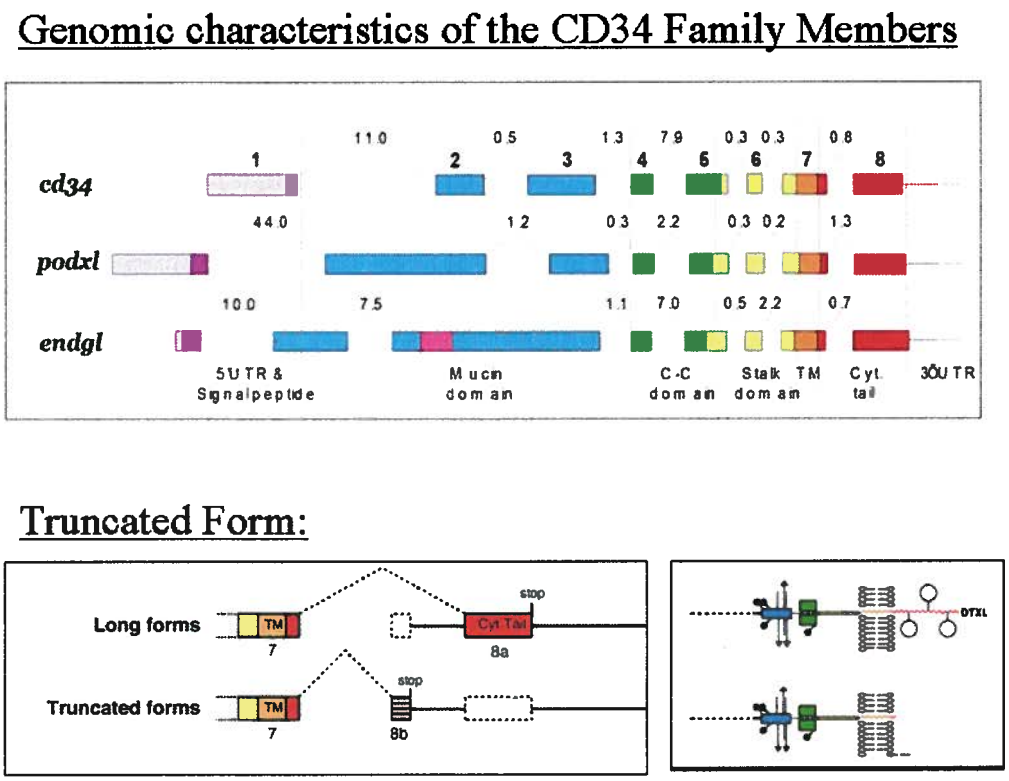


Figure 1.5 Genomic characteristics of the CD34 Family Member and an illustration of its cytoplasmic truncated form.

The CD34 family members have similar numbers of exons and they exist in two isoforms; full length and cytoplasmically truncated form which lack most of the C-terminal tail.

In addition, Podocalyxin, Endoglycan and CD34 share similar genomic organization in that they each have eight exons that encode similar regions of the protein (Figure 1.5) (Furness et al. 2006). Besides HSCs, CD34 family members are also expressed on the luminal surface of vascular endothelia in the lymph nodes known as high-endothelial venules (HEVs) (Baumheter et al. 1993; Puri et al. 1995; Sassetti et al. 1998; Fieger et al.

2003). At the HEV the members function as a ligand for L-selectin (Baumheter et al. 1993; Puri et al. 1995; Sassetti et al. 1998; Sassetti et al. 2000; Fieger et al. 2003).

HEVs are specialized venules that support the recruitment of lymphocytes to the lymphoid tissues (Girard et al. 1995). Despite the members' roles in the HEV, there have been other proposed functions for these proteins. CD34 and Podocalyxin have been shown to function as anti-adhesion molecules in mast cells and kidney podocytes, respectively (Somasiri et al. 2004; Drew et al. 2005; Nielsen et al. 2007).

1.6.1 CD34

CD34 was first discovered as an antigenic epitope detected by monoclonal antibodies specifically to recognize human bone marrow cells (Kohler et al. 1975; Civin et al. 1984). CD34 is the most commonly used marker to enrich for human HSCs from the bone marrow for clinical transplant and research (Verstegen et al. 2003). In the bone marrow, there are two distinct populations of CD34-positive and CD34-negative cells. It is accepted that cells which express the highest levels of CD34 contain the majority of immature hematopoietic progenitor cells (Andrews et al. 1989; Krause et al. 1994).

CD34 is also highly conserved *between* species and thus suggesting evolutionary importance. The highest level of homology is located at exon 8 encoding the cytoplasmic domain (Figure 1.5). Exons 2 and 3 code for the extracellular regions that are heavily glycosylated similar to the leukocyte antigen, CD43, which is also expressed by HSCs with a role in leukocyte adhesion and activation (Killeen et al. 1987).

Previously, our group has shown that CD34 is not only a selective marker of HSCs but is also a marker of murine mature mast cells (Drew et al. 2002). Mast cells are inflammatory cells which contain granules rich in histamines and heparins. They are best known for their role in allergy and anaphylaxis and they play an important role in wound healing and defense against pathogens (Prussin et al. 2003). We have shown that CD34 is the only member of this family expressed by mast cells and we provide data to suggest that it may function as an anti-adhesin. For example, deletion of CD34 in mast cells leads to an enhanced cell aggregation and impaired homing (Drew et al. 2005). This aggregation is reversible by ectopic re-expression of full length CD34. Cell-cell adhesion is blocked most effectively by the naturally-occurring splice variant, which lacks most of cytoplasmic domain (Drew et al. 2005).

To date, only two binding partners were found that associate with the cytoplasmic region of CD34: v-crk sarcoma virus CT10 oncogene homolog-like (CrkL) (Felschow et al. 2001) and protein kinase C – delta (PKC-δ) (Fackler et al. 1990). These two proteins may play a role in modulating CD34 function at the cytoplasmic region.

Although it is important as a HSCs marker, CD34 knock-out mice are viable and fertile (Suzuki et al. 1996). There are no obvious hematopoiesis defects or differences in HSCs mobilization rates into the circulation.

1.6.2 Podocalyxin

Podocalyxin was first identified by Kerjaschki, Sharkey and Farquhar (Kerjaschki et al. 1984) as a 140kDa sialomucin found at the inter-digitating foot processes of podocytes, which are large cells that form a crucial component of the glomerular filtration barrier. The glomerular capillaries ensure that protein and blood remain in the bloodstream and small molecules such as water and ionic salts filter through to produce urine (de Zoysa et al. 2005). We have shown that Podocalyxin deficient mice die perinatally due to improper development of podocytes in the kidneys (Doyonnas et al. 2001). Consequently, this deficiency results in high blood pressure and eventually perinatal death (Doyonnas et al. 2001).

Similar to CD34, Podocalyxin has been shown to play a role as an anti-adhesion molecule. Over-expression of Podocalyxin in Chinese hamster ovary (CHO) and Mardin-Darby canine kidney (MDCK) cells blocks cell aggregation and the proper formation of cell-cell junctions (Takeda et al. 2000). In a study conducted by Somasiri et al., over expression of Podocalyxin in a breast cancer cell line, MCF-7 perturbs proper formation of cell junctions. In addition, Podocalyxin is dramatically up-regulated in a subset of highly *metastatic* human breast cancers (Somasiri et al. 2004) and thus makes it a suitable marker for prognosis.

Podocalyxin is also expressed on murine HSCs but its expression in the hematopoietic compartments during embryogenesis is transient. Essentially, 97% of cells in the yolk sac at E10 are Podocalyxin positive; however, the level of expression gradually decreases

over the next few days of development and seems to increase again at E15 to further gradually decrease until birth (Doyonnas et al. 2005). For the duration of the mouse's adult life, Podocalyxin is expressed only on a rare subpopulation in the bone marrow (e.g. LSK).

Since Podocalyxin expression corresponds to the commencement of hematopoiesis during embryogenesis, we were excited to discover that Podocalyxin proteins play a role in the quality of HSCs engraftment. By performing serial transplantations using wild type bone marrow derived LSK cells segregated by Podocalyxin expression, we found that Podocalyxin positive HSCs tend to engraft lethally irradiated host better than HSCs that lack the protein (Doyonnas et al. 2005). In addition to engraftment, we show that fetal liver derived Podo$^{-/-}$ cells have impaired migration (20-30% less efficient than wild type cells) in an in vivo short-term homing assay (Doyonnas et al. 2005).

To date, NHERF-1 and NHERF-2 are the only known *cytoplasmic* ligands for Podocalyxin. NHERF proteins associate with Podocalyxin at the C-terminal PDZ docking region. Podocalyxin and NHERF-1 are expressed in murine primary LSK cells and in an IL-3 dependent cell line, FDC-P1 (Tan et al. 2006). The work in this thesis investigates Podocalyxin's function in the homing process.

1.6.3 Endoglycan

Endoglycan is structurally and genomically similar to CD34 and Podocalyxin, except that it contains an extra cysteine residue in the stalk domain that presumably allows the molecule to form a disulphide-dependent dimer (Fieger et al. 2003). It is a type I transmembrane protein with a highly conserved cytoplasmic region and has similar tissue expression as the other members (Sassetti et al. 2000). Although it binds L-selectin like CD34 and Podocalyxin, it employs a different type of binding mechanism than the other two (Fieger et al. 2003).

Endoglycan binds to its ligand at its highly acidic amino terminal via two tyrosine residues and an O-linked sialyl Lewis-x (sialyl LeX, sLeX), which is a tetrasaccharide carbohydrate attached to O-glycans on the surface of cells (Takada et al. 1993). O-linked sialyl Lewis-x plays a vital role in cell-cell recognition processes. Endoglycan shares a common cytoplasmic ligand with Podocalyxin – NHERF-1 (Tan et al. 2006). Unlike CD34 and Podocalyxin, very little is known about Endoglycan with only ten articles listed in the Pub Med database as of July 2008.

1.7 NHERF Proteins

Our group has identified NHERF-1 as a ligand for two members of CD34 family: Podocalyxin and Endoglycan (Tan et al. 2006). Briefly, by using both biochemical and mass spectrometry techniques, we determined NHERF-1 as a ligand for Podocalyxin in FDC-P1 cells. The co-association and co-localization of both proteins were dependent on

IL-3 stimulation. NHERF proteins are cytosolic adaptors that possess three domains: two tandem PDZ domains and one Ezrin, Radixin and Moesin (ERM) binding domain. ERM regions bind to ezrin to indirectly link proteins to the actin cytoskeleton (Shenolikar et al. 2004).

PDZ domains are structurally defined domains that specifically recognize C-terminal amino acid sequences of their ligand. There are currently three classes of PDZ binding proteins: Class I specifically recognizes X - S/T – X –Φ; Class II specifically recognizes X - Φ – X – Φ; and Class III recognizes X – D/E/R/K – X – Φ (where X is any amino acid and Φ is any hydrophobic residue) (Doyle et al. 1996; Rubinson et al. 2003). NHERF-1 and NHERF-2 are protein isoforms that share approximately 60% sequence identity overall and 94% identity between defined protein domains.

A key difference between these isoforms is that NHERF-1 has been shown to have more potential phosphorylation sites in mammalian cells than NHERF-2 (Weinman et al. 1998). At least seven phosphorylation sites have been identified in NHERF-1 by mass spectrometry. However, the functional role for these phosphorylation sites is still unclear. Biochemical studies by several groups have established that NHERF proteins bind several types of transmembrane proteins, ion exchangers and cytoskeletal elements (Sabolic et al. 2002; Shibata et al. 2003; James et al. 2004; Tan et al. 2006).

Murine NHERF-1 and NHERF-2 were first discovered as ligands for the Na^+/H^+ exchanger isoform 3 (NHE3). This ion transporter is found in the proximal convoluted

tubule of the kidney (Bomberger et al. 2005) and it is responsible for the homeostasis of sodium and hydrogen ions exchange during blood filtration. We have shown that NHERF-1 is present in the re-populating subset of bone marrow referred to as LSK cells and thus, we speculate that it may have a role in HSC biology (Tan et al. 2006).

Both NHERF-1 and NHERF-2 have been found to indirectly link NHE3 to the cytoskeleton via ERM molecules (Bomberger et al. 2005), which anchor transmembrane proteins indirectly to the cytoskeleton; forming multi-protein complexes with other regulatory proteins (Nawrot et al. 2004). Experiments by Weinman and colleagues show that ezrin functions as a protein kinase A (PKA) anchoring protein and that the ERM-binding domain of NHERF is critical in mediating the phosphorylation and activity of NHE3. Presumably, there is a redundant mechanism for regulating the activity of NHE3 in cells that express both NHERF isoforms (Bomberger et al. 2005).

1.8 FDC-P1: Our Model Cell System

FDC-P1 (*F*actor-*d*ependent *C*ontinuous cell line - *P*aterson Institute-*1*, *F*actor-*d*ependent *c*ell-*P*atterson-*1*) is a cell line generated from the long term culture of marrow cells derived from B6D2F$_1$ mouse strains (C57BL/6 x DBA/2)F$_1$ (Dexter et al. 1980). Long-term cultures were established by flushing the marrow from a single mouse femur, resuspending the cells in medium containing hydrocortisone sodium succinate (facilitates the maintenance *in vitro* hematopoiesis (Greenberger 1978)) and subsequently maintained with WEHI-3 conditioned media containing IL-3. In soft agar colony assays,

FDC-P1 cells mainly display cells with pro-yelomonocytic morphologies and therefore, FDC-P1 are characterized as myelomonocytic progenitors.

FDC-P1 cell system is useful to study HSC biology because it has some of the characteristics, which are representative of multipotent cells: capability to self-renew (under appropriate conditions), and terminally differentiate.

1.9 Hypotheses

Previous to these studies, our research group identified Podocalyxin and NHERF-1 in the hematopoietic cell system. We further confirmed the presence of both proteins in the murine hematopoietic system using FDC-P1 cells, which we will be using as a model system for all *in vitro* experiments.

General hypothesis: Podocalyxin and NHERF-1 proteins are necessary for HSC homing.

Specific hypotheses are:

(N.B. Prior to my commencement as a PhD candidate in Dr. Kelly McNagny's group, he had already identified NHERF-1 as a potential ligand for Podocalyxin by screening chicken hematopoietic cDNA library. My subsequent work is based on its identification.)

1) Association of Podocalyxin and NHERF-1 proteins in FDC-P1 cells is factor dependent.
2) Podocalyxin or NHERF-1 plays a role in adhesion to matrix in FDC-P1 cells.
3) Podocalyxin or NHERF-1 plays a role in chemotaxis of FDC-P1 cells through transwell.
4) Podocalyxin or NHERF-1 plays a role in uropod formation in FDC-P1 cells.

1.10 Research Questions

The following are specific research questions which are addressed in this thesis.

Specific hypothesis 1: Association of Podocalyxin and NHERF-1 proteins in FDC-P1 cells is factor dependent.

A) Does Podocalyxin associate with NHERF-1 via its cytoplasmically conserved PDZ docking domain?
B) Is Podocalyxin and NHERF-1 association and subsequent re-localization at the cell surface membrane dependent on IL-3 stimulation?
C) Is Podocalyxin re-localization at the membrane NHERF-1 dependent?

Specific Hypothesis 2: Podocalyxin or NHERF-1 plays a role in adhesion to matrix in FDC-P1 cells.

A) Does the lack of Podocalyxin or NHERF-1 lead to a deficiency in adhesion to extracellular matrix such as fibronectin?
B) Does the lack of Podocalyxin or NHERF-1 lead to an adhesion defect to M210B4 stromal cells?

Specific Hypothesis 3: Podocalyxin or NHERF-1 plays a role in chemotaxis of FDC-P1 cells through transwell.

A) Does a Podocalyxin or NHERF-1 deficiency lead to a migration defect towards a chemoattractant gradient such as CXCL12 and SCF?

B) Does reduction in Podocalyxin and NHERF-1 expression lead to an impaired CXCR4 expression?

C) Does Podocalyxin associate with CXCR4?

D) Two key events that take place during migration (which will be addressed in this thesis) are the initiation of key signaling pathways and changes in cell morphology. Activation of AKT is one of the key molecules in the initiation of migration via CXCL12 and SCF. Is the phosphorylation of AKT affected as a result of shPodocalyxin or shNHERF-?

Specific Hypothesis 4: Podocalyxin or NHERF-1 plays a role in uropod formation in FDC-P1 cells.

A) Does a deficiency in Podocalyxin or NHERF-1 expression lead to a decrease in uropod or uropod-like structures?

   i) at steady state

   ii) after stimulation on fibronectin.

B) Does a loss of Podocalyxin affect cell size?

C) Do uropod-expressing shPodocalyxin cells lead to a migration defect towards a chemoattractant gradient in a lateral migration assay?

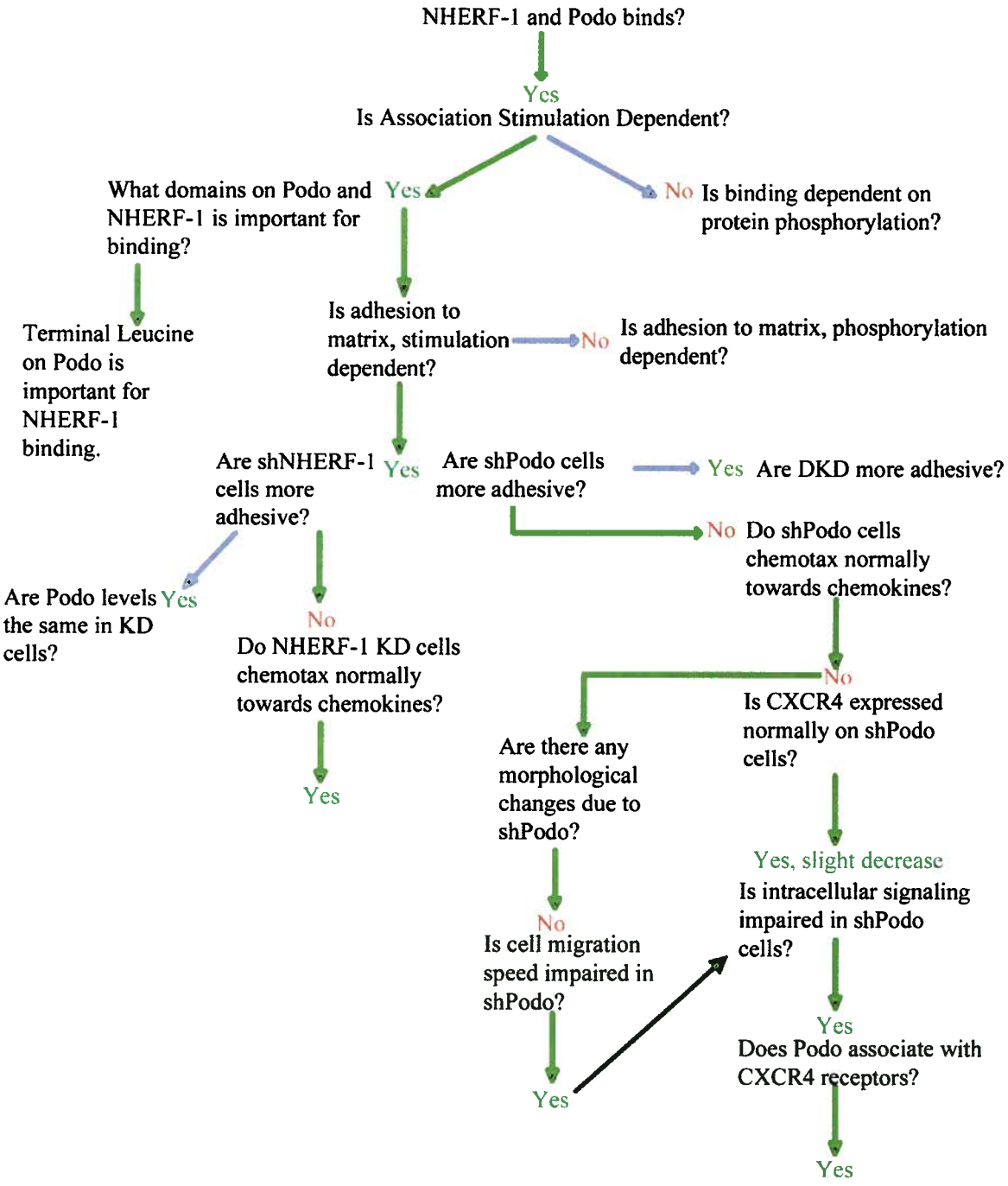


Figure 1.6 Experimental "road map" for investigation of the function of Podocalyxin and NHERF-1 in HSCs adhesion and migration.

This is a road map, which I used during the course of studies that helped me form testable research questions. Green arrows: decision route; Gray arrows: plan B; KD: knock-down; DKD: double knock-down.

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Chapter 2 : Na^+/H^+ Exchanger Regulatory Factor-1 is a Hematopoietic Ligand for a Subset of CD34 Stem Cell Surface Proteins[1]

2.1 Introduction

CD34, Podocalyxin and Endoglycan comprise a family of hematopoietic and vascular-restricted sialomucins (Sassetti et al. 2000; Doyonnas et al. 2001; Nielsen et al. 2002). CD34 is the highest profile member of this family due to its widespread use as a marker of hematopoietic stem cells (HSC) (Krause et al. 1996; Lanza et al. 2001). The members of this family share several biochemical motifs including: a heavily-glycosylated mucin domain, a disulfide-bonded globular domain, membrane proximal stalk domain, transmembrane region and a highly conserved cytoplasmic tail of 70-80 amino acids (aa) with consensus phosphorylation sites for PKC and CKII (Doyonnas et al. 2001; Nielsen et al. 2002). Their classification as a gene family is supported by a similar exon/intron structure in their respective genomic loci and similar patterns of alternative splicing that give rise to mRNAs, encoding protein isoforms that lack or maintain the bulk of the cytoplasmic domain(Nakamura et al. 1993; McNagny et al. 1997; Doyonnas et al. 2001).

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All three proteins are expressed on HSC/progenitors but their function on these cells has remained enigmatic (Krause et al. 1996; McNagny et al. 1997; Sassetti et al. 1998; Hara et al. 1999; Sassetti et al. 2000; Kerosuo et al. 2004; Doyonnas et al. 2005). When expressed by specialized endothelial cells in the lymph nodes, called High Endothelial Venules (HEV) (Baumheter et al. 1993; Sassetti et al. 1998; Sassetti et al. 2000), CD34, Podocalyxin and Endoglycan are modified with an unusual type of carbohydrate moiety that permits their recognition by a leukocyte-specific carbohydrate-binding receptor called L-selectin (Rosen 2004). Leukocyte- or **L**-selectin is expressed by newly formed lymphocytes and is used by these cells to bind to the appropriately glycosylated CD34 family members expressed on HEV. This initial binding is the first key step in a process that culminates in leukocyte immigration into the peripheral lymph nodes (Rosen 2004).

Although this is a well-documented function for CD34-type proteins on HEV, several observations suggest that this pro-adhesive function is an important exception rather than a general rule. Most notably, the binding of CD34-type proteins by L-selectin is highly dependent on the modification of these proteins with HEV-specific carbohydrate moieties. These modifications, however, have not been detected on virtually any other vascular endothelial cell type or on hematopoietic cells.

In contrast to their pro-adhesive function on HEV, it has been demonstrated that ectopic expression of Podocalyxin, in CHO or MDCK cells leads to a block in cell aggregation and in cell-cell junction formation, respectively (Takeda et al. 2000). Similarly, we have

shown that Podocalyxin expression is naturally up-regulated on a subset of human breast carcinomas and that this leads to disruption of cell-cell junctions (Somasiri et al. 2004). Finally, deletion of the Podocalyxin-encoding gene in mice leads to increased adhesion between kidney podocytes and this results in a lack of urine production, kidney failure, and perinatal death (Doyonnas et al. 2001).

On hematopoietic cells, too, there is recent evidence that these molecules may function as anti-adhesins. We have shown that CD34 is a selective marker of murine mast cells (Drew et al. 2002) and that deletion of CD34 or the related mucin, CD43, leads to enhanced aggregation of mast cells and impairment in mast cell homing due to enhanced adhesion (Drew et al. 2005). This block in adhesion is reversible by the ectopic re-expression of CD34 and, intriguingly, adhesion is blocked most effectively by the naturally-occurring splice-variant of CD34 that lacks most of the cytoplasmic domain (Drew et al. 2005).

In summary, the data suggest that although CD34-type proteins can function as pro-adhesive molecules when appropriately glycosylated, under most conditions they function as molecular "Teflon" to block non-specific adhesion and cell-cell junction formation. The data also suggest that the cytoplasmic domain may fine-tune this effect (Drew et al. 2005).

The observation that isoforms of CD34-type proteins lacking most of the cytoplasmic domain are more effective in blocking cell adhesion, has led us to speculate that the

members of this family can regulate their anti-adhesive properties dynamically by association with cytoskeletal elements that enhance or inhibit their localization at the sites of cell-cell or cell-matrix attachment. A number of observations in the literature are consistent with this hypothesis. It has been demonstrated that activation of the PKC pathway in cells leads to a rapid phosphorylation of the cytoplasmic tail of CD34 (Fackler et al. 1990; Sutherland et al. 1992). It has also been shown that activation of vascular endothelial cells leads to the re-localization of CD34 on these cells (Fackler et al. 1992; Delia et al. 1993). Similarly, it has been shown that ectopic expression of Podocalyxin in MDCK cells leads to an apical localization of the molecule, activation of RhoA and polymerization of actin at the sites of expression (Schmieder et al. 2004). These effects are likely to be regulated by cytosolic binding proteins. But, to date, the only known ligands for members of this family are the cytoplasmic adaptor protein, CrkL, which binds to the membrane proximal domain of CD34 (Felschow et al. 2001), and the podocyte-specific PSD-95/Drosophila Discs large/ZO-1 (PDZ) and Ezrin/Radixin/Moesin (ERM) domain-containing protein, NHERF-2, which binds to the tail of Podocalyxin (Takeda et al. 2001; Sabolic et al. 2002; Liedtke et al.).

As a first step toward understanding the role of cytoplasmic binding proteins in the regulation of CD34-type protein function in hematopoietic cells, we have used genetic screens and biochemical approaches to identify hematopoietic ligands for the cytoplasmic tail of Podocalyxin. Here we identify NHERF-1, a homologue of NHERF-2, as a binding protein for Podocalyxin and Endoglycan, but not CD34. We show that Podocalyxin and NHERF-1 are co-expressed in normal HSC and that they co-localize upon Podocalyxin

clustering. Furthermore, this clustering is enhanced by cytokine treatment. Our results suggest that NHERF-1 is a ligand for a subset of CD34-type proteins and that it may play a role in regulating their anti-adhesive properties in HSCs.

2.2 Materials & Methods

2.2.1 Cells and Media

FDC-P1 cells (Dexter et al. 1980) were maintained in RPMI (Gibco; Ont. Canada; http://www.invitrogen.com), 2mM L-glutamine, penicillin/streptomycin, 10% fetal bovine serum and WEHI-3B conditioned media containing interleukin -3. Bone marrow cells were obtained by flushing femurs and tibias of 8-10 week old C57BL/6 mice, with room temperature PBS using a 25-gauge needle. Thymus, spleen, Peyer's patches and lymph node were dispersed into single cell suspensions by passing through a 45μm nylon cell strainer.

2.2.2 Antibodies

Rabbit anti-NHERF-1 antibody ab3452 (Abcam; USA; http://www.abcam.com) was used for all fluorescent assays and rabbit anti-NHERF-1 antibodies APZ-006 (Alomone Laboratories; Israel; http://www.alomone.com) and ab3452 were used for immunoblot analyses. Rat anti-mouse Podocalyxin antibody MAB1556 (R&D; MN, USA; http://www.rndsystems.com) was used for all staining, immunoprecipitation and immunoblot studies. Secondary antibodies were goat anti-rabbit AlexaFluor 488

(Molecular Probes; Ont. Canada; http://www.invitrogen.com), goat anti-rat AlexaFluor 568 (Molecular Probes Ont. Canada; www.invitrogen.com) and goat anti-rat PE (Pharmingen; CA, USA; http://www.bdbiosciences.com/pharmingen). Isotype controls were rabbit IgG (H&L) (Vector Laboratories; CA, USA; http://www.vectorlabs.com) and rat IgG2a (Cedarlane; Ont. Canada; http://www.cedarlanelabs.com). Directly conjugated lineage-specific antibodies Terr119, CD3, Gr-1, Mac-1 and B220 and Sca-1 and c-Kit were purchased from R&D Laboratories.

2.2.3 Peptides

Peptides were made and biotinylated by the in-house peptide facility at the Biomedical Research Centre.

Peptides were:

1) **Podo-1-73**, (C-terminal 73 aa, avian thrombomucin; accession #CAA74311(McNagny et al. 1997)):

HQRFSQKKSQQRLTEELQTMENGYHDNPTLEVMETGSEMQEKKVNLNGELGDS WIVPLDTIMKEDLEEEDTHL.

2) **Podo-15-73**, (C-terminal 58 aa):

ELQTMENGYHDNPTLEVMETGSEMQEKKVNLNGELGDSWIVPLDTIMKEDLEE EDTHL.

3) **Podo-53-73**, (C-terminal 20 aa):

WIVPLDTIMKEDLEEEDTHL.

4) **Podo-53-72,** (C-terminal 20 aa, less the terminal leucine):

WIVPLDTIMKEDLEEEDTH.

5) **Endo**, (C-terminal 26 aa, murine Podocalyxin-like 2; accession #AAH33384(Strausberg et al. 2002)):

SSWSALMGSKRDPEDSDVFEEDTHL.

6) **CD34**, (C-terminal 72 aa, murine CD34; accession #NP_598415 (Brown et al. 1991):

RRSWSPTGERLGEDPYYTENGGGQGYSSGPGASPE

TQGKANVTRGAQENGTGQATSRNGHSARQHVVADTEL.

2.2.4 Phage Screens

Phage screens for Podocalyxin-binding proteins were performed essentially as described previously (Li et al. 2002). Peptides Podo-1-73, Endo, and CD34 were used to probe an avian early hematopoietic progenitor library (Graf et al. 1992; McNagny et al. 1996; McNagny et al. 1997). Briefly, phage-infected XL-1 Blue MRF *E.coli* were plated on 30mm LB plates at a density of 20,000 plaques/plate. As visible plaques appeared, IPTG (Fermentas; Ont. Canada; www.fermentas.com)-soaked nitrocellulose membranes (Bio-rad; Ont. Canada; http://www.bio-rad.com) were overlaid and allowed to incubate for 8-12 hours. Filters were washed 4x 20 minutes (0.1% Triton-X/PBS), and blocked overnight at 4°C (2% bovine serum albumin/PBS/0.02% sodium azide).

Biotinylated peptides (25pmol/ml in wash buffer) were complexed with streptavidin-alkaline phosphatase (SAP, 1μg/ml) for 20 minutes prior to incubation with filters in blocking buffer. Biotin/SAP complexes (1μg/μl) were added as a non-specific blocking reagent. After overnight incubation at 4°C on an orbital shaker, the filters were washed 4x 15 minutes prior to 5 minutes incubation with SAP developing buffer (Roche; Quebec,

Canada, www.roche-applied-science.com). Filters were dried on Whatman 3MM paper, positive plaque lifts were aligned and phage plugs removed and transferred to microfuge tubes containing 500µl of SM buffer and 4% chloroform. Each plug was subcloned, re-screened and purified phagemids were excised *in vivo* using ExAssist protocols recommended by the manufacturer (Stratagene; CA, USA; http://www.stratagene.com), prior to automated sequencing (Lone Star Labs; Texas, USA; http://www.lslabs.com).

2.2.5 Confocal Microscopy and Flow Cytometry

Immunofluorescent staining was performed as described previously (McNagny et al. 1997). For intracellular staining cells were fixed with 4% PFA at room temperature for 15 minutes, washed 4x (1% BSA/PBS), permeabilized with 0.1% Triton/PBS for 15 minutes, washed 4x and blocked (1%BSA/10% goat serum/PBS) for 30 minutes. These were then incubated with secondary alone, isotype control or primary antibody for 30 minutes, washed 4x, incubated with FACS buffer or secondary antibody for 30 minutes, washed 4x and analyzed by flow cytometry (FACSCalibur, Beckton Dickinson). For confocal microscopy cells were re-suspended in Fluormount G, (Southern Biotechnologies; AL USA; www.southernbiotech.com) prior to imaging on a confocal microscope (Biorad Radiance 2000, Nikon Eclipse TE300 microscope with MaiTai Sapphire laser, 60x objective, 2.5x zoom, 166 lines/second, BioRad Lasersharp 2000 software or on an Olympus FluoView 1000 system, Olympus IX81 microscope, 60x objective, 1.7x zoom, 10µs/pixel using FluoView 1000 software).

2.2.6 Cell Stimulation, Counting and Analysis

FDC-P1 cells were IL-3-starved for 2 hours before stimulation with IL-3 or 100nM of phorbol 12-myristate 13-acetate (PMA) for 10, 20, 30, 60, or 120 minutes prior to staining. After staining, eight random fields were captured from each time point to assess Podocalyxin distribution in the plasma membrane. Three cell phenotypes were present: punctate, capped and global staining of Podocalyxin. Three counts of 100-250 cells each were made for every time point, and the average percentage of each cell phenotype was obtained.

2.2.7 Affinity Purification and Mass Spectrometry

All washes and buffers were at 4°C, centrifuge steps were conducted at 2000rpm for 30 seconds in a bench top microfuge (Hoeffer). Streptavidin-sepharose resin (Amersham Biosciences; Que. Canada; http://www.amershambiosciences.com), 25µL bed volume, was pre-equilibrated in TBS wash buffer (25mM Tris pH7.9, 138mM NaCl, 2.7mM KCl) plus 0.15% NP-40, 6% glycerol, 66nM EDTA, 500nM $MgCl_2$, 1mM PMSF, 1x Protease inhibitors (Sigma). 25µg of each peptide were incubated with 25µL of streptavidin-sepharose (Amersham Biosciences) on a rotator for 30minutes in 1.5mL of this buffer. 3.5mg of whole cell extract (TBS, 0.5% NP-40, 20% glycerol, 200nM EDTA, 1.5mM $MgCl_2$, 1mM PMSF, 1x Protease inhibitors) was diluted to the same concentration as wash buffer with TBS and pre-cleared for 30 minutes against 25µL of streptavidin-sepharose. Unbound peptide was removed with 500µL washes (3x) of wash buffer. Pre-cleared lysate was added to peptide binding reactions and incubated for 120 minutes on a rotator. Non-specific proteins were removed by washing the resin bed 7x with wash

buffer. Bound material was eluted by boiling in SDS-loading buffer, and specific interactors were identified by Coomassie staining of SDS-PAGE, in-gel tryptic digest and LC-MS/MS.

2.2.8 Immunoblotting, Affinity Purification and Immunoprecipitation

Immunoblotting was conducted using standard protocols (Drew et al. 2002) with antibodies at 1μg/ml in 1% BSA (ab3542) or 5% non-fat milk (MAB1556) in TBS-T overnight. Affinity purification was carried out essentially as above except 5μg of peptide were used for each purification along with 200μg of whole cell extract. Immunoprecipitation was performed using standard techniques.

2.3 Results

2.3.1 Identification of Cytoplasmic Podocalyxin binding partners

Cytoplasmic domains of CD34-type proteins exhibit a remarkably high degree of sequence conservation, which could reflect the binding of highly conserved intracellular ligands (McNagny et al. 1997; Doyonnas et al. 2001; Lanza et al. 2001). To identify these potential ligands, we screened an early hematopoietic progenitor cDNA expression library cloned into lambda phage (McNagny et al. 1996) for binding partners to the cytoplasmic tail of Podocalyxin. Briefly, a 73 aa peptide was synthesized with an N-terminal biotin affinity-tag and incubated with immobilized proteins from lambda phage

plaques, and filter-bound peptides were detected using streptavidin-coupled alkaline phosphatase (SAP) and a chromogenic substrate as described previously (Li et al. 2002) (Figure 2.1). In our initial screen of $1x10^6$ clones we identified several reactive phage plaques, but only one was consistently reactive after several rounds of screening. This clone was sequenced and found to encode the C-terminal 232 aa of the tandem PDZ-domain-containing protein, NHERF-1 (Weinman et al. 1997) (Figure 2.1).

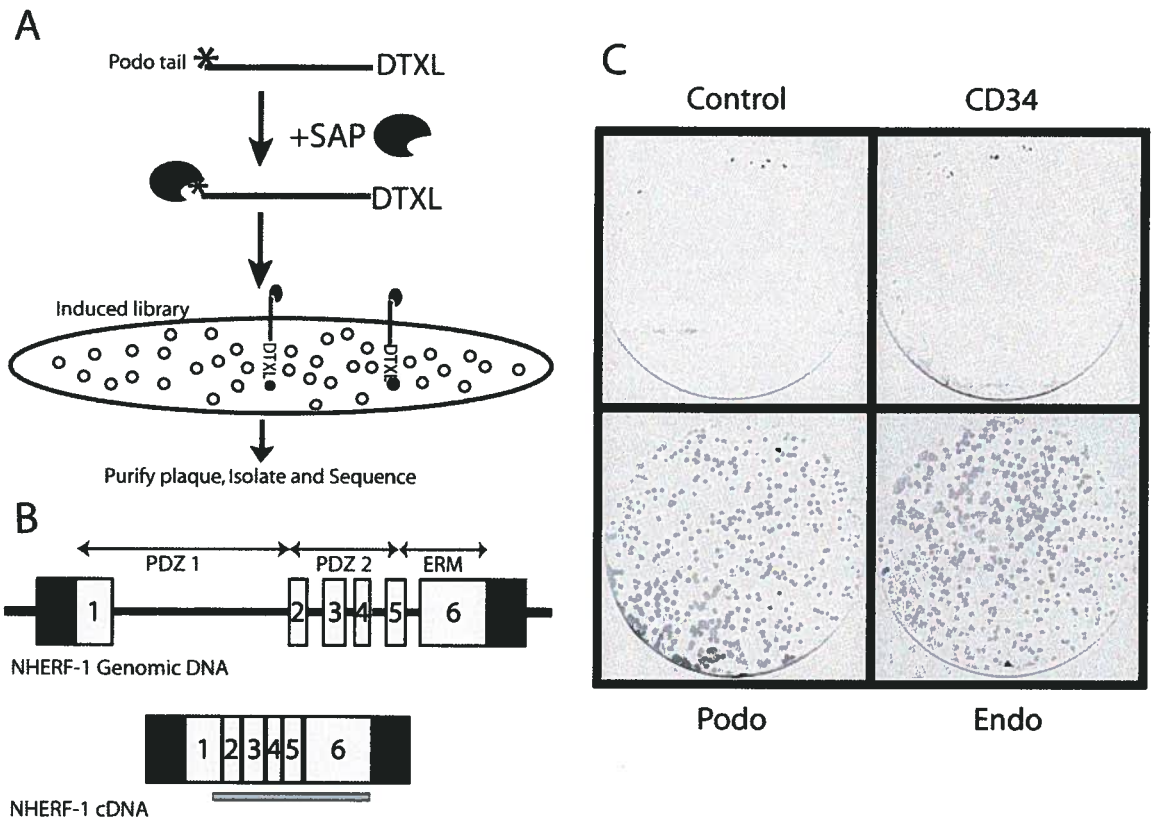

Figure 2.1 Phage screen identifying NHERF-1 as ligand for Podocalyxin and Endoglycan.

A) Schematic of library screening strategy for identification of Podocalyxin-binding proteins. Biotinylated peptides corresponding to the intracellular domain of avian Podocalyxin were complexed with SAP and incubated with phage-expressed cDNAs immobilized on nylon filters (see Materials and Methods). Positive plaques were isolated, purified and cDNA inserts were sequenced. Sequencing data suggest that one clone, 1-56#2, is a 5'-truncated version of avian NHERF-1 encoding 232 of the 333 aa full length NHERF-1. B) Schematic structure of the murine NHERF-1 gene. NHERF-1 contains six exons (large boxes) and is made up of two tandem PDZ binding domains and an ERM domain. White boxes represent coding sequences whereas black boxes denote untranslated exonic sequences. The grey line indicates homologous region of NHERF-1 contained within the avian cDNA clone of phage 1-56#2). C) NHERF-1 binds Podocalyxin and Endoglycan but not CD34. Purified phage were plated and screened for the ability to bind to the C-terminal sequences of CD34, Podocalyxin, Endoglycan or a control peptide encoding the CXCL12 molecule.

To determine whether this clone also had affinity for other members of the CD34 family, we tested its ability to bind peptides corresponding to the cytoplasmic domains of CD34 and Endoglycan or to CXCL12 as an irrelevant control (Figure 2.1C). NHERF-1-expressing phage plaques bound to the tails of Podocalyxin and Endoglycan but, not to CD34. It is noteworthy that the tails of Endoglycan and Podocalyxin show a much higher degree of sequence similarity to each other than they do to CD34. This is also true of the very C-terminal sequence of the proteins, which contain a putative docking site for NHERF-type PDZ-domains (Takeda et al. 2001; Li et al. 2002). In Podocalyxin and Endoglycan this C-terminal docking sequence is DTHL whereas in CD34 it is DTEL.

2.3.2 CD34-type proteins bind NHERF-1 from hematopoietic progenitor cell extracts.

To validate the specificity of NHERF-1 for a subset of CD34-type proteins and to search for additional interactors that may not have been identified in the phage screen, we affinity-purified CD34-family binding proteins from progenitor cell extracts and identified them via mass spectrometry. As a source of binding proteins we chose the IL-3-dependent hematopoietic murine progenitor cell line FDC-P1 since this line has been well studied (Dexter et al. 1980) and expresses high endogenous levels of Podocalyxin (unpublished results). Binding proteins from FDC-P1 lysates were affinity-purified using synthetic peptides corresponding to the cytoplasmic tails of CD34, Podocalyxin and Endoglycan. Affinity-purified proteins were eluted, separated by SDS-PAGE, and

resolved bands of interest were excised and subjected to in-gel tryptic digestion followed by LC-MS/MS.

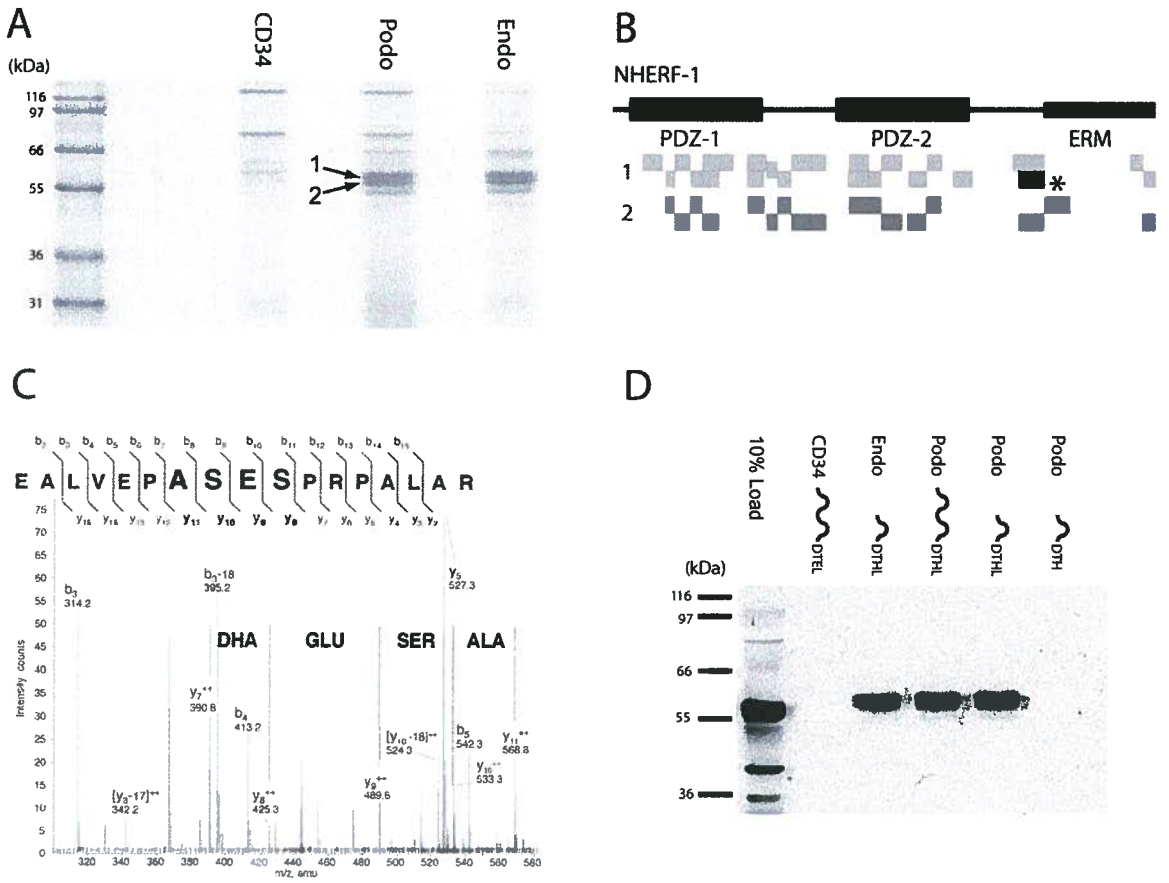

Figure 2.2 Endogenous NHERF-1 interacts with Podocalyxin and Endoglycan but not CD34.

A) Whole cell lysates from FDC-P1 were affinity-purified using biotinylated CD34 family peptides on strepavidin-sepharose resin. After extensive washing, proteins were eluted by boiling in SDS load buffer and resolved by SDS-PAGE. The bands indicated (1 and 2) were excised, subjected to in-gel tryptic digestion and analyzed by LC-MS/MS.

B) Schematic showing the NHERF-1 protein and the peptides covering the corresponding regions identified by mass spectrometry. Shown with * (and in black) is the phosphorylated peptide identified by mass spectrometry, shown in 2C and Table 1.

C) Partial spectra from tandem MS/MS sequencing of the triple-charged phosphorylated peptide EALVEPASESPRPALAR (m/z 624.98) shows phosphorylated residue to be Ser275. Double-charged y ion series containing dehydroalanine (Dha) in position 275

indicates a 98Da, beta-elimination, neutral loss of phosphoric acid. The boundary of the neutral loss is coincident with phosphorylated Ser275 and unmodified Ser273.

D) Specificity of NHERF-1 for CD34 family members. Proteins that bind to the CD34 family C-terminal peptides were affinity-purified, resolved by SDS-PAGE, blotted to nylon membranes and NHERF-1 reactive proteins were identified via Western blotting.

Table 1 shows the resulting mass spectrometry analyses of two excised bands shown in Figure 2.2A. Both bands (22 and 16 independent peptide analyses in two separate experiments, Figure 2.2B) correspond to murine NHERF-1, confirming that NHERF-1 interacts specifically with both Podocalyxin and Endoglycan but not CD34. Since NHERF-1 has been reported to undergo cell-cycle-dependent phosphorylation(He et al. 2001) and contains numerous potential phosphorylation sites, one possible difference between the higher and lower molecular weight isoforms we detected is that they correspond to phosphorylated and non-phosphorylated forms of the protein. This notion is supported by mass spectrometry data that reveals a mass sequence consistent with phosphorylation of Ser275 (Table 1 and Figure 2.2B), which has not been previously reported. Phosphorylation of this residue was confirmed by tandem MS/MS sequencing (Figure 2.2C).

Although this phosphorylation is naturally occurring, it is not required for the binding of NHERF-1 to Podocalyxin and Endoglycan peptides since these molecules bind NHERF-1 in phage plaques, which lack these modifications. In summary, both phage screens and biochemical analyses suggest that NHERF-1 is a *bona fide* hematopoietic ligand for a subset of CD34-type proteins.

Table 1. Peptide sequences obtained by LC-MS/MS and matched to murine NHERF-1					
Band 1					
Amino Acid Position	Observed mass/charge	Expected Mass	Calculated Mass	Change in Mass	Sequence/Modification
20-32	471.57	1411.69	1411.66	0.03	GPNGYGFHLHGEK
35-40	360.21	718.41	718.41	0	VGQFIR
41-50	513.79	1025.56	1025.54	0.02	LVEPGSPAEK
51-58	394.72	787.43	787.42	0.01	SGLLAGDR
59-69	615.33	1228.65	1228.63	0.02	LVEVNGENVEK
59-78	574.31	2293.2	2293.17	0.03	LVEVNGENVEKETHQ QVVSR
88-98	643.33	1284.64	1284.66	-0.01	LLVVDPETDER
88-100	509.63	1525.88	1525.84	0.04	LLVVDPETDERLK
101-107	386.76	771.51	771.5	0.01	KLGVSIR
108-116	381.21	1140.62	1140.61	0	EELLRPQEK
117-139	799.03	2394.07	2394.02	0.05	SEQAEPPAAADTHEAG DQNEAEK
154-167	511.93	1532.77	1532.74	0.03	KGPNGYGFNLHSDK
155-167	469.23	1404.67	1404.64	0.03	GPNGYGFNLHSDK
168-175	311.52	931.55	931.52	0.02	SKPGQFIR
176-189	692.84	1383.67	1383.66	0.01	AVDPDSPAEASGLR
194-205	667.83	1333.65	1333.64	0.01	IVEVNGVCMEGK
194-205	675.82	1349.62	1349.63	-0.02	IVEVNGVCMEGK / Oxidation (M)
206-215	527.3	1052.58	1052.56	0.02	QHGDVVSAIK
223-235	528.29	1581.86	1581.83	0.03	LLVVDKETDEFFK
Table 1. Peptide sequences obtained by LC-MS/MS and matched to murine NHERF-1					
262-282	778.06	2331.17	2331.12	0.04	ESSREALVEPASESPRP ALAR/ Phospho (S)

266-282	598.33	1791.98	1791.95	0.03	EALVEPASESPRPALAR
266-282	936.96	1871.91	1871.91	-0.01	EALVEPASESPRPALAR Phospho(S)
339-347	559.8	1117.59	1117.53	0.05	RAPQMDWSK
348-355	482.76	963.51	963.5	0.01	KNELFSNL
Band 2					
35-40	360.21	718.42	718.41	0	VGQFIR
35-40	360.21	718.42	718.41	0	VGQFIR
41-50	513.78	1025.55	1025.54	0.01	LVEPGSPAEK
51-58	394.72	787.43	787.42	0.01	SGLLAGDR
59-69	615.32	1228.63	1228.63	0	LVEVNGENVEK
88-98	643.34	1284.67	1284.66	0.02	LLVVDPETDER
101-107	386.76	771.5	771.5	0	KLGVSIR
108-116	381.22	1140.62	1140.61	0.01	EELLRPQEK
117-139	799.04	2394.09	2394.02	0.07	SEQAEPPAAADTHEAG DQNEAEK
155-167	469.23	1404.67	1404.64	0.02	GPNGYGFNLHSDK
168-175	466.77	931.52	931.52	-0.01	SKPGQFIR
176-189	692.85	1383.69	1383.66	0.03	AVDPDSPAEASGLR
194-205	667.83	1333.65	1333.64	0.02	IVEVNGVCMEGK
194-205	675.84	1349.66	1349.63	0.03	IVEVNGVCMEGK / Oxidation(M)
206-215	527.29	1052.57	1052.56	0.01	QHGDVVSAIK
266-282	598.33	1791.97	1791.95	0.02	EALVEPASESPRPALAR
283-299	891.41	1780.8	1780.76	0.04	SASSDTSEELNSQDSPK
348-355	482.77	963.52	963.5	0.02	KNELFSNL

To further characterize the interaction between NHERF-1 and the cytoplasmic tails of the CD34 family members, biotinylated peptides corresponding to the final 20 amino acid of

Podocalyxin, as well as these final 20 aa minus the C-terminal leucine, were evaluated for their ability to bind NHERF-1 from FDC-P1 extracts by small-scale affinity purification and anti-NHERF-1 immunoblotting. The C-terminal 20 aa of Podocalyxin and Endoglycan were sufficient for NHERF-1 binding, while truncation of the C-terminal leucine residue ablated binding of NHERF-1, consistent with previous reports suggesting the importance of this residue in the recognition of proteins by PDZ-domains (Takeda et al. 2001; Li et al. 2002)

2.3.3 NHERF-1 associates with Podocalyxin *in vivo*

The interaction of NHERF-1 with Podocalyxin was confirmed using two additional methods: co-localization via confocal microscopy and direct co-immunoprecipitation from cell lysates. FDC-P1 cells were surface stained for Podocalyxin, and then cytoplasmically stained for NHERF-1.

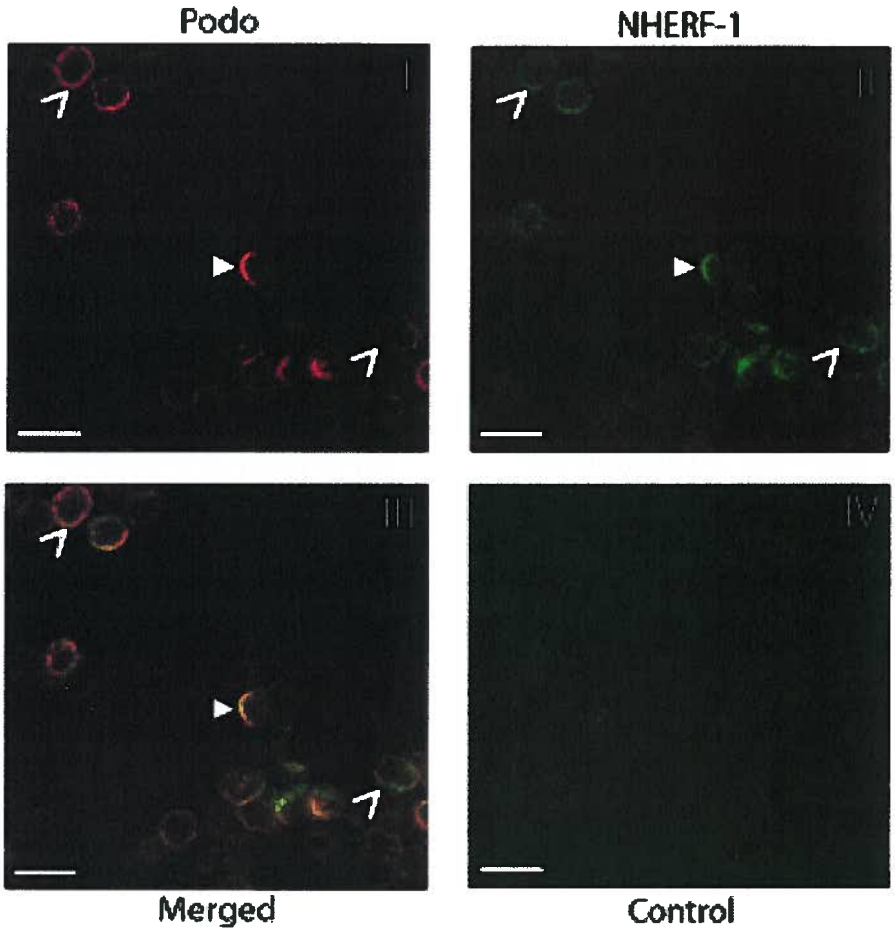

Figure 2.3 NHERF-1 co-localization with Podocalyxin in FDC-P1.

FDC-P1 cells were fixed, stained with NHERF-1 and Podocalyxin antibodies prior to confocal microscopy analysis. Open arrows indicate cells with global expression of Podocalyxin and closed arrows show more localized expression of Podocalyxin on one side of the cell. Scale bars, 10um.

As shown in Figure 2.3, although most FDC-P1 cells express both Podocalyxin (panel I) and NHERF-1 (panel II), only a subset of these cells exhibited strong co-localization of these antigens (panel III). The strongest co-localization correlated with polarized capping of Podocalyxin on the cell membrane; cells uniformly expressing Podocalyxin on their surface showed only weak co-localization, whereas cells displaying asymmetric

localization of Podocalyxin on their surface showed high overlap with NHERF-1. Since FDC-P1 cells require IL-3 to proliferate, capping of Podocalyxin on a subset of these cells may reflect cells that have been more potently activated with cytokines. To test this hypothesis, we performed confocal analyses of Podocalyxin distribution in the plasma membrane of IL-3-starved or in IL-3-stimulated cells (Figure 2.4). The majority of IL-3-starved cells displayed a uniform "halo" of Podocalyxin and only rarely, could cells be found with Podocalyxin capped on one pole (Figure 2.4, 0 hours). Within minutes of IL-3-stimulation Podocalyxin capping increased and this correlated with NHERF-1 co-localization and reached a steady state maximum at 30 to 60 minutes.

To further explore this activation-dependent re-localization of Podocalyxin, we tested whether it was specific to cytokine signaling (Jak/Stat pathway) or could be mimicked by PMA-stimulation as an activator of protein kinase C (PKC) pathways. PMA failed to induce strong capping of Podocalyxin and gave reduced co-localization with NHERF-1 when compared with IL-3-stimulation. Instead, PKC activation led to the redistribution of Podocalyxin into a more "punctate" or "speckled" pattern on the surface of these cells (Figure 2.4B). This reached a maximum after 30 minutes and then gradually declined to the background levels.

To further confirm that stimulation of the PKC and Jak/Stat pathways results in the differential association of Podocalyxin with NHERF-1, we immunoprecipitated Podocalyxin-bound complexes using Podocalyxin antibodies after: 1) IL-3-starvation for two hours, 2) IL-3-stimulation for one hour or 3) PMA-stimulation for one hour. As a

specificity control, lysates from the Podocalyxin-negative cell line, FD5, were precipitated. Endogenous NHERF-1 co-immunoprecipitated weakly with Podocalyxin in IL-3-starved cells and this was only mildly enhanced by PMA treatment, but was potently enhanced by stimulation with IL-3 (Figure 2.4).

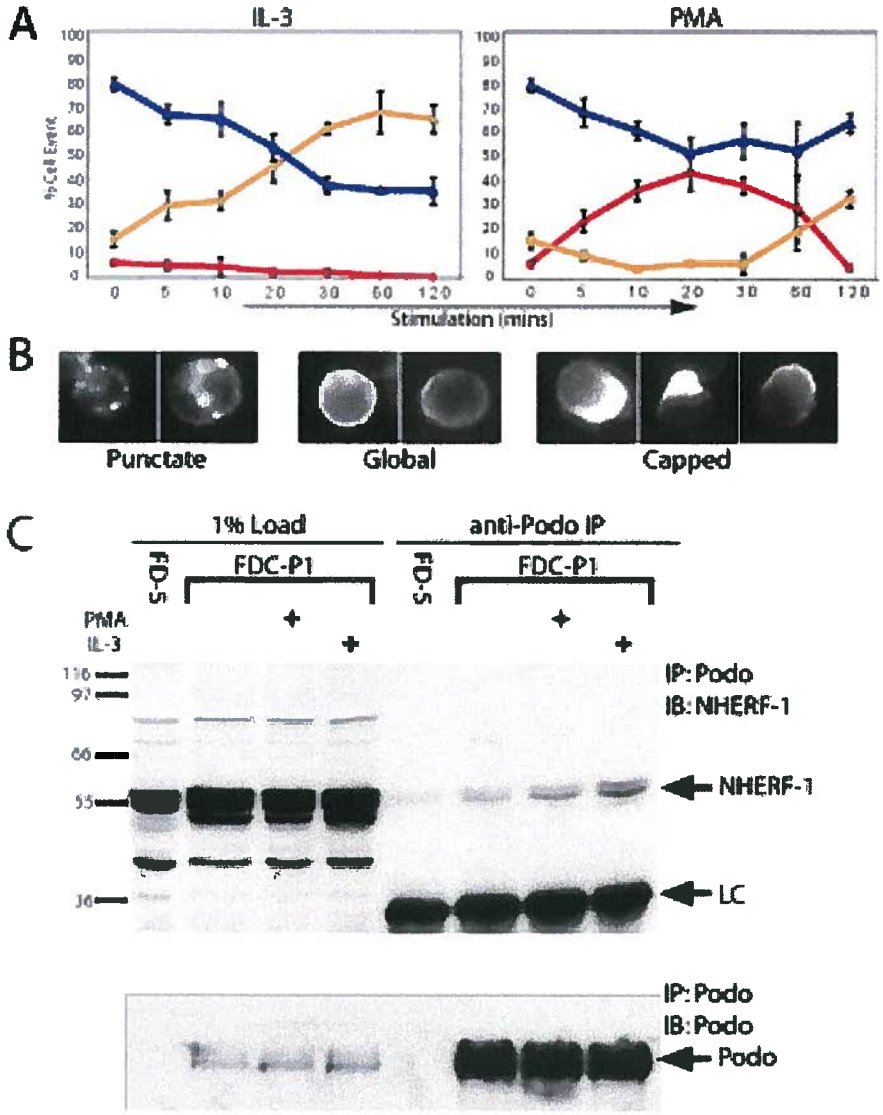

Figure 2.4 IL-3 and PMA differentially regulate localization of Podocalyxin and NHERF-1 in FDC-P1.

(A,B): Graph show the kinetics of Podocalyxin and NHERF-1 re-localization in response to IL-3 and PMA. Three cell phenotypes were scored for each time point: 1) punctate, 2) capped and 3) uniform surface expression of Podocalyxin. Cells were starved for 2 hours (time = 0 hour) prior to IL-3 or PMA stimulation. Blue lines, uniform expression of Podocalyxin on cells, orange lines, capped/clustered staining; red lines, punctate staining. Three counts of >100 cells each were averaged at each time point. C) Immunoblot of immunopurified Podocalyxin complexes from FDC-P1 cells. FD5 cell line

(Podocalyxin-negative) was used as a negative control. Upper blot, anti NHERF-1 and lower blot, anti-podocalyxin.

In summary, our results reveal differential association of NHERF-1 with Podocalyxin in response to activators of the Jak/Stat and the PKC pathways; cytokine stimulation (JAK/Stat pathway) leads to a very distinctive capping of Podocalyxin on the surface of cells and that this correlates with the formation of a complex with NHERF-1, while activators of the PKC pathway led to only minimal capping of Podocalyxin and this resulted in correspondingly lower association with NHERF-1.

2.3.4 NHERF-1 is expressed by mature hematopoietic cells and by cells with an HSC phenotype

Although the expression of NHERF-1 by kidney cells and epithelial cells has been described previously (Wade et al. 2003), its hematopoietic distribution has never been examined. We therefore performed a detailed flow cytometric survey of hematopoietic tissues for NHERF-1 expression by staining permeabilized cells (Figure 2.5). The MDA-231 cell line, which lacks significant levels of NHERF-1 (Ediger et al. 1999; Wade et al. 2003), served as a negative control (not shown). NHERF-1 was broadly expressed by essentially all cells in hematopoietic tissues (bone marrow, thymus, spleen, and peripheral lymph nodes) and the highest levels were observed in T cell precursors in the thymus (Figure 2.5A). Two peaks of expression were found in spleen and mesenteric lymph nodes, one bright and corresponding to the frequency of T cells in these tissues (30% and 50%, respectively) and one with lower intensity corresponding to the frequency

of B lineage cells. Consistent with the flow cytometric analyses, immunoblotting revealed NHERF-1 expression by all hematopoietic tissues and the highest levels in thymocyte lysates (Figure 2.5B). Bone marrow, which showed the lowest levels of NHERF-1 expression by flow cytometry, was also found to have the lowest expression levels by immunoblot; to obtain near-equivalent detection of NHERF-1 approximately 13-fold more bone marrow protein extracts had to be loaded per lane (Figure 2.5B). Again, specificity of the antibody reactivity for NHERF-1 was confirmed by blotting extracts from kidney and MDA-231 breast cancer cells, which express or lack NHERF-1, respectively(Ediger et al. 1999; Wade et al. 2003).

We also performed a detailed 2-color immunofluorescence analysis of NHERF-1 expression by lineage-restricted precursors in the bone marrow. Cells stained for lineage-specific markers B220 (B lineage cells), CD3 (T lineage cells), Mac-1 (myelomonocytic cells), Gr-1 (granulocytes) and Ter119 (erythroid cells), were fixed and stained for cytoplasmic NHERF-1. NHERF-1 was detected in the majority of cells expressing lineage-restricted markers, although it was low-to-negative on distinct subsets of B lineage and erythroid lineage cells (B220 and Ter119 stains, Figure 2.5C).

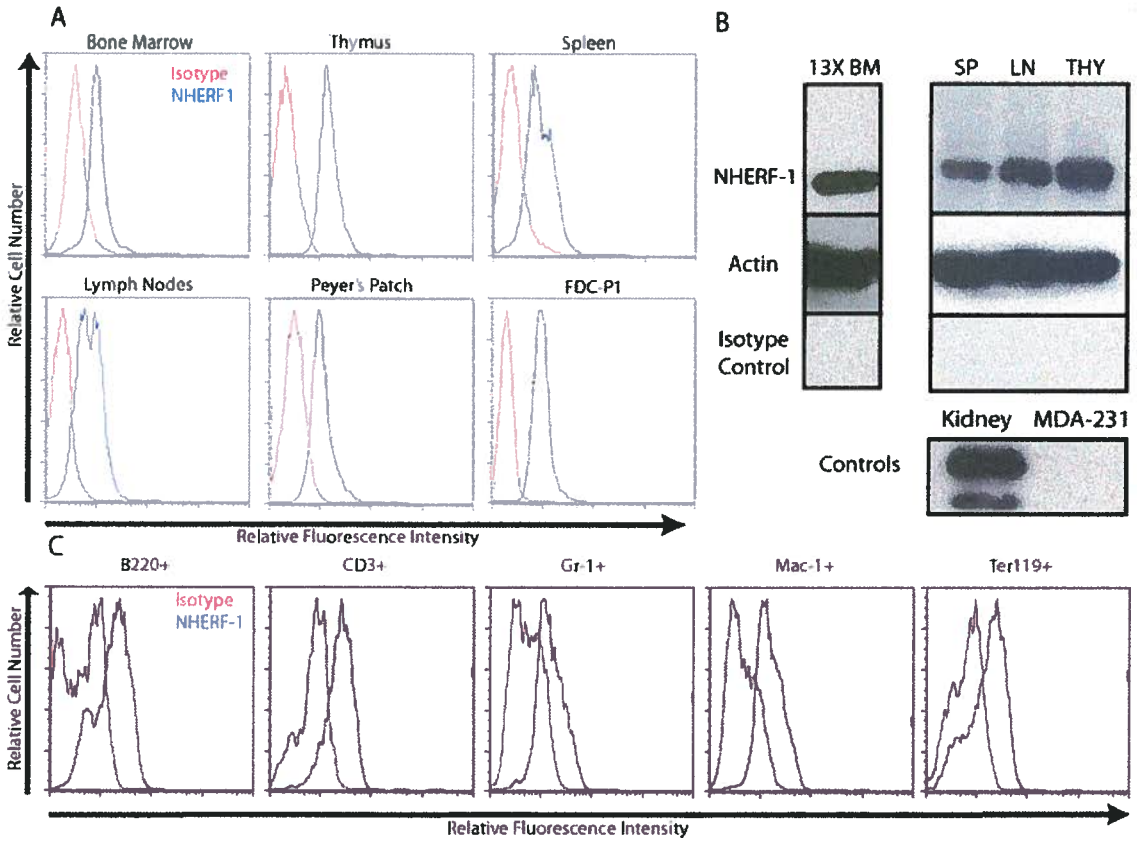

Figure 2.5 Hematopoietic distribution of NHERF-1.

A) Single cell suspensions from the indicated tissues or cell lines were isolated, fixed stained with NHERF-1 (blue) or control antibodies (red), and subjected to flow cytometric analyses. Note that the thymus has the highest and whole bone marrow lysates the lowest expression for endogenous NHERF-1 proteins. B) Immunoblot analysis of whole cell lysates for NHERF-1 expression or actin as a control. MDA-231 and murine kidney cells were used as a negative and positive controls, respectively. C) Two-colour flow cytometry analysis of NHERF-1 expression by bone marrow cells. In each profile cells were gated to show only the lineage marker-positive fraction indicated. Abbreviations: BM, bone marrow; LN, lymph nodes; SP, spleen; Thy, thymus.

Previously, it has been reported that Podocalyxin is expressed by human cells with an HSC phenotype (Kerosuo et al. 2004) and we have shown that murine Podocalyxin is expressed by the c-**kit**$^{+}$/**L**ineage marker^{-}/**S**ca-1^{+} (KLS) fraction of cells in bone marrow and that these cells can reconstitute all hematopoietic lineage cells in lethally irradiated recipients (Doyonnas et al. 2005). To confirm that NHERF-1 is co-expressed by HSCs, we performed multicolor flow cytometric analyses to identify this rare KLS population and look for co-expression of NHERF-1. NHERF-1 was expressed by all cells bearing Sca-1 and c-Kit on their surface and lacking expression of lineage-restricted markers (Figure 2.6). Since these cells have previously been shown to contain all HSC activity(Spangrude et al. 1991) and to express Podocalyxin(Doyonnas et al. 2005), we conclude that NHERF-1 is a *bone fide* ligand for Podocalyxin HSCs.

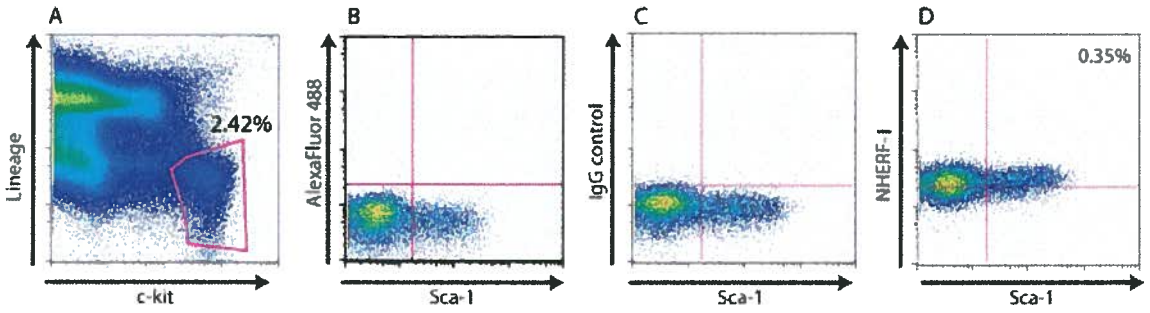

Figure 2.6 NHERF-1 distribution on Lin-, c-Kit+, and Sca-1+ cells.

Single-cell suspension of bone marrow were stained and analyzed by flow cytometry. A) Flow cytometry results of gated cells that were c-Kit+ high and Lin- (2.42% of the total bone marrow). These cells were further gated for SCA-1+ cells. (B,C) Bone marrow cells were stained with all three hematopoietic markers or with isotype control (C). Profile shows only those cells that were positive for c-Kit and negative for lineage markers. (D): Same as (C) but stained with NHERF-1 specific antibodies.

2.4 Discussion

Until recently, the role of CD34-type proteins in hematopoiesis and development has remained obscure. Attempts to link their surface expression to intracellular signaling pathways has led a number of groups to search for intracellular ligands for these molecules. Here we describe the identification of the cytosolic protein NHERF-1 as a hematopoietic ligand for a subset of CD34-type proteins and show that it is expressed by cells with an HSC phenotype. These results have important implications for the function of CD34-type molecules in hematopoiesis and in a variety of non-hematopoietic cell types.

2.4.1 Function of CD34-type molecules

Despite CD34's widespread use over the past 20 years as a clinical marker of human HSCs its functional role on hematopoietic lineage cells has remained enigmatic. Although there has been speculation that this antigen may play a role in blocking hematopoietic cell maturation, enhancing proliferation or act as a homing receptor, there are only minor defects in mice lacking this protein (Cheng et al. 1996; Suzuki et al. 1996). The recent discovery of two additional CD34-related proteins (Podocalyxin and Endoglycan) suggests that the lack of defects in these mice may be due, in part, to functional redundancy with these new family members (McNagny et al. 1997; Sassetti et al. 1998; Sassetti et al. 2000; Doyonnas et al. 2001). A corollary to this hypothesis is that the most profound defects in mice lacking these molecules would be in tissues where they are expressed singly and lack the capacity for functional compensation. With this

concept in mind, we (and others) have characterized defects in cell types where these molecules are aberrantly expressed or in mice where the encoding genes have been disrupted. Consistently, we have found that these molecules play an important role in blocking cell adhesion and cell-cell contact. For example, we have shown that mast cells lacking CD34 show an increased propensity to aggregate and adhere *in vitro* and that they exhibit impaired homing and migration *in vivo* (Drew et al. 2005). This enhanced adhesion is reversible by the ectopic re-expression of CD34 and is more potently reversed by the expression of the naturally occurring short form of CD34 lacking most of the cytoplasmic domain. Likewise, we have found that mice lacking Podocalyxin on their kidney podocytes (where it is, normally, abundantly expressed) there is a striking increase in cell-cell adherens and tight junctions, which leads to a block in urine production and perinatal death (Doyonnas et al. 2001).

Finally, it has been shown that ectopic expression of Podocalyxin in epithelial cells leads to decreased cell-cell adhesion and that up-regulation in tumors correlates with metastatic behavior (Takeda et al. 2000; Schopperle et al. 2003; Somasiri et al. 2004). These data suggest that the principle role of CD34-type molecules is to block cell adhesion and increase invasiveness.

One important caveat to this anti-adhesive hypothesis is that, under many circumstances, the cells that normally express this family of molecules are able to adhere to basement membranes and substrates. In this regard, all of the models showing anti-adhesive roles for these molecules involve either the deletion of the encoding genes (complete loss-of

function) or high-level ectopic over-expression of these molecules (potent gain-of-function). Thus, the ability of normal cells to overcome the anti-adhesive properties of CD34-type proteins may reflect a tight control over the levels of expression of these anti-adhesins or the ability to tightly regulate their subcellular localization in an activation-dependent manner. The latter model correlates well with our observation that a naturally occurring splice variant encoding a cytoplasmically-truncated form of the CD34 (which, presumably, has lost the ability to be actively redistributed in the plasma membrane) is a dominant blocker of adhesion (Doyonnas et al. 2005; Drew et al. 2005).

2.4.2 CD34-family ligands

As a first step toward revealing the mechanisms underlying this latter hypothesis, several groups have pursued intracellular ligands for this family of molecules. For example, the adapter molecule CrkL was recently identified as a cytoplasmic ligand for CD34 (Felschow et al. 2001). Although its role in CD34 function has not been resolved, CrkL has been implicated in linking a number of extracellular signaling pathways to cytoskeletal rearrangements, cell migration and differentiation. Thus, it is one likely candidate for regulating the localization of CD34 during adhesion. Similarly, two groups have identified the PDZ and ERM domain containing protein, NHERF-2, as a cytoplasmic ligand for Podocalyxin in kidney podocytes (Takeda et al. 2001; Li et al. 2002) and (Weinman 2001). Moreover, it has been show that NHERF-2 and Podocalyxin co-localize with ezrin and actin in an apical domain of kidney podocytes and that loss of this complex correlates with the pathological loss of foot processes in disease models (Takeda 2003). It is unlikely, however that NHERF-2 is a hematopoietic ligand of

CD34-type proteins in HSCs and vascular endothelia since its expression is relatively restricted to podocytes and other rare cell types in non-hematopoietic tissues (Wade et al. 2003). Correspondingly, we have failed to detect NHERF-2 as a ligand in hematopoietic tissues and cells by either mass spectrometry or functional screens (data not shown).

Instead we have identified NHERF-2's close relative, NHERF-1, as a hematopoietic ligand for these proteins and shown that NHERF-1 has specificity for Podocalyxin and Endoglycan but not CD34. Although NHERF-1 has been postulated to be a ligand for Podocalyxin, based on its similarity to NHERF-2, to our knowledge, this is the first demonstration of a naturally occurring interaction between Podocalyxin and this protein (Li et al. 2002; Takeda 2003). In addition, there is reason to believe that there may be differences in the function of NHERF-1 and NHERF-2. Although they share an overall sequence identity of 50%, there are a number of significant differences between these proteins including a much greater number of potential phosphorylation sites in NHERF-1.

It has also been noted that in proximal tubules in the kidney (one of the few cell types where these molecules are co-expressed) these molecules display differences in their subcellular localization, with NHERF-1 residing in the apical regions of microvilli and NHERF-2 more closely associated with vesicle rich domain at the base of microvilli (Wade et al. 2003). Thus, there may be functional heterogeneity within the NHERF family.

NHERF-1 has strong affinity for Podocalyxin and Endoglycan but not CD34. The cytoplasmic domains of Podocalyxin and Endoglycan show a much higher degree of sequence similarity to each other than to CD34 and this includes an amino acid substitution in the C-terminal PDZ-domain-docking site from DTHL (Podocalyxin and Endoglycan) to DTEL (CD34). This is the first clear demonstration of functional heterogeneity in this family of sialomucins and may indicate the existence of an independent PDZ-domain-docking protein for CD34.

2.4.3 Functional Significance of CD34 family proteins and NHERF-1

Although NHERF-1 was first described as a specific regulator of transmembrane Na+/H+ exchangers, it is thought to act as a broad-based scaffolding protein for linking membrane proximal proteins with the actin cytoskeleton and thereby regulating their subcellular localization and, potentially, their stability and internalization (Voltz et al. 2001). NHERF-1 has been shown to bind to the C-terminus of a large number of cytosolic proteins and transmembrane receptors via its two tandem PDZ-domains including: β_2AR, CTFR, PDGF-R, $P2Y_1$, Trp4 and Trp5, and PLCβ isoforms. These are then linked to the cytoskeleton by virtue of NHERF-1s ability to oligomerize and to bind members of the ezrin/radixin/moesin family of cytoskeletal proteins (Voltz et al. 2001). With regard to the CD34 family, we speculate that their ability to block hematopoietic adhesion may be intimately associated with their degree of clustering in the plasma membrane by NHERF-like proteins. Previously we noted that Podocalyxin is selectively up-regulated in a subset of human breast carcinomas and that its expression strongly correlates with poor

patient outcome *in vivo* and with a loss of tumor cell polarity *in vitro* (Somasiri et al. 2004). Strikingly, the cells with the greatest loss of polarity and highest metastatic behavior, also show a loss of NHERF-1 expression (Ediger et al. 1999; Dai et al. 2004) (Roskelley, Huntsman, McNagny, unpublished results).

These data would suggest that up-regulation of Podocalyxin and loss of NHERF-1 are required for a dominant loss of cell contact/adhesion and that the ability to interact with NHERF-1 affords cells the ability to clear these anti-adhesins from pro-adhesive molecules and establish apical and basolateral domains. This is further supported by a recent report showing Podocalyxin is involved in establishing the apical (non-adhesive) domain on epithelial cells and that this is critically dependent on the C-terminal PDZ-docking site and correlates with a co-localization of NHERF-2 (Meder et al. 2005). The fact that this domain is established prior to cell adhesion is consistent with a model in which Podocalyxin clustering permits establishment of a Podocalyxin-free and adhesion-molecule-rich basolateral domain.

Previously we have shown that Podocalyxin and CD34 play a role in blocking hematopoietic cell adhesion *in vivo* and *in vitro* and that truncation of the cytoplasmic domain increases the effectiveness of this block (Doyonnas et al. 2005; Drew et al. 2005). By analogy with their documented role in epithelial cell polarization (Meder et al. 2005) we propose that, in hematopoietic cells NHERF-1 serves as a potent regulator of this phenomenon by actively redistributing Podocalyxin to non-adhesive domains and thereby permitting cell adhesion. This is consistent with our observation that Podocalyxin and NHERF-1 show the most dramatic co-localization on cells exhibiting a polarized cap of

Podocalyxin and that this is enhanced with IL-3-stimulation. This active redistribution would permit rapid changes in the adhesive properties of cells in the absence of a need for *de novo* protein synthesis. In this light, it is noteworthy that in a recent survey of gene allelic variants that most closely correlated with HSC turnover, the IL-3R locus was found to be one of the four most tightly-linked quantitative trait loci associated with this behavior (Bystrykh et al. 2005).

An interesting possibility is that Podocalyxin, as a downstream modulator of adhesion, may be a mediator of IL-3R-dependent HSC mobilization or turnover. Clarification of this model will be facilitated by the expression of dominant-negative forms of NHERF-1 in HSCs and the generation of null backgrounds for assessing their function.

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Chapter 3 : Podocalyxin Modulates SCF and CXCL12 Mediated Migration of Myeloid Progenitor Cells[1]

3.1 Introduction

Blood cells are derived from the multi-potent hematopoietic stem cell (HSC). Quiescent HSCs reside in the adult bone marrow and are triggered to mobilize into the circulation for maintenance of blood homeostasis or response to host injury (Nervi et al. 2006). HSCs have an intrinsic homing (Whetton et al. 1999) mechanism to facilitate their localization to the bone marrow. This is a process known as homing. Successful homing is important for stem cell transplantation and involves a coordination of processes such as mobilization, migration, adhesion and engraftment to the HSC niche. Homing of intravenously transplanted cells begins with a chemo-attraction to the bone marrow, extravasation from the blood vessel and subsequently transmigration through the stromal layer through to the sub-endosteum (Hart et al. 2004; Kaplan et al. 2007).

CXCL12 plays a central role as a chemoattractant for HSC cells to the niche. In addition, this chemokine also regulates motility, homing and retention, survival and proliferation in the bone marrow (Aiuti et al. 1997; Aiuti et al. 1999). CXCL12 is a ligand for CXCR4, a seven transmembrane G-protein-coupled receptor that is expressed by HSCs (Weismann). Recently an additional chemokine receptor CXCR7 was identified on the surface of HSC. CXCR7 is also able to bind CXCL12 but has been shown to regulate mainly HSC

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proliferation (Balabanian et al. 2005; Burns et al. 2006), thus, CXCR4 still is the predominant receptor necessary for migration.

In addition to CXCL12, stem cell factor (SCF) plays a major role in HSC migration. SCF is produced by stromal cells in the bone marrow, and aids not only in the homing and maintenance of HSCs within the niche but also in the survival and proliferation of HSCs (Hart et al. 2004). SCF can be found both in membrane bound and soluble forms and it is the sole ligand for the receptor c-Kit, which is also essential for hematopoiesis (Blechman et al. 1993; Broudy 1997)

In the initial process of homing, several adhesion molecules become important. When in circulation, HSCs sense a chemotactic gradient and as a consequence will tether to the endothelium. Upon tethering, the cell then begins to roll, and then halt to establish firm adhesion. This adhesion is aided by integrins. Integrins are made up of a heterodimer of α and β subunits. They help anchor the cell firmly to the endothelium and also have been shown to participate in HSC extravasation through the stroma (Williams et al. 1991; Papayannopoulou et al. 1997). Although adhesion is important in the homing process, it is critical that this integrin mediated event is well coordinated in a way that allows the cells to be mobile and migrate. Briefly, an equilibrium is established between activated and non-activated integrins at the cell surface to propel the cell forward during this process. We hypothesized that Podocalyxin may play a role as an anti-adhesive molecule since expression of CD34 molecules on mast cells decreases cell-cell aggregation (Drew et al. 2002; Drew et al. 2005).

A similar affect has also been reported by over expressing Podocalyxin in breast cancer cell lines such as MCF-7. Normally, MCF-7 proliferates into cobblestone-patterned monolayers with tight junctions and appropriate organization of apical and basal domains. Upon expression of Podocalyxin, the cells exhibit disrupted cell junctions and display increased cell shedding from the monolayer (Somasiri et al. 2004). Increased cell shedding from the monolayer is associated with potential for tumour metastasis. Podocalyxin is highly expressed on a distinct set of invasive breast carcinomas in human patients and that its expression correlates with poor prognosis (Somasiri et al. 2004).

Podocalyxin is one of three members of the CD34 family. It is a transmembrane sialomucin with heavily N and O glycosylated extracellular region, a highly conserved (among species and family members) cytoplasmic region with a PDZ docking site. Due to alternative splicing, Podocalyxin exists in two distinct forms: 1) a full length protein with a cytoplasmic tail of 72 amino acids, and 2) cytoplasmically truncated form (generated due a premature stop codon) bearing only 10 intracellular amino acids.

Podocalyxin is expressed on a majority of cells, including LSK in mouse E15 fetal liver. We have demonstrated that a hematopoietic precursor population that lack Podocalyxin expression home less efficiently to the bone marrow and spleen in short term assays (Doyonnas 2005). To further investigate this finding, we performed biological and biochemical assays to study two key aspects of Podocalyxin's role in hematopoietic homing: 1) cell-adhesion and 2) cell migration. We found that suppression of Podocalyxin in a hematopoietic cell line and *Podxl*$^{-/-}$ E15 fetal liver cells attenuated migration toward a CXCL12 plus SCF gradient. We also show that Podocalyxin not only

co-associates with CXCR4 upon cell stimulation with CXCL12 plus SCF but that in the absence of Podocalyxin, CXCR4 activation of AKT is impaired. Our data suggest that Podocalyxin and CXCR4 association are important for the chemotactic process during HSC migration toward a CXCL12 and SCF cocktail.

3.2 Materials and Methods

3.2.1 Cells

Factor dependent cell-Paterson1 cells (FDC-P1) were maintained in RPMI (Hyclone) with 10% FBS (Gibco), 4mM L-Glutamine (Gibco), 1x Penicillin and Streptomycin (Gibco) and 10% 1X WEHI-3B (source of mIL-3 made in house) conditioned media. FDC-P1 infected with lentiviral shRNA were maintained in 1μg/ml of G418 (Sigma-Aldrich) drug selection and G418 was removed two weeks prior to experiments.

3.2.2 Antibodies

Podocalyxin and isotype antibodies were purchased from R&D systems, USA. All secondary antibodies (goat anti-rat 488, donkey anti-goat 568) for immunocytochemistry were from Molecular Probes, USA and HRP conjugates were from Dako. CXCR4 antibody for immunocytochemistry was purchased from Santa Cruz, antibody for immunoblotting was purchased from Abcam and antibody for flow cytometry staining was purchased from BD Biosciences. Phosphorylated AKT S473 antibody is from Cell Signaling.

3.2.3 Lentiviral shRNA Infection

Sequences were generated using PSI Oligomaker version 1.5 (http://web.mit.edu/jacks-lab/protocols/pSico.html). Briefly, the program generates a list of shRNA sequences and a set of algorithms calculates and scores the sequences with the highest probability of knocking-down the protein of interest, in our case this is Podocalyxin. We chose the shRNA sequences based on the calculated high scores that would correlate with highest probability of silencing Podocalyxin. The oligos were generated by Invitrogen and the constructs were used to generate functional lentivirus, which were then used to infect FDC-P1 cells (Figure 3.1). For a more detailed description of the silencing mechanism of shRNAs please refer to Appendix B.

The lentiviral expression protocol is adapted from Van Parijs and colleagues (Rubinson et al. 2003). pLL3.7kb+2.0kb spacer plasmid was a generous gift from Dr. Fabio Rossi. Predicted sequences for each of the shRNA oligos were purchased from Invitrogen and were annealed (in a PCR block) at 55°C for 40 cycles. Annealed oligos were ligated into pLL3.7 plasmids and propagated in *E.coli*. Plasmids were transfected into 293T along with packing plasmids (pVSVg, p-MDLgag/pol and p-RSV-rev) by calcium phosphate transfection, supernatant were collected after 36 hours post-transfection and used for re-suspending FDC-P1 cells and incubated for 48 hours to allow transfection to occur. FDC-P1 cells were sorted using GFP fluorescence and selected with 1μg/ml of G418. These cultures were maintained as outlined in section 2.2.

3.2.4 MTS Assay

Celltiter 96 © Aqueous MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium, inner salt) reagent powder (Promega) was prepared according to the manufacturer's instructions. MTS reagent is thawed at room temperature for approximately 90 minutes and phenazine methosulfate (PMS) (provided by manufacturer) is added fresh before each experiment. Cells were counted with a hemacytometer and re-suspended in complete tissue culture media. One thousand cells were placed into each well of a 96-well flat bottom Nunc plate in a set of quadruplicates. Using a repeater pipette, 20µl of MTS/PMS solution is dropped into each well. Plates were incubated for 1-4 hours at 37°C in a humidified 5%CO_2 atmosphere. Chemi-fluorescence were recorded at absorbance 490nm using Spectromax 3000 and SoftMax-pro software. Plots are corrected by subtracting wells with culture medium alone (blanks). Student's t-test was performed to determine statistical significance between different experimental conditions.

3.2.5 Fluorescence Activated Cell Sorting (FACS) and Immunocytochemistry.

FDC-P1 cells were fixed with 4% paraformaldehyde (Sigma) for 15 minutes at room temperature, washed four times with blocking buffer (1%BSA in PBS), permeabilized with 0.1% Triton-100X (Sigma) for 15 minutes at room temperature and washed four times with blocking buffer. After permeabilization, non-specific antibody binding was blocked with 10% goat serum for 20 minutes. Cells were then labeled with 2µg/ml of Podocalyxin for 15 minutes, cells were then washed four times with blocking buffer and

finally incubated with corresponding goat anti rat Alexa Fluor antibodies for 15 minutes and analyzed by a Becton Dickinson FACS Calibur. For immunocytochemistry, cells were allowed to settle on Poly-L-lysine coated slides for 30 minutes in the dark and then mounted with a cover slip for analysis on an Olympus confocal microscope.

3.2.6 Transwell Migration Assay

FDC-P1 cells were washed with sterile 37°C PBS twice and re-suspended in complete RPMI media without WEHI-3B conditioned media and incubated at 37°C with 5% CO_2 for 3 hours. Costar 5um transwells were coated with 100ug/ml fibronectin (Chemicon) or with 2.5 $x10^4$ M210B4 stromal cells (a generous gift from Dr. Connie Eaves) 24 hours prior. For transwells coated with fibronectin, wells were washed four times with PBS and blocked for 2 hours with 5% BSA in PBS at 37°C. After blocking, wells were washed four times and 1.0 $x10^6$ cells were added into the upper chamber and 500µl of media with 100nM CXCL12 (made in house from our peptide facility) or 200ng/ml SCF (R&D systems) was added to the lower chamber.

For transwells coated with stroma, each well was checked visually for viability and homogeneity of the layer. After verifying the quality of the stromal cells $1.0x10^6$ FDC-P1 cells were carefully added to the top chamber and migration was allowed to occur for 6 hours. Migrated cells were carefully re-suspended and were counted on a hemacytometer. For a more detailed description of the transwell migration assay please refer to Appendix C.

3.2.7 Harvest of Podxl$^{-/-}$ E15 Fetal Liver Cells

Podocalyxin knock-out fetal liver cells were obtained from timed-mating between *Podxl*$^{+/-}$ mice. At E15, pregnant females were sacrificed by CO_2 asphyxiation and embryos were carefully extracted. Sample tissue (tail snip) was obtained from each embryo for genotyping and in parallel; fetal livers were carefully removed from the embryo. Each fetal liver was re-suspended in sterile PBS to generate a single cell suspension. The cells were subsequently labeled with Ter119-PE antibodies (eBiosciences) for 15 minutes, washed and then incubated with magnetic anti-PE micro beads (Automacs). This cell suspension was run through a manual Macs column to deplete Ter119^{+} cells. Ter119^{-} cells are recovered from the column flow-through, washed two times with sterile RPMI media (Hyclone) supplemented with WEHI-3B culture media (source of IL-3). Cells are counted and re-suspended to the appropriate concentration before loading into the transwells. Transwell migrations were performed as mentioned above.

3.2.8 Adhesion Assay

Prior to the experiment, 96-well Nunc flat bottom plates were coated with 100μg/ml of fibronectin overnight at 4°C. Wells are washed four times with HBSS (Gibco) and blocked with 3% BSA in PBS for 2 hours at 37°C, after blocking wells were washed again three times with HBSS.

FDC-P1 cells were washed twice with HBSS and 1 x 10^6 cells were re-suspended in 1ml of RPMI media containing 3μg Calcein-AM (Calcein acetoxymethylester) (Molecular

Probes). These cells were incubated for 1 hour at 37°C with 5% CO_2. After labeling, cells were washed three times with HBSS and re-suspended in RPMI complete media without phenol-red (Gibco). Cells were starved for 3 hours, after starvation 2 x 10^5 cells were added in triplicates into pre-blocked wells and stimulated with 200ng/ml of SCF or 100nM of CXCL12 for various times. At each time point, a pre-wash and a post-wash were read at a wavelength of 450nm using a fluorimeter. Each well was carefully washed four times. Percent adhesion was calculated based on the post-washed fluorescence divided by pre-washed fluorescence.

3.2.9 Immunoblotting

SDS-page fractionation of cell lysates was performed as previously described (Tan et al. 2006). For immunoblotting, nitrocellulose membranes were blocked with 10% BSA or non-fat milk in TBS-Tween for 2 hours at room temperature. Membranes were incubated with primary antibodies overnight at 4°C and HRP conjugated antibodies for 1 hour. The blots were then detected with chemiluminescence reagent (ECL, Amersham).

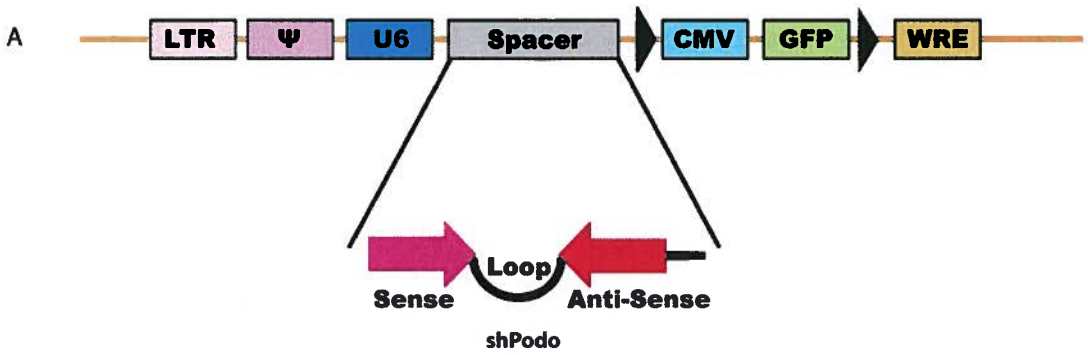
3.2.10 Analytical Tools

All statistical analyses were performed using student's paired t-test and Microsoft Excel program. FACS analyses were done with FlowJo and all cell image analyses were done with Fluoview 006.

3.3 Results

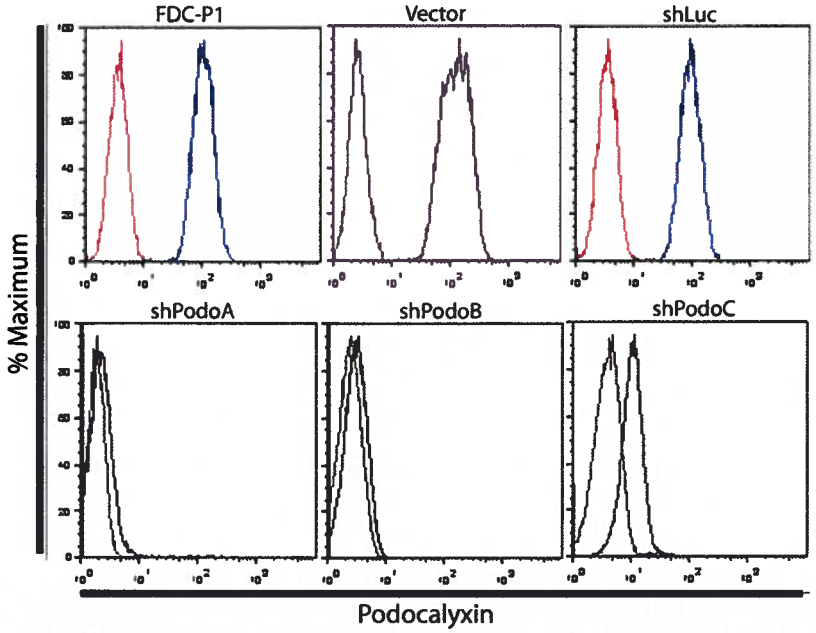
3.3.1 Silencing of Podocalyxin Proteins in FDC-P1 cells

To assess the importance of Podocalyxin in hematopoietic precursor function, we constructed lentiviral Podocalyxin-specific shRNA expression vectors and used them to infect FDC-P1 cells. Three independent Podocalyxin inhibitory lentiviral constructs were generated (Figure 3.1) (Rubinson et al. 2003).



B

Sequences	----------SENSE ----------LOOP----------ANTISENSE----------
shPodoA	5'-TGAGACTGGCCTATCATTTATTCAAGAGATAAATCATAGGCCAGTCTCTTTTTTC-3'
shPodoB	5'-TGTATTCTTGTGGTATAAGTTTCAAGAGAACTTATACCACAAGAATACTTTTTTC-3'
shPodoC	5'-TGAATGTAAATGTCTATTTATTCAAGAGATAAATAGACATTTACATTCTTTTTTC-3'



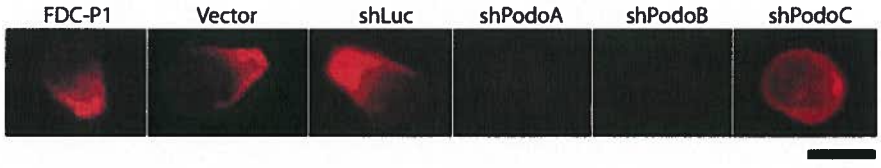


Figure 3.1 Schematic of lentivirus plasmid and sequences for stably silencing Podocalyxin in FDC-P1.

A) Schematic of lentiviral construct used to silence Podocalyxin expression. LTR: long-terminal repeat; ψ: HIV packaging signal; U6: promoter; CMV: cytomegalovirus promoter; GFP: green fluorescent protein; WRE: woodchuck hepatitis virus response

element B) Three sequences for shPodocalyxin as determined by Jackson program predictions. C) Flow cytometry analysis of Podocalyxin knock-down in shPodocalyxinA and shPodocalyxinB populations. Blue lines: Isotype control; Red lines: shPodocalyxinA. D) Confocal X-Y images of Podocalyxin staining in control cells and negative staining in shPodocalyxinA and B clones. Bar is 10um. Vector: lentiviral plasmid; shLuc: Luciferase knock-down; shPodo: Podocalyxin knock-down

We conclude that shPodocalyxinA and shPodocalyxinB shRNA constructs efficiently inhibited Podocalyxin expression. Control cells containing only the lentiviral plasmid or a non-specific shRNA for luciferase showed normal levels of Podocalyxin similar to the FDC-P1 parental line.

3.3.2 Suppression of Podocalyxin in FDC-P1 cells does not alter viability, growth or expression of several surface molecules.

We used an MTS assay to determine whether silencing Podocalyxin expression affected proliferation and viability of cells. As shown in Figure 3.2, we observed no major differences in the proliferation of shLuc and shPodocalyxin cells over 24 hours, 48 hours or 72 hours. shLuc and shPodocalyxin cells consistently exhibited >90% viability by trypan blue dye exclusion counting over the same time period (unpublished observations).

We also assessed wildtype, control and shPodocalyxin cells for the expression of a panel of cell surface antigens via flow cytometry (Figure 3.2). These included cell surface

sialomucins, CD43, CD45, CD34, Endoglycan and other transmembrane molecules such as CD180, CD106 and a fibronectin receptor, Syndecan-4. We observed no significant changes in the levels of surface expression of these markers suggesting that Podocalyxin knock down shRNA oligomers were highly selective for their target genes and do not influence expression of related cell surface markers.

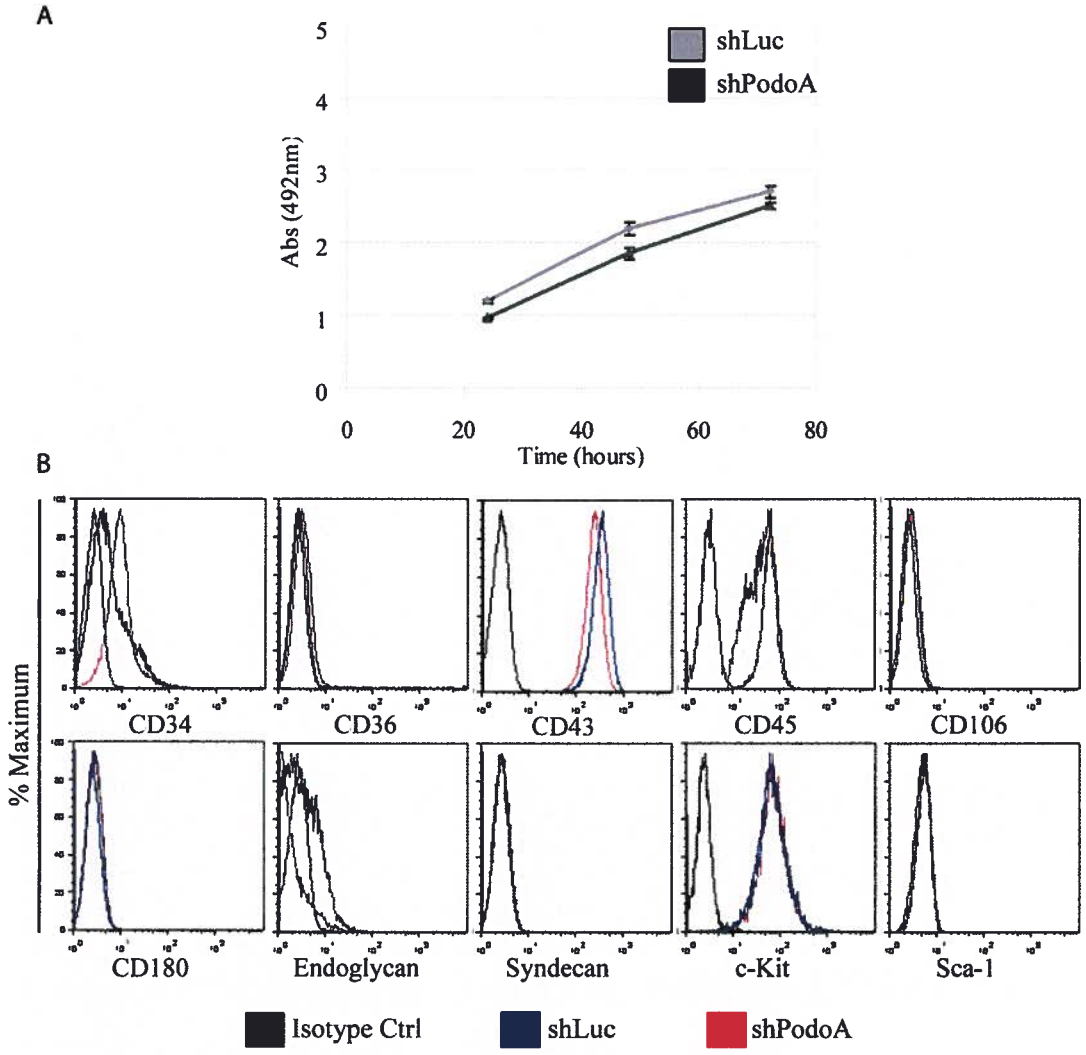


Figure 3.2 shPodocalyxin and shLuc cells show comparable proliferation, viability and express no significant difference of cell surface proteins.

A) MTS assay for cell viability/proliferation at 24 hours, 48 hours and 72 hours. There are no major proliferation differences between shLuc and shPodocalyxinA. B) shLuc and shPodocalyxin express normal levels of cell surface antigens. Isotype controls for shPodocalyxin is equal to isotype control for shLuc. shPodocalyxin-isotype control is omitted for clarity.

3.3.3 Podocalyxin Deletion Impairs Migration across a Stromal Cell Monolayer

We next evaluated the importance of Podocalyxin in migration via transwell assays. Stromal monolayers were grown on the surface of the filter in the upper chamber of each transwell. CXCL12 and SCF were added to the bottom chamber to generate a chemotactic gradient. Luciferase and Podocalyxin knockdown cells were then placed into the upper chamber and were allowed to migrate for 6 hours in a humidified 5% CO_2 incubator at 37°C. In four independent experiments, we found that shPodocalyxin cells were severely impaired (63% ± 2.2) in their ability to migrate across the stromal cell-coated transwells (Figure 3.3).

Migration of FDC-P1 was dependent on both CXCL12 and SCF since shLuc cells exhibited only minimal transwell migration in the presence of either factor alone. This requirement for both factors is consistent with previous reports suggesting that SCF increases CXCR4-dependent chemotaxis (Lapidot et al. 2002; Hart et al. 2004). Since there were no differences in levels of c-kit or CXCR4 expression in shPodocalyxin cells, impaired migration is not the result of lack of or decreased receptor expression (Figure 3.2 and Figure 3.10; immunoblot for CXCR4). Therefore, Podocalyxin may play an essential role in migration towards chemoattractant.

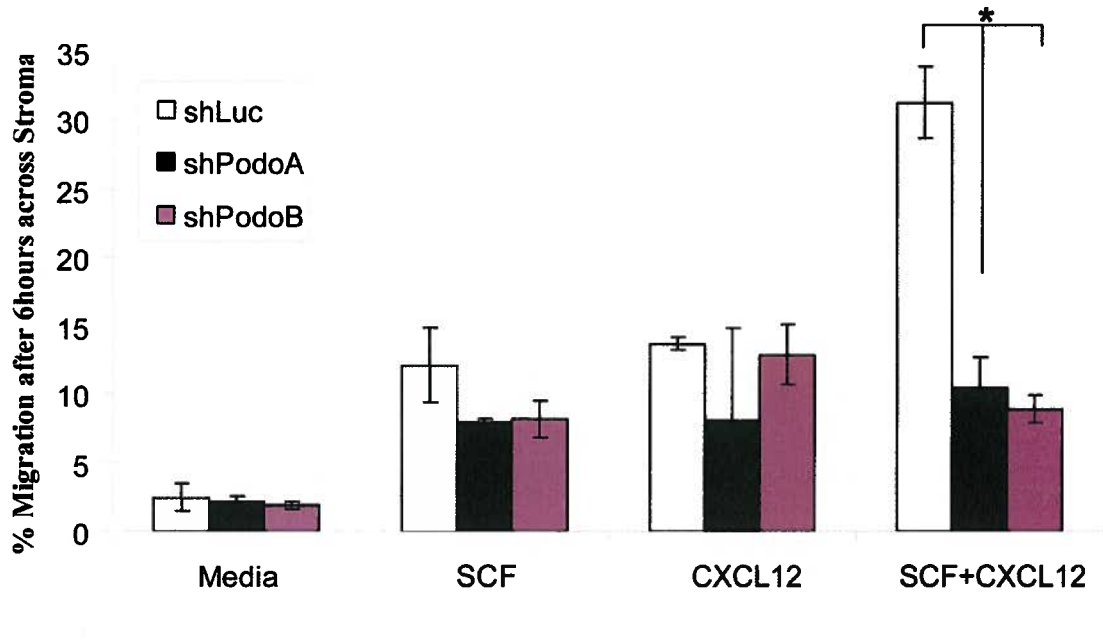


Figure 3.3 shPodocalyxin cells have impaired chemotaxis towards SCF and CXCL12 across *stromal monolayer*.

FDC-P1 cells were placed in stromal cell coated Costar transwells for a six hour migration towards SCF and CXCL12. There were little migration with SCF and CXCL12 alone; however, there is a dramatic increase upon stimulation with both stimulants. Migrated cells were counted and plotted as function of percent cells migrated of total cells loaded into the assay. Podocalyxin deficient cells were defective in migration. (* indicates significance by a Student's t-test $p<0.05$ and $n = 4$).

3.3.4 Podocalyxin Deletion Impairs Migration Across A Fibronectin Matrix.

In order to determine whether the effects of Podocalyxin on chemotaxis required factors modified or produced by stromal cells, or if an adhesive matrix alone was sufficient, we repeated these migration assays on fibronectin-coated transwells. Fibronectin is an extracellular matrix component secreted by cells in the HSC niche, and, previous work has suggested that the fibronectin receptor, $\alpha_4\beta_1$ integrin is key for HSC homing/migration (Williams et al. 1991; Papayannopoulou et al. 1993). Similar to stromal cell coated wells, we consistently found that shPodocalyxin cells were severely impaired (68% ± 2.2) in their ability to migrate across fibronectin coated transwells also (Figure 3.4). Thus, fibronectin is a sufficient adhesive substrate for Podocalyxin dependent migration.

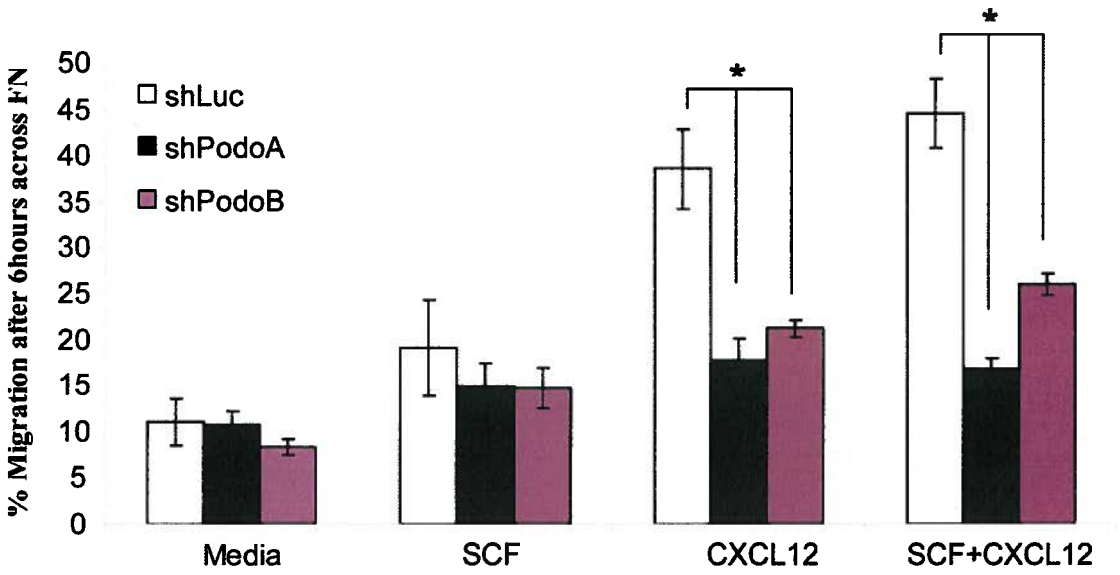


Figure 3.4 Loss of Podocalyxin impairs CXCL12 and SCF chemotaxis across *fibronectin matrix*.

FDC-P1 cells were placed in fibronectin coated Costar transwells for a six hour migration towards CXCL12 and SCF gradient. There was little migration with CXCL12 or SCF alone; however, there is a dramatic increase upon stimulation with both stimulants. Migrated cells were counted and plotted as a function of percent cells migrated of total cells loaded into the assay. shPodocalyxinA and shPodocalyxinB indicates two different successful knock-down for the proteins. Podocalyxin deficient cells were defective in migration. (* indicates significance with a Student's t-test $p<0.05$, $n = 3$).

To test whether Podocalyxin-dependent migration could be blocked at the protein level, we performed assays in the presence of a Podocalyxin-specific monoclonal antibody. Similar to our results with shPodocalyxin cells, we observed impaired chemotaxis of control FDC-P1 cells when these were pretreated with a Podocalyxin-specific monoclonal antibody for 15 minutes prior to the migration assay (Figure 3.5). We

therefore conclude that Podocalyxin is important for efficient chemotactic migration and that its activity can be inhibited by either blocking antibodies or the expression of Podocalyxin on the cell.

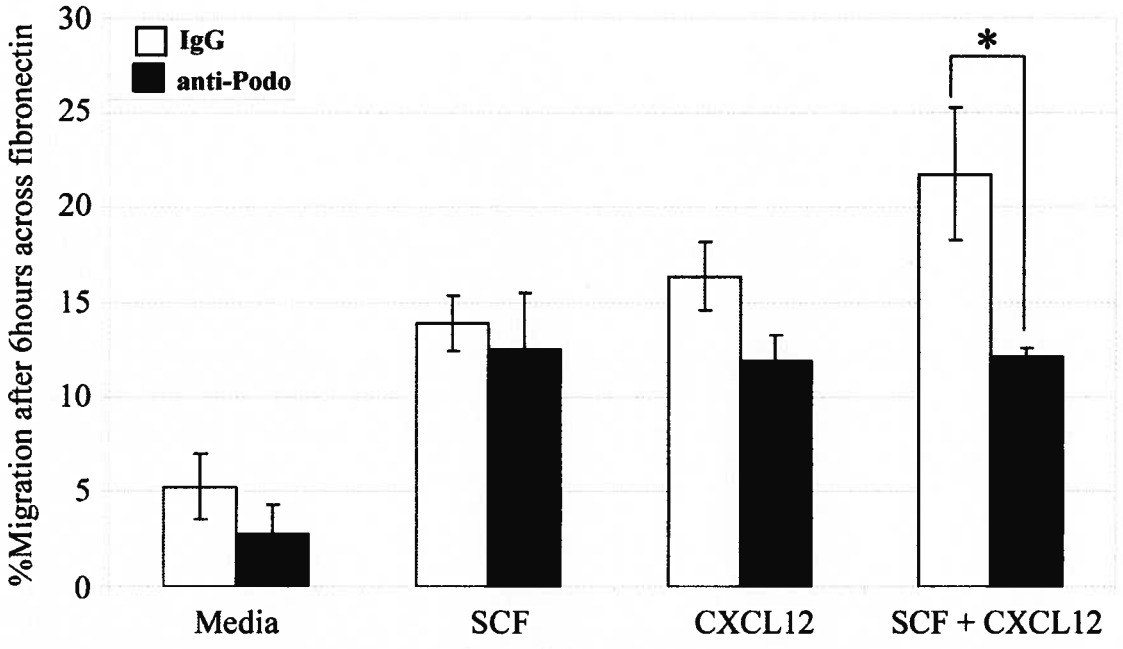


Figure 3.5 Podocalyxin antibody impairs CXCL12 plus SCF cell migration across fibronectin.

shLuc cells were placed in stromal cell coated Costar transwells for 6 hour migration towards CXCL12 and SCF. There were little migration with CXCL12 or SCF alone; however, there is a synergistic affect upon stimulation with both stimulants. Migrated cells were counted and plotted as function of percent cells migrated of total cells loaded into the assay. Podocalyxin deficient cells were defective in migration. (n = 2)

Previously we showed that in a short term homing assay with LSK cells derived from a wild-type mouse, a Podocalyxin-negative sub-population of cells had impaired migration (Doyonnas et al. 2005). To test if the observed migration defect in Podocalyxin-deficient FDC-P1 was also applicable to primary cells we repeated similar migration assays using

wildtype and *Podxl*$^{-/-}$ fetal liver cells derived from embryos at E15 - a site where the most primitive cells are found early in fetal development. (N.B. *Podxl*$^{-/-}$ fetal liver cells were used because *Podxl*$^{-/-}$ mice die one day after birth due to kidney defects). Fetal liver hematopoietic progenitors from wild type and Podocalyxin–deficient embryos were enriched by depletion of Ter119^{+} cells and these were then assessed for migration across fibronectin-coated transwells. As shown in Figure 3.6, *Podxl*$^{-/-}$ fetal liver cells, like Podocalyxin-deficient FDC-P1 cells, displayed a clear reduction (53.5% ± 2.4) in their ability to migrate towards a gradient of CXCL12 and SCF (shown is one out of two similar experiments). We conclude that, as with cell lines, primary hematopoietic cells in mouse fetal liver require Podocalyxin expression for efficient migration.

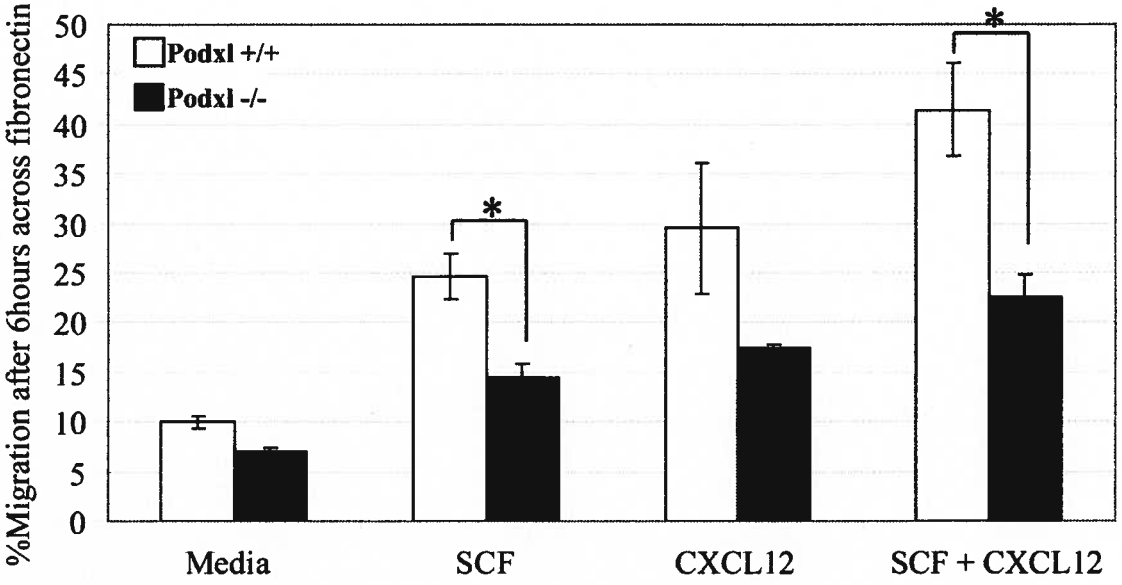


Figure 3.6 Ter119 depleted Podocalyxin knock-out E15 fetal liver cells have impaired chemotaxis towards CXCL12 and SCF across fibronectin.

Ter119+ Podocalyxin knock-out fetal liver cells have impaired migration across fibronectin matrix towards a SCF and CXCL12 gradient. * indicates statistical significance; t-test; $p<0.05$, $n = 2$.

3.3.5 Podocalyxin Deletion Does Not Impair Cell Adhesion to Fibronectin Matrix

Previously, we and others have shown that when over expressed by adherent tumour cell lines, Podocalyxin acts as an anti-adhesive molecule and results in reduced cell aggregation, an increased in apical domain expansion and a disruption of cell-cell junctions (Somasiri et al. 2004; Nielsen et al. 2007). Subsequently, we hypothesize that a loss of Podocalyxin from hematopoietic precursor cells would result in excessive

adhesion to fibronectin or stroma – a potential mechanism that could explain enhanced migration.

To address this issue we first examined shLuc and shPodo cells for expression levels of α4β1 integrin, a well known hematopoietic precursor receptor for fibronectin as well as two other hematopoietic adhesion molecules, PECAM1 and CD44. As shown in Figure 3.7, flow cytometric analyses revealed no major changes in the expression levels of any of these adhesion molecules.

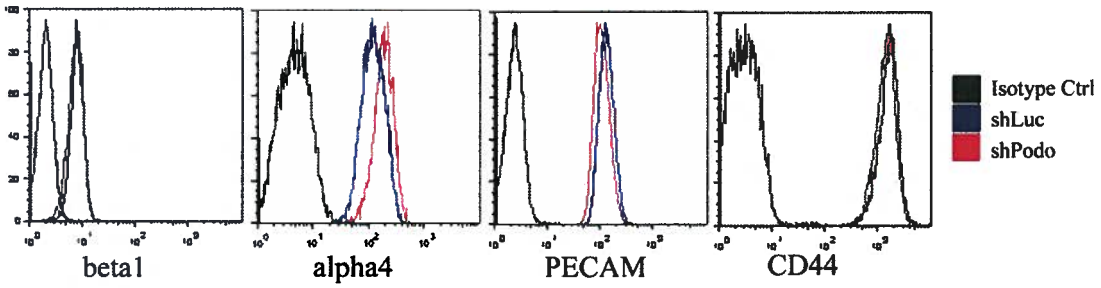


Figure 3.7 shPodocalyxin cells express normal cell surface adhesion antigens.

Alpha4beta1 is known receptor for fibronectin and are expressed on these cells. There is no significant difference between shLuc and shPodocalyxinA. In addition, other known adhesion molecules are also expressed at normal levels in both cell populations. FACS was used to analyze the expression of cell surface antigens. Black: Isotype control-shLuc; Blue: shLuc; Red: shPodocalyxinA. Isotype-shPodocalyxinA is omitted to prevent clutter but is identical to isotype-shLuc.

We next examined the ability of these cells to adhere to fibronectin. As shown in Figure 3.8, shPodocalyxin and shLuc cells did not exhibit any significant difference in the ability to adhere to fibronectin upon stimulation. Thus, we conclude that suppression of

Podocalyxin does not alter cell adhesion and that the defects we observe in shPodocalyxin FDC-P1 reflect a more specific defect in the ability to sense a chemotactic gradient.

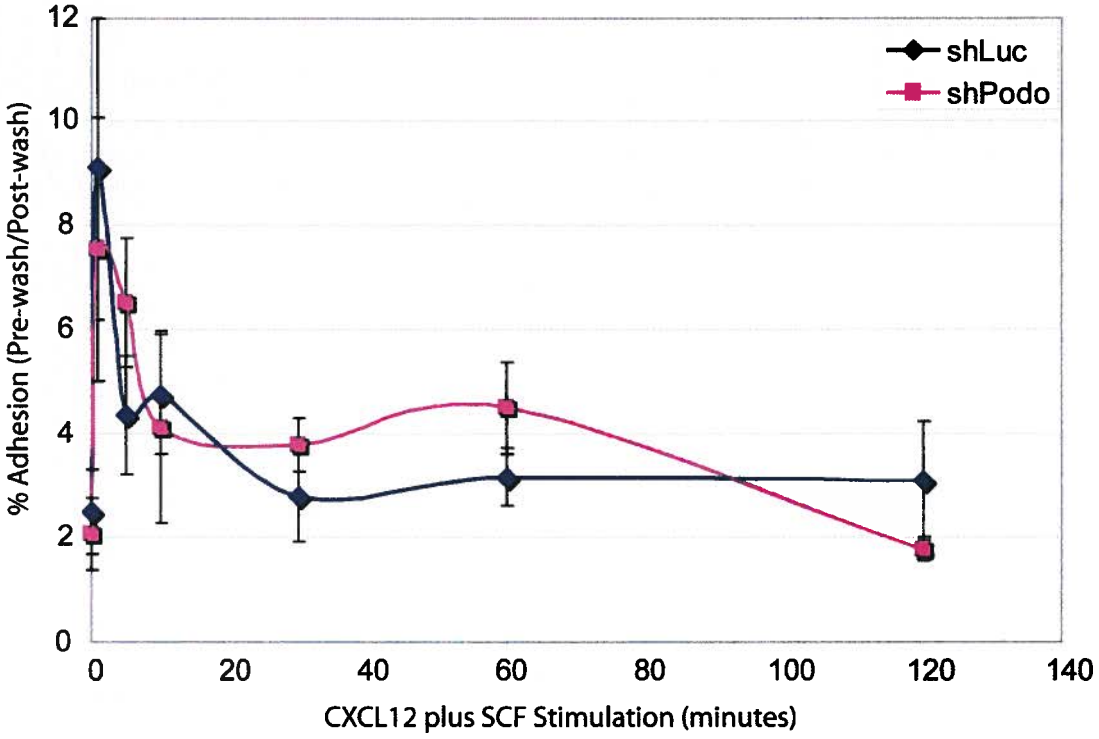


Figure 3.8 Loss of Podocalyxin does not affect CXCL12 and SCF stimulated cell adhesion on fibronectin matrix.

Cells were starved of IL-3 for 3 hours, stained with Calcein-AM and stimulated with CXCL12 and SCF for 1, 5, 10, 30, 60 and 120 minutes. There is no significant cell adhesion differences with shLuc and shPodocalyxin (Blue: Control; Red: shPodocalyxin; * indicates statistical significance of $p<0.05$ with student's t-test analysis, $n = 2$).

3.3.6 Podocalyxin Modulates c-kit and CXCR4 Downstream Signaling Pathways

In addition to possible defects in cell adhesion, we chose to measure CXCR4 receptor activity in shPodocalyxin cells. It has been demonstrated that sialomucins are capable of not only associating with chemokine receptors but that they can also regulate the activity of this receptor by modulating cell signaling (Forde et al. 2007; Veerman et al. 2007). To explore whether CXCR4 and Podocalyxin associates in stimulated cells, we performed confocal microscopy on FDC-P1 cells. To detect surface expression of CXCR4 on these cells, we used an antibody that is specific for the N-terminus region of the CXCR4 receptor. Levesque and colleagues demonstrated that in unstimulated cells, the N-terminus region of full-length CXCR4 ($CXCR4^{FL}$) undergoes proteolytic cleavage to form an inactive truncated version ($CXCR4^{TRUNC}$), which consequently abolishes the ability for CXCL12 binding and subsequently the activation of intracellular signaling (Brelot et al. 2000; Valenzuela-Fernandez et al. 2002; Levesque et al. 2003).

In unstimulated shLuc and shPodoclayxin cells, there was minimal staining for the active $CXCR4^{FL}$ possibly indicating that most of the CXCR4 on the cell surface are $CXCR4^{TRUNC}$ (Figure 3.9A). However, upon stimulation of shLuc cells with a combination of CXCL12 and SCF, active $CXCR4^{FL}$ surface proteins were re-distributed and colocalized with Podocalyxin. Upon one minute of stimulation with CXCL12 and SCF, active $CXCR4^{FL}$ receptor is polarized. As time progressed with both stimulants, $CXCR4^{FL}$ and Podocalyxin re-distribute and localizes with each other. To confirm that

the colocalization of $CXCR4^{FL}$ and Podocalyxin is specific, we also stained for CD43 – a molecule which has been shown to polarize on activated leukocytes. Despite the polarization of $CXCR4^{FL}$, CD43 remains globally expressed at the surface after CXCL12 and SCF stimulation. Furthermore, we used the N-terminus specific CXCR4 antibody and FACS analysis to determine surface expression of $CXCR4^{FL}$. Our FACS analysis further confirmed that upon stimulation, shLuc cells expressed relatively more $CXCR4^{FL}$. In shPodocalyxin cells, there was no detection of $CXCR4^{FL}$ even after persistent stimulation with both CXCL12 and SCF (Figure 3.9 – shPodocalyxin). However, CD43 is polarized at the surface and we speculate that it may be trying to act as a compensatory molecule in the absence of Podocalyxin in shPodocalyxin cells.

We further characterized this association by performing co-immunoprecipitation of stimulated cells with the N-terminus specific CXCR4 antibody and followed by immunoblotting for Podocalyxin (Figure 3.9). Consistent with the confocal data mentioned above, in shLuc cells, $CXCR4^{FL}$ and Podocalyxin are found in a co-immunoprecipitable complex (Figure 3.9B). As expected, in shPodocalyxin cells, Podocalyxin is not in the $CXCR4^{FL}$ co-immunoprecipitable complex. By using a CXCR4 antibody which detects full length protein total CXCR4 (tCXCR4) was determined and was found to be equivalent between each stimulation point including at steady and starved states. This indicates that tCXCR4 is present in starved cells and suggest that most of the receptor exists as $CXCR4^{TRUNC}$ (Figure 3.9B).

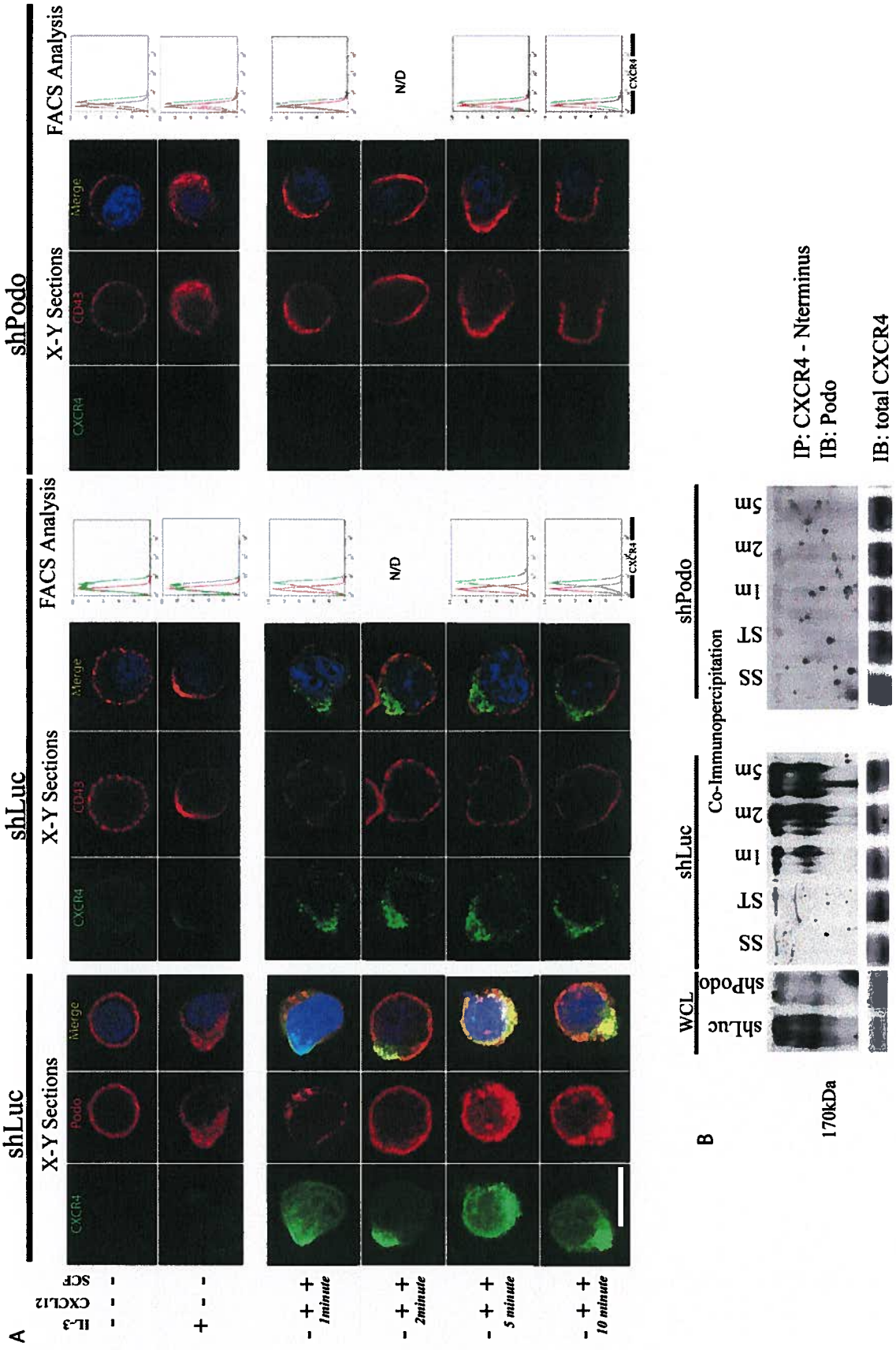


Figure 3.9 CXCR4 co-localizes and co-percipitates with Podocalyxin upon stimulation with CXCL12 and SCF.

Figure 3.9 CXCR4 co-localizes and co-percipitates with Podocalyxin upon stimulation with CXCL12 and SCF. A) Confocal images (X-Y sections) of stimulated shLuc show increased co-localization of both Podocalyxin and $CXCR4^{FL}$ as cells are subsequently stimulated however; there is no co-localization of CD43 and $CXCR4^{FL}$. In shPodocalyxin, little to no $CXCR4^{FL}$ is detected but there is strong polarization of CD43 in stimulated cells. FACS profiles show the difference in expression levels of active $CXCR4^{FL}$ in shLuc and shPodocalyxin. Red lines: Isotype control; Green lines: CXCR4 staining. B) Co-immunopercipitation with N-terminus specific CXCR4 antibody and immunoblot of Podocalyxin showing that there is a gradual increase of co-immunopercipitated Podocalyxin as stimulation progresses. (n = 2)

To gain further mechanistic insights into the importance of Podocalyxin for chemotaxis, we investigated the formation and activation of signaling complexes in wildtype and mutant cells. AKT is a known downstream mediator of chemotactic signals, particularly of CXCR4 and so we examined its activation in response to SCF and CXCL12 in knockdown cells. Cells were starved of IL-3 for three hours and then stimulated with IL-3, CXCL12 and SCF at different lengths of time prior to analysis of AKT phosphorylation.

We found that lower levels of phosphoAKT in steady state and IL-3 starved Podocalyxin knockdown cells and a complete lack of enhanced phosphorylation after CXCL12 and SCF stimulation (Figure 3.10).

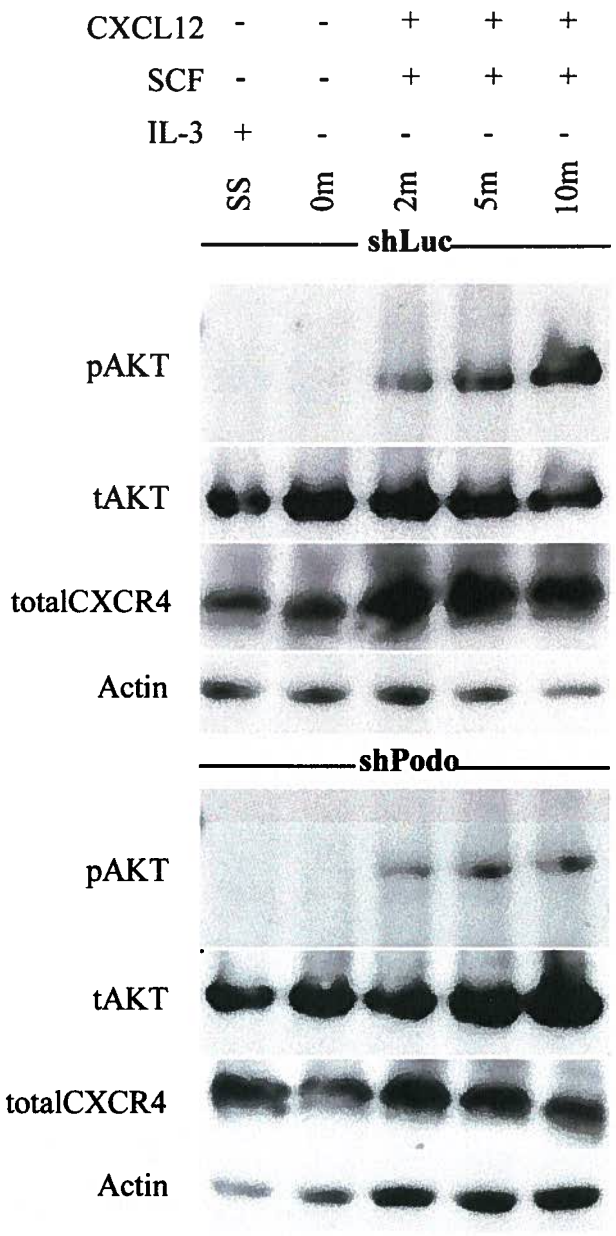


Figure 3.10 AKT phosphorylation is down regulated in shPodocalyxin cells upon stimulation with CXCL12 and SCF.

Cells were starved and then stimulated with CXCL12 and SCF at the indicated times. Blots were generated from cell lysates from each time point then detected with appropriated antibodies. Actin was used as a loading control. pAKT bands are indicated by open arrowheads. AKT phosphorylation is reduced in cells deficient for Podocalyxin. (n = 2)

Previously we showed that Podocalyxin is uniformly distributed on the surface of IL-3 starved FDC-P1 cells but that and that in response to IL-3 stimulation it rapidly accumulates in a polarized pattern (Tan et al. 2006). We found a similar redistribution of CXCR4 and Podocalyxin upon CXCL12 and SCF signaling. We and others have shown that SCF can act as a synergistic molecule responsible for up-regulating cell surface proteins such as CXCR4. However, in shPodocalyxin cells, active $CXCR4^{FL}$ expression is completely absent, suggesting that Podocalyxin may not only be required for the polarization of CXCR4 but also for efficient activation of the receptor as well. Subsequently, proper polarization and activation of CXCR4 in the presence of Podocalyxin may be required for effective chemotaxis.

To determine whether this effect is due to the formation of a complex between CXCR4 and Podocalyxin we performed co-immunoprecipitation assays before and after chemotactic signaling. As shown in Figure 3.9, immunoprecipitation of podocalyxin and blotting for CXCR4 revealed the formation of a distinct activation-dependent CXCR4/Podcalyxin complex. We did not find c-Kit in the co-immunoprecipitation complex (data not shown). We conclude that Podocalyxin is required for efficient signal transduction by being present in a complex with CXCR4 and its subsequent association with CXCR4 receptor facilitates chemotaxis towards a chemotactic gradient.

3.4 Discussion

We and others have previously shown that Podocalyxin modulates cell-cell adhesion in adherent cell lines (Takeda et al. 2000; Somasiri et al. 2004; Nielsen et al. 2007). We have shown that its relative, CD34 could act as an anti-adhesion molecule on mast cells and thus, we hypothesize that Podocalyxin may also have the same function (Drew et al. 2005). To test this hypothesis we used a lentiviral shRNA system similar to previously described protocols (Rubinson et al. 2003) to silence Podocalyxin. Subsequently, adhesion of FDC-P1 cells was assessed. We did not find any significant differences in adhesion of these cells to stromal monolayer or fibronectin matrix upon stimulation with both CXCL12 and SCF.

We found that FDC-P1 posses two receptors, CXCR4 and c-kit which have been widely published to play key roles in the HSC homing to the bone marrow (Broudy 1997; Papayannopoulou et al. 1997; Pituch-Noworolska et al. 2003; Nilsson et al. 2006). Since migration is one of the key steps to successful homing, we chose to look at how well cells lacking both proteins migrated across fibronectin matrix and stromal monolayer towards a chemotactic gradient. We found that migration is dependent on Podocalyxin. To further investigate this mechanism, we used biochemical methods to determine if there were any defects in the signaling pathways commonly used by CXCR4 and c-Kit. Our results indicate that pAKT, a key signaling molecule in the downstream pathway of both receptors, is not activated in Podocalyxin deficient cells. Therefore, it is possible that by altering the expression of Podocalyxin on FDC-P1 modulates the CXCL12 and SCF migratory function of myeloid progenitor cells.

There are many groups that have demonstrated that CXCL12/SCF and CXCR4/c-kit are critical players for homing and retention of HSCs at the bone marrow stromal niche. There has been other evidence that both CXCR4 and c-Kit cooperate with other receptors such as CD26 (Herrera et al. 2001) and CD45 (Fernandis et al. 2003) to mediate homing and modulate downstream components of certain signaling pathways. This suggests that a transmembrane molecule such as Podocalyxin could potentially modulate CXCR4 signaling pathways in a tissue dependent manner. Upon stimulation of CXCR4 with CXCL12, a molecule such as AKT is activated downstream via PI3K and subsequently activates further signaling events that include cell mobility. Our data show that in FDC-P1 cells, upon binding of CXCL12 with CXCR4 receptor increases phosphorylation of AKT and that our data suggests that Podocalyxin is required for the activation of this pathway in myeloid progenitor cells. This signaling cascade has been shown to be involved in cell adhesion and chemotaxis through cell polarization resulting in enhanced migration to different microenvironmental niches. We show that cells lacking Podocalyxin have no adhesion defects on stromal cells however, there is a significant ($p<0.05$) defect in migration. As mentioned, one other possible mechanism by which Podocalyxin may regulate CXCR4 signaling is by direct physical interaction of Podocalyxin and CXCR4 at the membrane.

There are several ways in which Podocalyxin could facilitate this synergistic homing. The fact that migration of shPodo cells were impaired, suggest that it plays a role in either the signaling with (discussed above), or the stable maintenance of CXCR4 on the

plasma membrane. The fact that we observe no polarized relocalization of the residual CXCR4 on the surface of Podocalyxin knockdown cells argues that Podocalyxin may play a role in lateral membrane transport of this chemokine receptor. Finally, as mentioned CXCR4 is subject to inactivation via amino terminal proteolysis. It is therefore conceivable that Podocalyxin, as a highly glycosylated mucin, may "protect" or "shield" CXCR4 from proteolysis when they are tightly associated in a complex. Further experiments will be required to determine which of these mechanisms play the most dominant role in Podocalyxin modulation of chemotaxis.

The interaction of Podocalyxin with CXCR4 is reminiscent of another similar sialomucin, CD164. This transmembrane sialomucin, also known as endolyn is an adhesion receptor that regulates the adhesion of $CD34^{+}/CD133^{+}$ human cord blood cells to the bone marrow stroma and subsequently the recruitment of these cells to the niche (Schopperle et al. 2003). In addition, knocking down CD164 via shRNA also resulted in a down-regulation of pAKT and dramatic decrease in migration. Both CD164 and Podocalyxin are type I transmembrane sialomucins with heavily glycosylated extracellular domains, with putative PDZ domain docking site (Sassetti et al. 1998; Forde et al. 2007). It is possible that CD164 is responsible for the residual homing seen with Podocalyxin knock-down cells.

SCF plays an important role in homing of HSCs. It is accepted that SCF plays a synergistic role with a multitude of proteins (Levesque et al. 1995; Miyazawa et al. 1995). It has been shown that cells exposed to SCF upregulates VLA-4, VLA-5 and

CXCR4 (Hart et al. 2004) and that HSC cells exposed for a very short time to SCF prior to transplantation engraft the recipient at a higher frequency (Okada et al. 1991; Simmons et al. 1994; Driessen et al. 2003). It is obvious that in our observations with shLuc and wildtype cells, migration only dramatically increases in the presence of both CXCL12 and SCF. It is possible that SCF alone may lead to expression of CXCR4 on the surface but without CXCL12 present, the receptor does not get activated and hence no migration occurs. Moreover, the binding of CXCL12 to endogenous levels of CXCR4 may not be optimal for stimulating migration but is sufficient for viability and thus the synergistic affects of both CXCL12 and SCF is necessary for the expression and activation of optimal levels of CXCR4 for migration to occur.

Our results suggest that Podocalyxin may function as a regulator of homing of myeloid progenitor cells by modulating migration of these cells towards a chemotactic gradient. Since Podocalyxin has recently been associated with an ever-widening array of epithelia tumors (Schopperle et al. 2003; Somasiri et al. 2004; Casey et al. 2006; Heukamp et al. 2006; Ito et al. 2007; Ney et al. 2007; Sizemore et al. 2007), it may become a useful marker for predicting poor outcome. In this regard, CXCL12 has also been show to play an important role in the bone and peripheral metastasis of these tumors. Thus, Podocalyxin upregulation on these tumors may reflect the acquisition of competence for CXCL12-dependent metastasis. The fact that Podocalyxin antibodies inhibit this behavior *in vitro* suggests that these antibodies may be of therapeutic benefit.

NB. Studies similar to Chapter 3 were also performed with shNHERF-1 FDC-P1 cells. Please refer to Appendix A for results.

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Chapter 4 : Informatics and Cell Shape Analysis: Role of Podocalyxin in Uropod Formation and Cell Migration[1]

4.1 Introduction

After the cells are infused into the host, HSCs ability to reconstitute a lethally irradiated host depends on crucial events that take place. One of the hallmarks of HSCs is the ability to home to its appropriate niche, lodge, differentiate and maintain its population throughout the host's lifetime. This homing process requires many functional steps, including cell migration.

Historically, HSC migration studies have been based on short-term *in vivo* homing assays to the bone marrow and *in vitro* chemotaxis that utilizes transwells or Boyden chambers to measure migration properties. Indeed these assays are useful when questioning chemotactic response abilities for the cells towards a known gradient. However, it offers little information about the dynamics of the cell as migration occurs. There are countless studies on the influence of cell morphology on cell mobility and most of them are based on the locomotion of leukocytes. Efficient accomplishment of these functions requires regulated cellular cytoskeleton re-organization, receptor localization, recruitment of signaling molecules and changes in the morphology of the cell (Vicente-Manzanares et al. 2004).

[1]A version of this chapter is in preparation of publication. Tan PC., Gamble D., Kelly L., Nezamoddin KN., Fiegth P., Bains J., McNagny KM., Jervis EJ. Informatics and Cell Shape Analysis: Role of Podocalyxin and NHERF-1 in Uropod Formation. (2008) Manuscript in preparation for submission August 2008.

The first step for leukocyte migration is to establish a polarized morphology. Prior to migration, polarization mediates re-distribution of receptors and molecules to their appropriate regions. Typically, polarization is triggered by chemoattractants such as CXCL12 and SCF, which induce changes in actin redistribution to a particular region in the cell and thus generating a polarized morphology (Coates et al. 1992).
The leading edge is the front of migrating cell and is the region with a concentration of chemokine and cytokine receptors. At this region, the membrane stretches out like the edge of a fan. The uropod is the back end of the cell and typically has an accumulation of adhesion molecules such as $\alpha_4\beta_1$ integrins, CD43 and PSGL-1 (Sanchez-Madrid et al. 1999).

It has been shown that HSCs exhibit many of the characteristics of leukocytes during their migration process towards the bone marrow niche (Francis et al. 2002). In addition to these structures, HSCs extend filopodia, which are sensory or guidance apparatuses for HSCs (Davenport et al. 1993; Francis et al. 2002). Unlike uropods, filopodia are deployed and retracted at the leading edge of the cell and upon retraction the filopodia leaves behind a part of the cell membrane at the point of contact (Francis et al. 2002). It is not known whether leading edges occur immediately after establishing filopodial connections or if they occur independently. Little is known about the dynamics of HSCs morphology during the homing process.

We have shown that Podocalyxin is important in HSC migration (Tan et al. Stem Cells 2008, submitted) and cell-cell adhesion (Nielsen et al. 2007). As mentioned, Podocalyxin

is a hematopoietic stem cell surface protein. It is a transmembrane sialomucin with heavily glycosylated extracellular regions and a highly conserved cytoplasmic region with a PDZ domain docking site. FDC-P1 cells are an IL-3 dependent murine myeloid progenitor cell line (Dexter et al. 1980). FDC-P1 cells are able to generate uropods and in a sub-population of cells, both NHERF-1 and Podocalyxin are co-associated at these structures.

Using a lentiviral system to silence NHERF-1 and Podocalyxin proteins, we were able to examine their effect on uropod dynamics cell migration rates. shLuc and shPodocalyxin populations were exposed to a variety of conditions for five hours and were observed with real-time imaging (Dykstra et al. 2006) In our study, cell phenotypes were examined under dynamic conditions by allowing cells to flow past a microscope objective lens at a constant velocity. Rates of uropod formations are measured manually by tracking cells using custom Matlab software. This process was extended by developing novel automated image segmentation algorithms that permit high-resolution cell identification (uropod formation) and subsequent cell tracking (migration rates). In particular, image analysis routines were used to classify the shapes of the cells and measure their velocity.

Imaging studies revealed that Podocalyxin knock-down cells have fewer and less persistent uropods upon stimulation with CXCL12. While these differences are statistically significant, only a fraction of each population exhibits these differences. We found that with CXCL12 and SCF stimulation in shPodocalyxin cells, there is no

statistical significance for the frequency of uropod formation. In addition, the system examines cell migration speeds after stimulation with CXCL12 and SCF on a fibronectin matrix. As time progresses, shPodocalyxin cells have slower migration speeds compared to shLuc cells. By using a test for significance, ANOVA, cells which demonstrated persistent uropods did not have an advantage in cell migration and thus, suggest that migration speeds are determined by the presence of chemoattractants instead.

4.2 Materials and Methods

4.2.1 Cells

Factor dependent cell-Paterson1 cells (FDC-P1) were maintained in RPMI (Hyclone) with 10% FBS (Gibco), 4mM L-Glutamine (Gibco), 1x Penicillin and Streptomycin (Gibco) and 10% WEHI-3B (made in house) conditioned media. Knock-down cells were maintained with 1μg/ml of G418 (Sigma-Aldrich) for selection and were removed two weeks prior to experiments.

4.2.2 Antibodies

Rabbit anti-NHERF-1 antibody ab3452 (Abcam; USA; http://www.abcam.com) was used for all fluorescent assays and rabbit anti-NHERF-1 antibodies APZ-006 (Alomone Laboratories; Israel; http://www.alomone.com) and ab3452 were used for immunoblot analyses. Rat anti-mouse Podocalyxin antibody MAB1556 (R&D; MN, USA; http://www.rndsystems.com) was used for all staining, immunoprecipitation and immunoblot studies. Secondary antibodies were goat anti-rabbit AlexaFluor 488

(Molecular Probes; Ont. Canada; http://www.invitrogen.com), goat anti-rat AlexaFluor 568 (Molecular Probes Ont. Canada; www.invitrogen.com) and goat anti-rat PE (Pharmingen; CA, USA; http://www.bdbiosciences.com/pharmingen). Isotype controls were rabbit IgG (H&L) (Vector Laboratories; CA, USA; http://www.vectorlabs.com) and rat IgG2a (Cedarlane; Ont. Canada; http://www.cedarlanelabs.com). CXCR4 antibodies were purchased from Santa Cruz and phosphor-AKT antibody is from Cell Signaling. All secondary HRP antibodies for immunoblotting were purchased from Dako.

4.2.3 Immunoblotting

SDS-page was performed as described (Tan et al. 2006). Nitrocellulose membranes were blocked with 10% BSA or non-fat milk in TBS-T for either two hours at room temperature or overnight at 4°C. Membranes were incubated with primary antibodies overnight at 4°C and HRP conjugated antibodies for one hour, one minute with ECL chemiluminecence reagent and exposed to hyperfilm.

4.2.4 Bio-Assembly, Mosaic Builder, and Information system (BAMBI)

Developed by a group of bio-engineers, which was led by Dr. Eric Jervis the BAMBI system is a combination of specially designed computer software, robotics, a high-resolution microscope and digital camera paired with cell culture chambers. Its development is a bioinformatics solution for bridging the gap between conventional cell biology and biochemical techniques.

4.2.5 Imaging Chamber and the Videotracking System

Previously published techniques were integrated into the current study to image cell phenotypes (Dykstra et al. 2006; Moogk et al. 2007). Briefly, cells were cultured in imaging chambers constructed by cutting a microscope slide into 2mm by 8mm rectangle pieces, subsequently these pieces were glued to a glass coverslip. Following this procedure, a 15mm length of 12mm diameter glass tubing was glued to the surface of the coverslip to form up to three rectangular wells. Next, the wells were autoclaved and rinsed in PBS. Prior to culture assays, the chambers were coated with 5% BSA and 100μg/ml of fibronectin at 37°C with 5% CO_2 for at least four hours and rinsed with sterile PBS. A volume of 3-6ul of $5x10^5$ FDC-P1 cells was placed into each chamber.

The cell suspension was added by lifting the coverslip and then lowered carefully, relying on capillary action to consistently fill the chamber. Subsequently, each chamber reservoir was topped with 1ml of media and covered with a second coverslip to avoid evaporation and to maintain sterility. The entire apparatus was tilted at 2 degree angle to generate an incline, which allowed cells to flow at a constant velocity across the imaging unit. Imaging commenced immediately after stimulation with CXCL12 and SCF and different cell phenotypes were recorded for 3.5 hours

Imaging was performed on an inverted microscope (Axiovert 200, Zeiss Germany). Minimizing photo-toxicity involved the use of a shutter, which ensured that incident light from the microscope only reached samples while images were being acquired. A digital camera was used to capture images at three minute intervals (XCD-SX910, Sony).

Initially, the microscope was manually focused and locked. The apparatus was periodically checked to correct for any focal plane drifts during the entire imaging process. These procedures are elaborated in a detailed article by Moogk et al (Moogk et al. 2007).

4.2.6 Image Analysis: Cell Segmentation Model – An Automated process

Following the collection of images taken at three minute intervals, cell phenotypes were quantified using a cell segmentation computer algorithm generated in Dr. Eric Jervis's laboratory. This algorithm measures the different "segments" or cell structures that exist between steady state and stimulated cells. Based on a single image, the model was able to predict and measure the presence of cell boundaries. Cell boundaries were measured based on surface area and the degree of uniformity within the boundary. By applying a probabilistic cell model to phase contrast microscopic images, a map of cell centers were obtained for each frame. The map calculates and quantifies cells that were spherical or exhibited uropods to which it calculated and displayed a graph as a function of time. Cell centers were calculated for each frame and an aspect ratio was generated for the amount of eccentricity of each cell. A ratio of 1 represents a spherical cell and thus a ratio less than 1 deviates from the spherical shape and was assumed to exhibit a non-spherical shape (i.e. a uropod). Next, depending on the experiment, the average values of each dataset was compiled as a function of time or speed.

4.3 Results

4.3.1 Knock-Down Cells Display Uropods.

Our recent study found that Podocalyxin localized to a uropod-like cytoplasmic protrusion (Tan et al. Stem Cells 2008, submitted, Tan et al. 2006). Therefore, we hypothesized that with the absence of Podocalyxin, uropod formation would be inhibited. To test our hypothesis, we utilized shPodocalyxin FDC-P1 cells (generated using a lentiviral based system and as discussed in Chapter 3) as our cells of interest. Both, shPodocalyxin and shLuc cell groups were sent overnight to Dr. Eric Jervis for long-term live-cell imaging (LTLCI) and phenotype analysis using the BAMBI system.

Under identical conditions, the three different cell populations were imaged for approximately five hours. Next, raw image data were imported into custom Matlab software, which allowed the manual cell tracking and scoring of phenotype over the duration of the experiment. Tracking data, including time, position, uropod state, and lineage relationships were recorded in a Microsoft Access database. The database enabled rapid *ad hoc* hypothesis testing on the effects of the gene knock-down on uropod formation. Several parameters were investigated for their effect on uropod presentation including cell speed and acceleration, tracking duration, lineage relationships, and culture time dependent phenomena. The current chapter of this thesis will mainly focus on the affects of the presence and absence of Podocalyxin on 1) uropod formation and 2) cell migration speeds.

As shown in Figure 4.1, both cell populations exhibit different types of morphologies. Under steady state conditions, these uropod-like cytoplasmic protrusions occur transiently and a cell expresses them more than once as imaging progresses. In addition, knock-down cells exhibited these structures and therefore it was tempting to speculate that uropod formation under steady state is Podocalyxin independent.

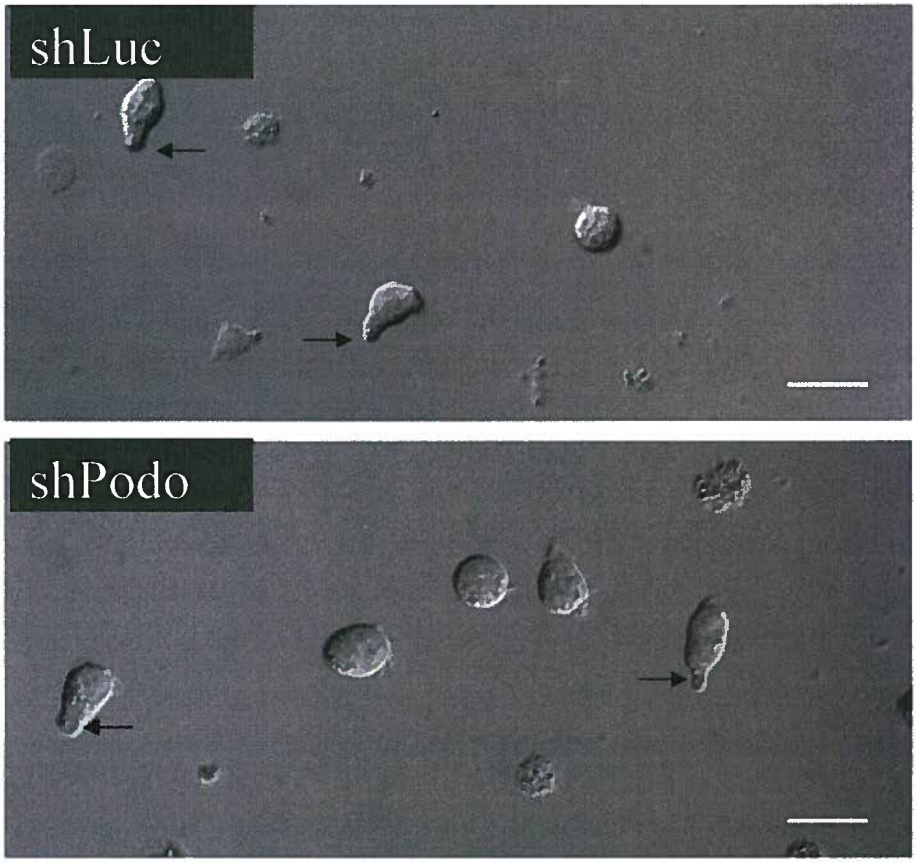

Figure 4.1 Uropod structures are present on shPodocalyxin cells cultured in IL-3.

Representative images from each population are shown with arrows indicating scored number of uropods. Cells were maintained in complete tissue culture media and WEHI-3B. All scale bars are 20μm.

4.3.2 Cells Lacking Podocalyxin Have Fewer Uropods with CXCL12 Stimulation.

Initially, cells were starved of IL-3 for three hours, followed by stimulation with CXCL12, and subsequently imaged for up to 3.5 hours. During the starvation state, 10% of shLuc cells exhibited phenotypes with uropods. This is significantly greater than shPodocalyxin, which showed uropods less than 5% of the time. Closer analysis revealed that as starvation persisted, the frequency of uropods steadily declined with shLuc and shPodoclayxin cells. Before the addition of CXCL12, the frequency of uropods remains between the ratio of approximately 0.035 and 0.07 (Figure 4.2 - black arrow) and after the addition of CXCL12, the number of uropods increases dramatically from baseline. The fraction of cells with uropods increased with shLuc and remained relatively low with shPodocalyxin cells (Figure 4.2). In shLuc cells, the proportion of cells that display a uropod increased 3.5 fold, from a baseline ratio of 0.07 to 0.24. In contrast to shPodocalyxin which increased 2.5 fold, from a baseline ratio of 0.035 to 0.09.

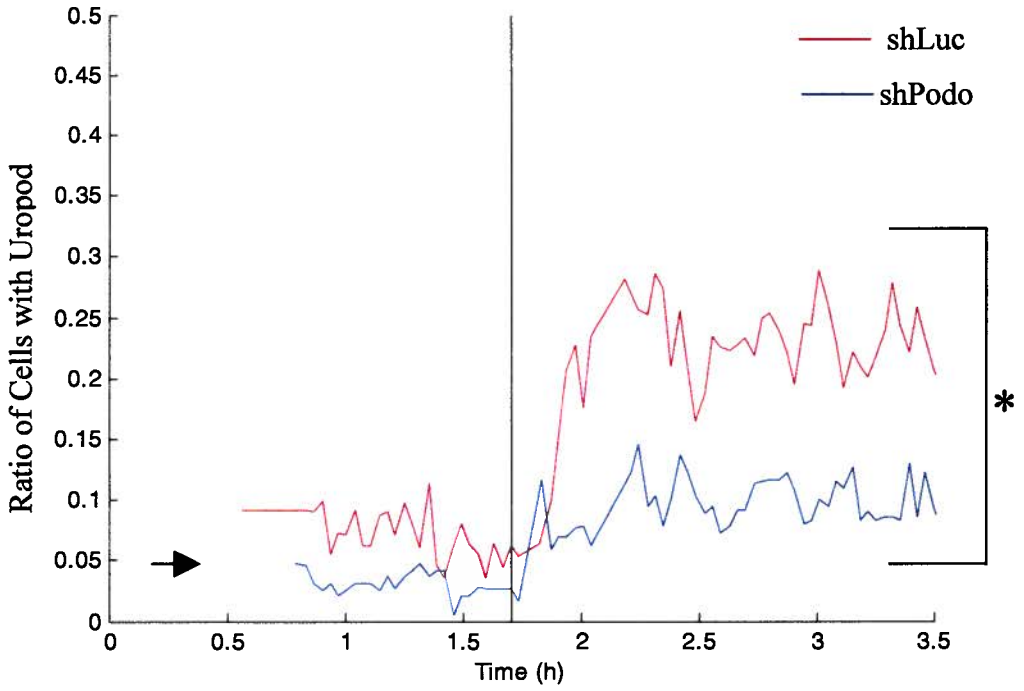

Figure 4.2 Cells lacking Podocalxyin tend to have fewer frequencies of uropods with CXCL12 stimulation.

Vertical black line signifies the addition of CXCL12; images were captured at three minute intervals between 0 and 3.5 hours. Cell images were manually scored for the presence and absence of uropod. Red: shLuc cells and Blue: shPodocalyxin cells. (* $p<0.05$, ANOVA analysis, $n = 3$)

4.3.3 Cells Lacking Podocalyxin have equivalent rates of migration under CXCL12 stimulation

As mentioned in Chapter 3, cells were treated similarly to look for any migration speed defects using the BAMBI system. There is no difference in migration speeds between shPodocalyxin and shLuc. In addition, findings revealed that CXCL12 stimulation alone is not sufficient to stimulate migration. Thus, migration speeds remained at

approximately 0.05μm/s before and after stimulation with CXCL12 (Figure 4.3). It is possible, that independently, CXCL12 is sufficient for stimulating a response, but is insufficient to trigger cell mobility. The initial spike with CXCL12 generates false migration due to a problem with fluidics and is disregarded in the final analysis (Figure 4.3, ++).

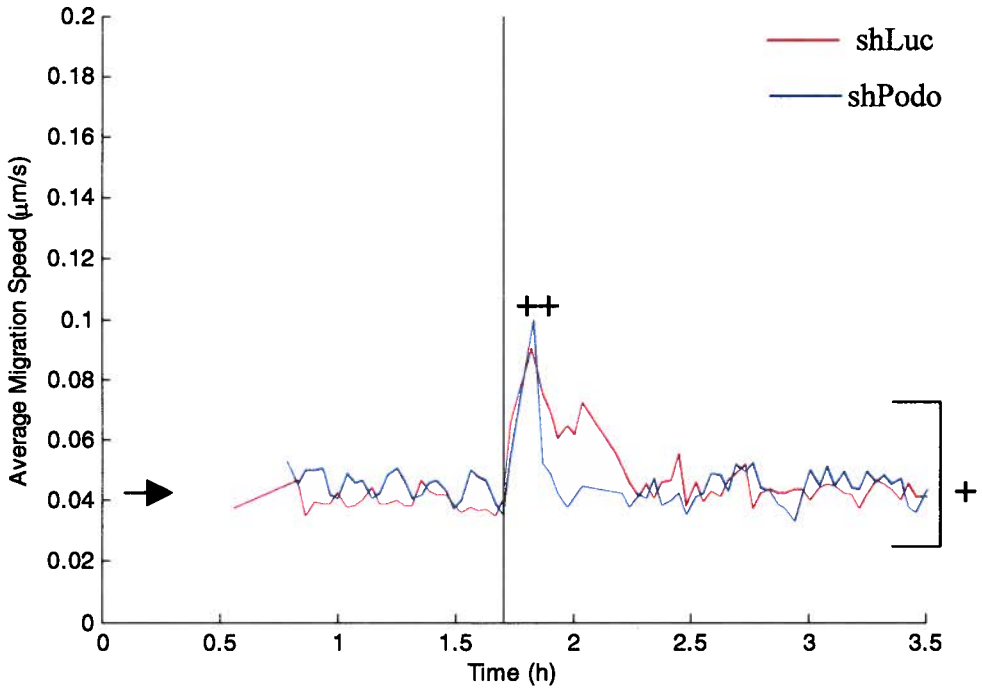

Figure 4.3 Cells lacking Podocalyxin have "normal" migration speeds with CXCL12 stimulation.

Cells were starved and then stimulated with CXCL12 only (black vertical line) and images were acquired and migration speed was measured. FDC-P1 cells lacking Podocalyxin did not have any significant migration differences in velocity from shLuc cells compared to baseline (black arrows). Blue line: shPodocalyxin and red line: shLuc cells. (+ $p>0.05$ ANOVA analysis, $n = 3$)

4.3.4 shPodocalyxin Cells Have Equal Frequency of Uropod Formation when Stimulated with Both CXCL12 and SCF

In contrast to findings in 4.3.2, shLuc and shPodocalyxin cells stimulated with *both* CXCL12 and SCF exhibit the same frequency of uropod – ratio from 0.04 to 0.25, a 6.25 fold increase. This suggests that uropod formation is independent of Podocalyxin and thus imply that, uropod formation is dependent on the presence of cytokine (Figure 4.4). Therefore, we suggest that in shPodocalyxin cells, SCF may be pertinent for the formation of uropods. It is possible that this phenomenon is attributed to SCF and c-kit's role as activators, since the downstream signaling of c-kit activates the PI3-kinase pathway, which is a known activator of molecules involved in the dynamics of cell morphology.

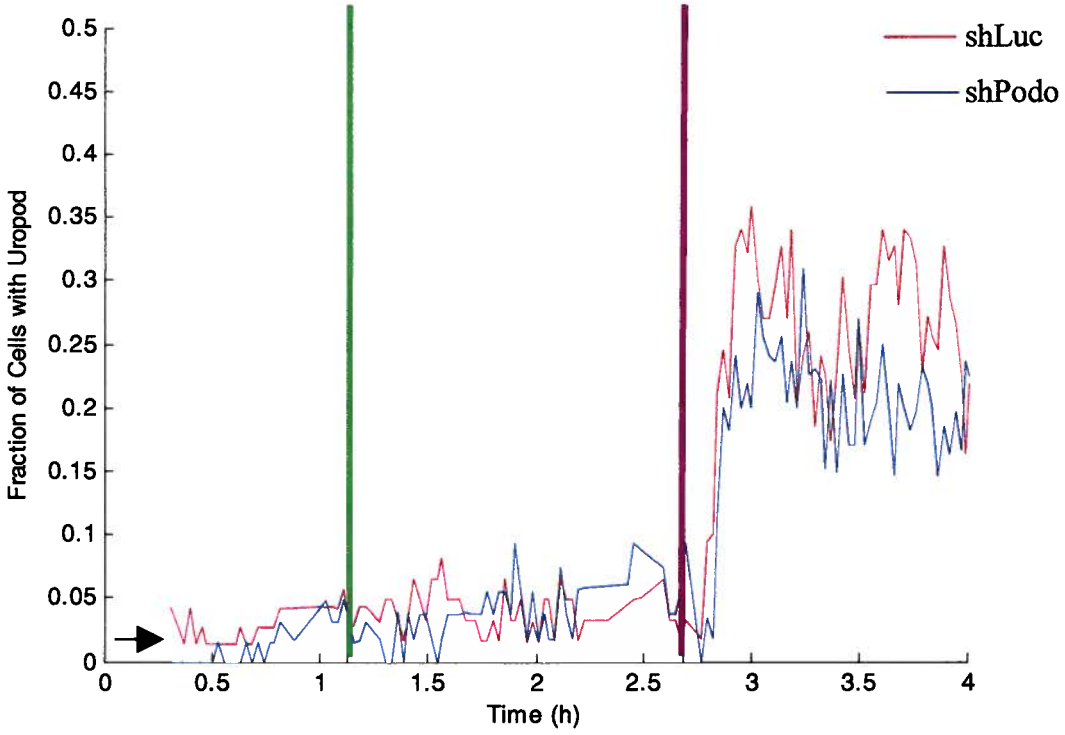

Figure 4.4 Podocalyxin deficient cells have equal frequency of uropods after CXCL12 and SCF stimulation.

Cells were stimulated with CXCL12 and SCF (purple line). As a control, cells were also spiked with media alone (green line). Red: shLuc; Blue: shPodocalyxin. There is no difference in the frequency of uropods after stimulation with media alone and the fraction of cells that exhibited uropods at this time was the same as baseline; before media stimulation at a ratio of approximately 0.035 (black arrow). Stimulation with a combination of CXCL12 and SCF causes the frequency of uropods (for both shLuc and shPodocalyxin cells) to increase within 30 minutes of stimulation and appear to steadily decrease as time persisted (n = 3).

In addition to uropod frequencies, we also ask whether migration speeds are affected in shPodocalyxin cells after stimulation with CXCL12 and SCF. To address this issue, shPodocalyxin and shLuc cells were starved and then stimulated with both CXCL12 and SCF. We found that with shLuc cells there was a two-fold increase in cell migration

speeds, from 0.04 to 0.08μm/s. However, in shPodocalyxin cells, migration speeds stayed relatively constant from 0.04 to 0.047μm/s (Figure 4.5). These observations are in contrast to what we observed in Figure 4.3. It has been previously shown that CXCL12 and SCF act in a coordinate fashion to modulate changes in cell mobility (Kijima et al. 2002). Therefore, it is possible that in FDC-P1, both chemokine and cytokine is necessary for triggering cell migration across fibronectin matrix.

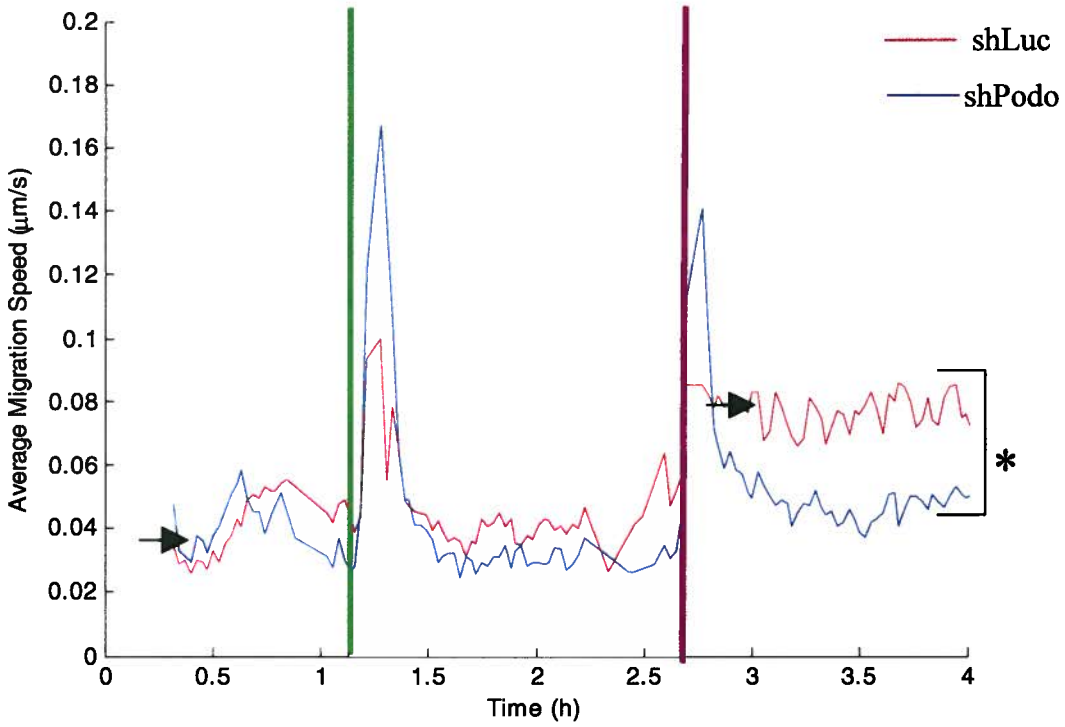

Figure 4.5 Cells lacking Podocalyxin migrated at slower speeds with CXCL12 and SCF stimulation.

Cells were stimulated with CXCL12 and SCF (purple line) and media as a control (green line). After stimulation with media as control, cell fluidics returned to average baseline speeds of approximately 0.035µm/s and increases after CXCL12 and SCF stimulation (black arrows). Cells lacking Podocalyxin have slower migration speeds compared to shLuc cells. shPodocalyxin cells are 2.0 times slower than shLuc. Initial spikes immediately after the addition of media or chemoattractants are a result of fluidics within the culture chamber. Red: shLuc; Blue: shPodocalyxin. (* $p<0.05$), $n = 3$.

4.3.5 The Presence of Uropods Does Not Correlate With Cell Migration Speeds

The effect of uropod frequency on migration was analyzed. As shown in Figure 4.6 CXCL12 stimulation alone does not affect the migration speeds of either control or shPodocalyxin cells. Following stimulation with CXCL12 alone, we observed that during migration, cells exhibiting a uropod did not have an advantage (i.e. uropods do not affect migration speeds).

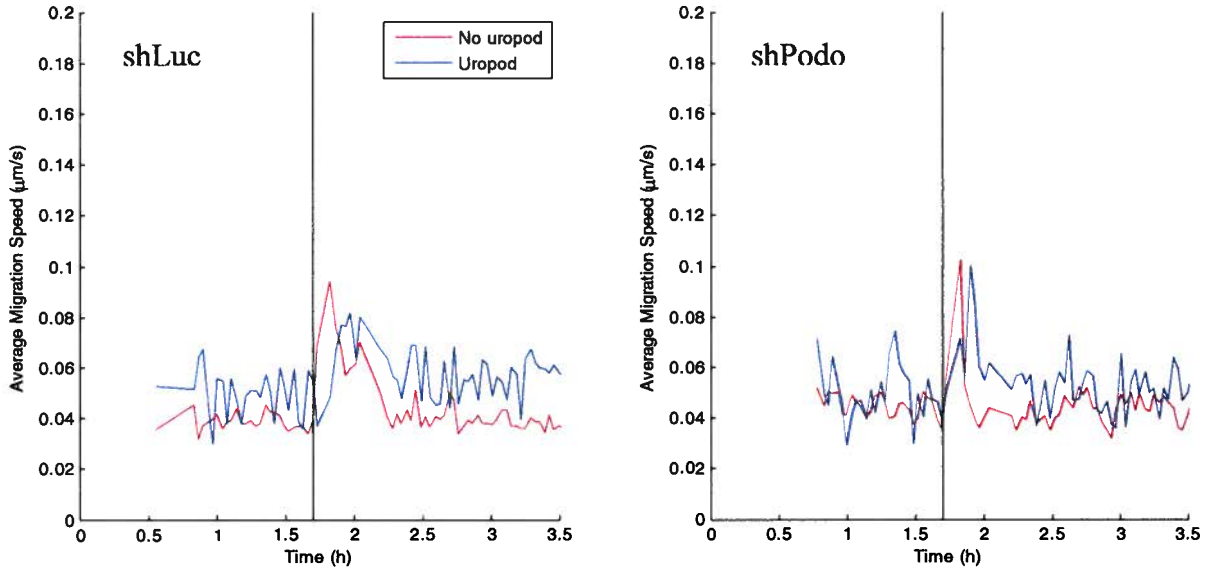

Figure 4.6 The presence of uropods does not correlate with cell migration speeds after CXCL12 stimulation.

Migration speed is charted as a function of uropod frequencies. There is no difference in migration speeds between cells that exhibit or lack uropods within each cell population: A) shLuc and B) shPodocalyxinB. Black line indicates the addition of CXCL12. (n = 3)

Next, shLuc and shPodocalyxin cells were stimulated with *both* CXCL12 and SCF. Although there is statistical difference between the overall migration speeds of shLuc (0.095um/s) and shPodocalyxin cells (0.05um/s), cells exhibiting uropods did not have an advantage in migration (Figure 4.7). Thus, this suggests that migration speeds is more likely dependent on the presence of cytokines.

The first spike seen in the graph is due to the addition of media and is omitted in final analysis (Figure 4.7). After stimulation with a both CXCL12 and SCF (Figure 4.7, second spike) shLuc displayed an increased in migration speed when compared to stimulated shPodocalyxin cells. In contrast, stimulation of shPodocalyxin cells did not increase cell migration when compared to its baseline. However, the presence or absence of a uropod does not correlate with cell migration speeds. For example, by looking within each knock-down population, cells having a uropod do not appear to have an advantage in cell migration.

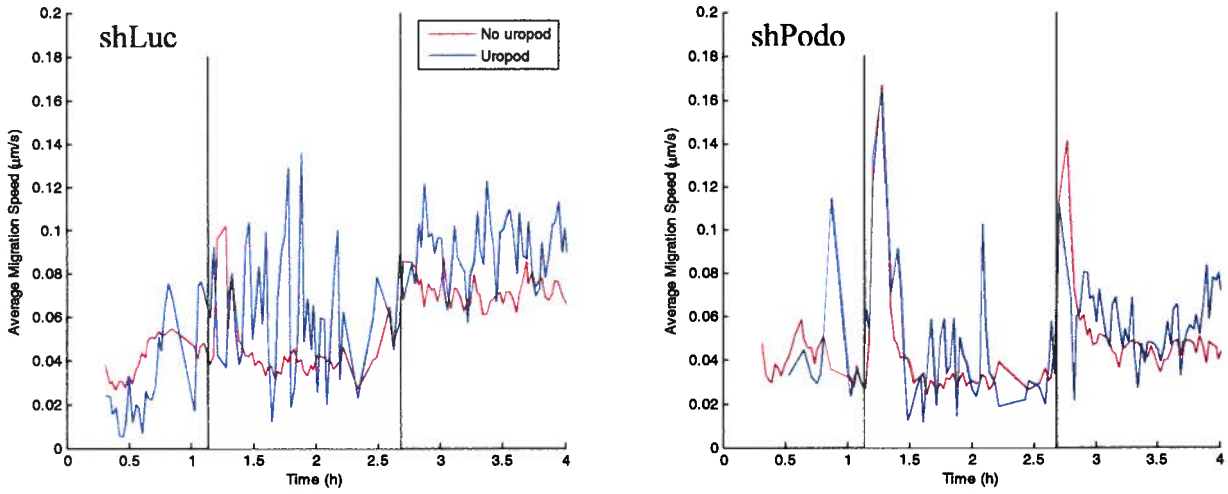

Figure 4.7 The presence of uropods does not correlate with cell migration speeds after CXCL12 and SCF stimulation.

Migration speed is charted as a function of uropod frequencies. A) shLuc cell migration speeds divided into populations with or without uropods. B) Migration speed of shPodocalyxin cells divided into population with our without uropods. (n = 3)

A test with ANOVA suggests that migration speed is dependent on a combination of SCF and CXCL12 stimulation and is affected by the lack of Podocalyxin. Cells exhibiting or lacking uropods, does not appear to correlate with cell migration speeds (Table 2). However, with CXCL12 stimulation the presence or absence of uropods is dependent on Podocalyxin expression.

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Population	0.4123	1	0.41233	299.69	0
Period	0.3239	2	0.16195	117.71	0
Uropod	0.0623	1	0.0623	45.28	0
Population*Period	0.2923	2	0.14616	106.24	0
Population*Uropod	0.0093	1	0.00933	6.78	0.0092
Period*Uropod	0.0061	2	0.00303	2.21	0.1102
Population*Period*Uropod	0.0056	2	0.0028	2.03	0.1308
Error	68.1794	49554	0.00138		
Total	79.5517	49565			

Table 2: Analysis of Variance between different attributes of shLuc and shPodocalyxin cell populations. Test of significance between different attributes of shLuc and shPodocalyxin. Population: shLuc and shPodocalyxin, period: starved, media only stimulation, SCF and CXCL12 cocktail, uropod: cells exhibiting and lacking a uropod, sum sq: sum of squares, df: degrees of freedom, mean sq: mean of squares, F: F ratio, Prob>F: p-value)

4.4 Discussion

As mentioned, uropods are commonly found on migrating cells especially during leukocyte recruitment. We found that under normal steady state conditions, FDC-P1 cells are capable of forming uropods transiently (unpublished observations). Additionally, we have shown shPodocalyxin cells have impaired cell migration across fibronectin towards a CXCL12 and SCF gradient (Chapter 3 and Tan et al. Stem Cells 2008, submitted). Therefore, we asked whether these cells have decreased frequencies of uropods. By using the BAMBI system, we were able to image individual cell morphology during stimulation with CXCL12 and SCF. For the duration of the stimulation, we did not find major differences in the frequency of uropod formation with

shPodocalyxin and shLuc cells. The presence of uropod did not seem to correlate with cell migration speed.

To further investigate the defect in cell migration in transwells, we used the BAMBI system to monitor lateral cell migration on fibronectin coated chambers. Briefly, lateral cell migration is performed by coating glass slides with fibronectin, followed by placing cells onto the matrix and subsequently stimulating them with a combination of CXCL12 and SCF. Individual cells are imaged, analyzed and catalogued based on the migration speed across the matrix. We found that shPodocalyxin cells stimulated with CXCL12 and SCF have overall, a lower migration speed compared to shLuc cells. Therefore, we speculate that the impairment in cell migration with transwell assay (Chapter 3) may be due to decreased migration speeds of shPodocalyxin. We are currently analyzing images of individual cells to detect any defect in cell morphology during this lateral migration process. This procedure may help to explain why lateral migration speeds across fibronectin is slower and therefore, affecting migration across transwells with shPodocalyxin cells.

In addition to cell migration, uropod-like structures have been attributed to the engrafting potential of purified HSCs. Recently, Jervis showed that the activity of highly purified populations of murine HSCs correlated inversely with the frequency of uropod formation (i.e. increased uropod formation, through *in vitro* studies, is an indicator of poor ability to engraft after transplantation. Furthermore, the group found that cells exhibiting uropods are likely to have more frequent cell cycle rates, which supports the generally accepted

rule that HSC tend to cycle less frequently relative to mature cell types (Dykstra et al. 2006). As a result, cells which display a higher frequency of uropods had low HSC activity and therefore higher frequency of cell cycle times. Since this is an on-going project, we are also interested in using the BAMBI system to perform intricate lineage tracing of shPodocalyxin cells to determine whether the cell cycle is affected by the lack of Podocalyxin.

In conclusion, we believe that shPodocalyxin cells have impaired migration in transwells possibly due to reduced lateral migration speeds and this reduction may be due to impaired signaling events that we previously observed (Chapter 3).

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Chapter 5 : Conclusions and Future Directions

5.1 Summary of Findings

Chapter 2: NHERF-1 is a Hematopoietic Ligand for a subset of CD34 Stem Cell Surface Proteins.

- Podocalyxin and NHERF-1 are present in murine LSK cells,
- NHERF-1 associates with Podocalyxin's C-terminal via the PDZ docking site (-DTHL),
- Podocalyxin and NHERF-1 association is induced following IL-3 stimulation,
- Podocalyxin and NHERF-1 co-localizes in a "polarized" fashion.

Chapter 3: Podocalyxin Modulates CXCL12 and SCF Mediated Migration of Myeloid Progenitor Cells.

- Successful knock-down of Podocalyxin expression in FDC-P1 cells by lentiviral-delivered shRNA gene silencing,
- shPodocalyxin cells have "normal" viability and express normal levels of other cell surface antigens,
- shPodoclayxin cells have impaired migration towards CXCL12 and SCF chemogradient,
- active $CXCR4^{FL}$ and Podocalyxin co-localize and associate in control cells,
- in shPodocalyxin cells there is no obvious re-localization of CXCR4,
- shPodoclayxin cells have decreased pAKT in response to activation by CXCL12 and SCF.

Chapter 4: Informatics and Cell Shape Analysis: Role of Podocalyxin in Uropod Formation and Cell Migration.

- CXCL12 and SCF stimulated shPodocalyxin cells have equivalent uropod frequencies however,
- CXCL12 and SCF stimulated shPodocalyxin cells have slower migration speeds on fibronectin coated surfaces,
- There is no correlation between uropod frequencies and cell migration in both shLuc and shPodocalyxin cells.

5.2 Conclusions

We have evaluated Podocalyxin and NHERF-1 as possible new players in the molecular mechanisms regulating part of the homing process. Since its discovery in the HSC system, Podocalyxin's function remains debatable. For the scope of this thesis, we chose to study Podocalyxin and its cytoplasmic ligand NHERF-1 in a murine HSC system by using FDC-P1 as our cell based model.

In FDC-P1, Podocalyxin associates with NHERF-1 via a four amino acid sequence at the C-terminal region of Podocalyxin (Figure 2.1and Figure 2.2) and this association is induced following IL-3 stimulation (Figure 2.4). Previous studies have shown that NHERF-1 has a role as a scaffolding protein in that it associates with membrane proteins at the cell surface to localize and subsequently activate signaling intermediates through these associations. We hypothesized that Podocalyxin may play a role as an anti-

adhesion molecule and that its function may be regulated by NHERF-1. To address this hypothesis, we chose to knock down both proteins in FDC-P1 and subsequently, cell adhesion effects were measured with adhesion assays. Briefly, we used lentiviral – driven shRNA system to silence Podocalyxin and NHERF-1 in FDC-P1 cells (Figure 3.1). Successful knock-downs were verified by flowcytometry, immunobloting and immunocytochemistry (Figure 3.2). Since a lentiviral infection may affect multiple factors in a cell, we also checked for proliferation, viability and normal cell surface expression of antigens. We found that all three attributes were normal and comparable between shLuc and shPodocalyxin cells (Figure 3.2).

Following stimulation with both CXCL12 and SCF, our findings indicated that there were no significant difference in adhesion between shLuc and shPodocalyxin on fibronectin matrix (Figure 3.8). In addition, the expression levels of different adhesion molecules remained the same, indicating that a lack of Podocalyxin expression does not alter the expression of cell adhesion molecules and thus, maintains adhesion abilities (Figure 3.8).

We found that shPodocalyxin (but not shNHERF-1, please see Appendix A) had significant impaired migration across a stroma monolayer (Figure 3.3) and fibronectin matrix (Figure 3.4). In addition, we observed a greater defect in migration with shPodocalyxin when compared to shLuc upon stimulation with both CXCL12 and SCF. To further confirm that Podocalyxin may play a role in chemotaxis, we performed a blocking experiment using a Podocalyxin specific antibody. The results from this assay

showed similar defects in cell migration as well (Figure 3.5). In order to extrapolate our cell migration data from FDC-P1 to primary cells, we chose to use E15 Podocalyxin knock-out ($Podxl^{-/-}$) fetal liver cells (the majority of wild-type fetal liver cells at E15 are Podocalyxin positive). In the context of a mouse genetic model, Podocalyxin-deficient fetal liver cells demonstrated a similar defect in chemotaxis after stimulation with both CXCL12 and SCF (Figure 3.6).

So, what causes the impairment to migrate towards a chemotactic gradient? To answer this question, we used FDC-P1 as our model system to observe whether Podocalyxin associated with CXCR4. To detect surface expression of CXCR4 on these cells, we used an antibody that is specific for the N-terminus region of the CXCR4 receptor. As mentioned in Chapter 3, Levesque and colleagues demonstrated that in unstimulated cells, the N-terminus region of full-length CXCR4 ($CXCR4^{FL}$) undergoes proteolytic cleavage to form an inactive N-terminally specific truncated version ($CXCR4^{TRUNC}$), which consequently abolishes the ability for CXCL12 binding and subsequently, the activation of intracellular signaling (Brelot et al. 2000; Valenzuela-Fernandez et al. 2002; Levesque et al. 2003). Analysis by confocal microscopy showed that in shLuc that not only the active $CXCR4^{FL}$ form is present but that it co-localizes with Podocalyxin in a polarized fashion at the cell membrane shortly after stimulation with CXCL12 and SCF (Figure 3.9). However, in shPodocalyxin cells we could not detect $CXCR4^{FL}$ following stimulation and we predict that in the absence of Podocalyxin two possible scenarios may occur: a) in Podocalyxin deficient cells, there is an absence of CXCR4/Podocalyxin co-complex and thus, the absence of signaling or b) in the absence of Podocalyxin,

$CXCR4^{FL}$ is constitutively being cleaved to the inactive $CXCR4^{TRUNC}$ form and thus, the absence of signaling. To further confirm CXCR4 association with Podocalyxin, we used the N-terminal specific CXCR4 antibody to co-immunoprecipated Podocalyxin from lysates prepared from CXCL12 and SCF stimulated cells. Association of $CXCR4^{FL}$ and Podocalyxin increased over a time course of stimulation (Figure 3.9).

In light of these results, we further speculate that without Podocalyxin's association with CXCR4, signaling pathways regulating migration in response to this chemokine are impaired. We show that shPodocalyxin cells have decreased magnitude and altered kinetics of AKT phosphorylation associated with activation (Figure 3.10).

To further assess our transwell migration studies, we analyzed cell migration through real-time imaging (collaboration with Dr. Eric Jervis from the University of Waterloo). As discussed in Chapter 4, the BAMBI system was used to visualize the behaviour of individual cells before and after stimulation with both CXCL12 and SCF. We were excited to find that on fibronectin matrix, shPodocalyxin cells have slower lateral migration speeds compared to shLuc cells (Figure 4.5). This result corroborates the decrease in chemotaxis in a transwell migration assay (results from Chapter 3) and thus suggests that the migration defect may be due to the velocity of migration rather than cell adhesion defects.

In addition to cell migration speed, we analyzed the dynamics of cell morphology using the BAMBI system. We hypothesized that cells undergoing chemotaxis may exhibit

morphological changes, which have been postulated to be necessary for chemotaxis. From our analysis, we conclude that shPodocalyxin cells exhibit uropod-like cytoplasmic protrusions but that these structures do not appear to play a role in enhancing chemotaxis (Figure 4.6 and Figure 4.7).

The results from this thesis have, for the first time, uncovered mechanistic understanding of how Podocalyxin can regulate cell migration. Since Podocalyxin is present on highly invasive human breast cancer cells with poor prognosis (Somasiri et al. 2004; Nielsen et al. 2007), the results from this thesis have important applications in understanding metastatic conversion of cancer and thus, may begin to provide valuable insights for new therapeutic targets. Since Podocalyxin has a role in murine HSCs migration (Doyonnas et al. 2005), it would be critical to understand how Podocalyxin expression is regulated during this process to improve the success rates of bone marrow transplantation therapies.

Previously, we have shown that Podocalyxin may play a role in cell-cell adhesion in epithelial cells (Nielsen et al. 2007). Our results indicate that Podocalyxin plays a significant role in chemoattractant-dependent cell migration as well.

Cell migration may be beneficial (e.g. bone marrow transplantation) or detrimental (e.g. cancer metastasis) depending on various circumstances. The efficient homing of stem cells to the bone marrow niche is an important factor in determining successful bone marrow transplantation since it aids in repopulating the host (Papayannopoulou et al. 1997; Whetton et al. 1999; Wright et al. 2002; Nilsson et al. 2004; Chute 2006). During

transplantation, only a small percentage of stem cells efficiently home to the bone marrow (Lapidot et al. 2002; Lapidot et al. 2005). In addition, the lack of CXCR4 expression or activity is major a obstacle in the homing process. Lapidot and colleagues have found that human CXCR4 deficient $CD34^+$ cells fail to efficiently migrate back to the bone marrow after transplantation (Lapidot et al. 2002; Tavor et al. 2004). Furthermore, their study revealed that enhanced migration occurred when these cells were stimulated with SCF prior to the assay. SCF enhanced CXCR4 expression on the surface and thus, enhancing cell migration with *in vivo* and *in vitro* assays (Wang et al. 2000; Kijima et al. 2002; Pituch-Noworolska et al. 2003; Kucia et al. 2004; Tavor et al. 2004; Lapidot et al. 2005). This indicates that SCF and CXCL12 have a synergistic affect on chemoattractant-dependent cell migration. The widely accepted dogma states that SCF and CXCL12 are pertinent molecules for the process of migration. My studies provide mechanistic insights into Podocalyxin's role in the homing process. In addition, my work also demonstrates that Podocalyxin's association with CXCR4 is necessary for efficient migration to occur.

Although migration is important during bone marrow transplantation, it is potentially detrimental under inappropriate conditions. With specific types of cancers, poor prognosis is typically associated with tumour reoccurrence and metastasis (Burger et al. 2003; Phillips et al. 2003). For example, breast cancer is characterized by metastasis to particular organs in the body. There is evidence to demonstrate that sites of metastasis are determined by the microenvironment and chemoattractant molecules present in each organ, which can promote homing of tumour cells to these areas (Ulivi et al. 2004).

CXCR4 has been shown to be involved in homing of metastatic breast cancer cells to the bone via a CXCL12 gradient. Podocalyxin is highly up-regulated on a subset of invasive breast cancers. Since Podocalyxin deficient cells have a defect in migration, it is possible that Podocalyxin upregulation aids in tumour progression by enhancing migration to the bone, which results in poor prognosis.

Results from this thesis raise an important question: ***how*** does Podocalyxin play a role in cell migration in either situation? The following is a proposed model for Podocalyxin's role in this process (Figure 5.1).

Since Podocalyxin is associated with CXCR4 upon stimulation with CXCL12, Podocalyxin may be necessary for stabilizing CXCR4 at a specific location at the cell membrane (e.g. at the leading edge or apical regions). As result of this stabilization, CXCR4 is activated following CXCL12 binding through G-proteins and thus, activates PI3K and subsequently pAKT in human hematopoietic stem cells. However, in shPodocalyxin cells, we observe a decrease amount of pAKT further confirming that Podocalyxin may be pertinent for the activation of a cell signaling cascades via CXCR4 during the migration process. One of these signaling cascades downstream of CXCR4 triggers RhoGTPase activity, which is implicated in cell migration. CXCR4 activates RhoGTPases through its G-proteins subunits; therefore we predict that the activation of RhoGTP would also be defective, subsequently affecting the migration process.

It is possible that CXCR4's stabilization is characterized by its active versus non-active state. Since Podocalyxin can be localized to a specific region at the cell surface, its presence may regulate the cleavage process of CXCR4 and thus, determine if the receptor is active or not.

The following is a model that illustrates ***how*** Podocalyxin modulates CXCR4 signaling.

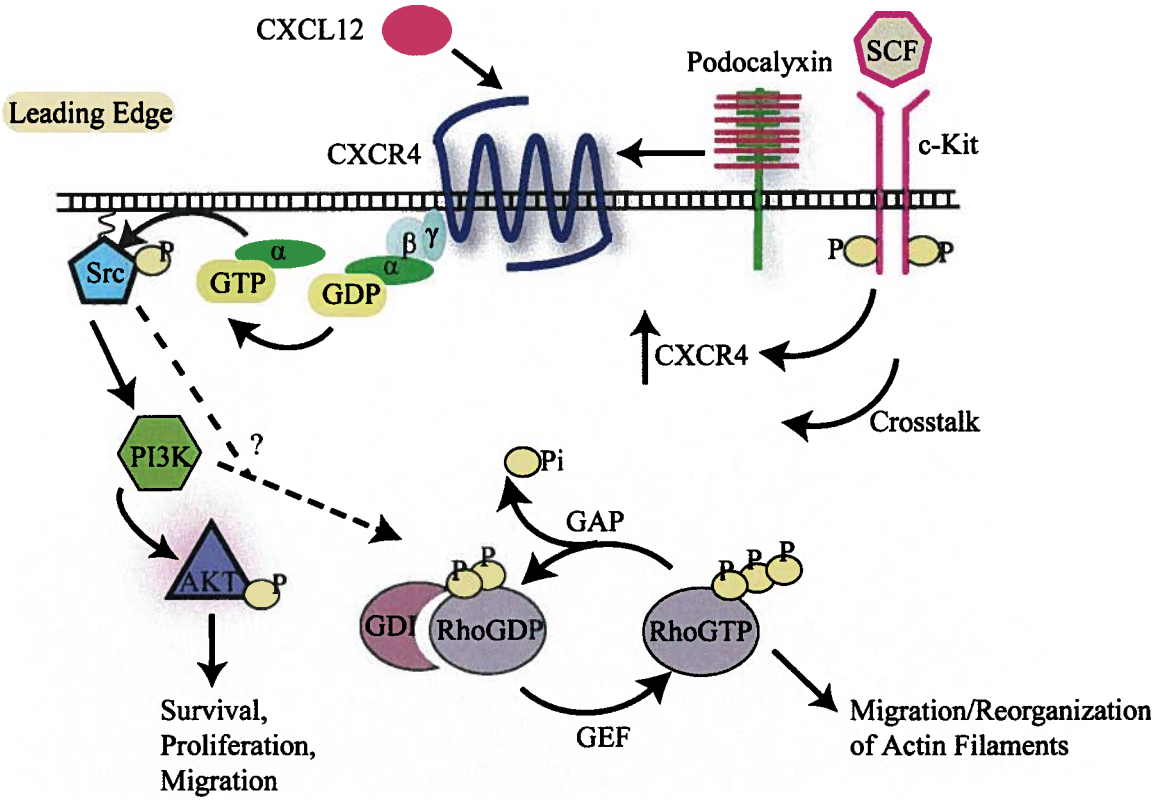

Figure 5.1 Model for the role of Podocalyxin during AKT and RhoGTPase activation in chemotaxis.

Podocalyxin associates and stabilizes CXCR4 at the leading edge of migrating cells. Chemoattractant binds to G-protein coupled receptor, CXCR4, which leads to the translocation and activation of Src kinases to the membrane, subsequently leading to the

activation of PI3K. Activated PI3K catalyzes the phosphorylation of AKT for the regulation of downstream molecules involved in cell survival, proliferation and migration. RhoGEF has been shown to be an effector of PI3K, which lead to the accumulation of RhoGTP. The activation and localization of CXCR4, PI3K and RhoGTP at the leading edge is necessary for cell migration. In addition, activation of c-kit increases CXCR4 expression to further enhance the activity of the receptor and consequently signaling downstream.

5.3 Proposed Future Experiments

Throughout my studies I have gained significant understanding concerning the functions of Podocalyxin, which include its role in development and its affects on cell migration. Although, my results hint at the function of Podocalyxin, many more questions arise from these findings.

I would like to suggest future experiments that will contribute to a greater understanding of how Podocalyxin regulates cell migration. Since Podocalyxin deficient cells have decreased pAKT activity, I encourage future studies to analyze for changes in PI3K signaling, since pAKT is downstream of PI3K. We would also speculate that in migration defective shPodocalyxin cells, GAP or GEF activity is affected and thus, RhoGTPase activity is also compromised.

Experiments and analysis for Chapter 4 are ongoing. Since Podocalyxin does not seem to play a role in uropod dynamics, we chose to focus on cell migration. The next step is to identify cell migration differences in gap chambers instead of two-dimensional

fibronectin matrix. Gap chambers are specially designed for the BAMBI system to observe and measure the migration speeds of cells towards a chemoattractant gradient such a combination of CXCL12 and SCF. The gap chamber system more accurately reflect "directional" migration similar to results in Chapter 3, which measures cell migration through transwells. We will be placing both shLuc and shPodoclayxin in these chambers to look for changes in migration speeds towards both CXCL12 and SCF.

I have designed a "road-map" of future experiments, which provides assistance in answering these questions and would lead to asking new ones (Figure 5.2). Hopefully, this road-map serves a useful purpose for the next graduate student who will continue to investigate the function of Podocalyxin.

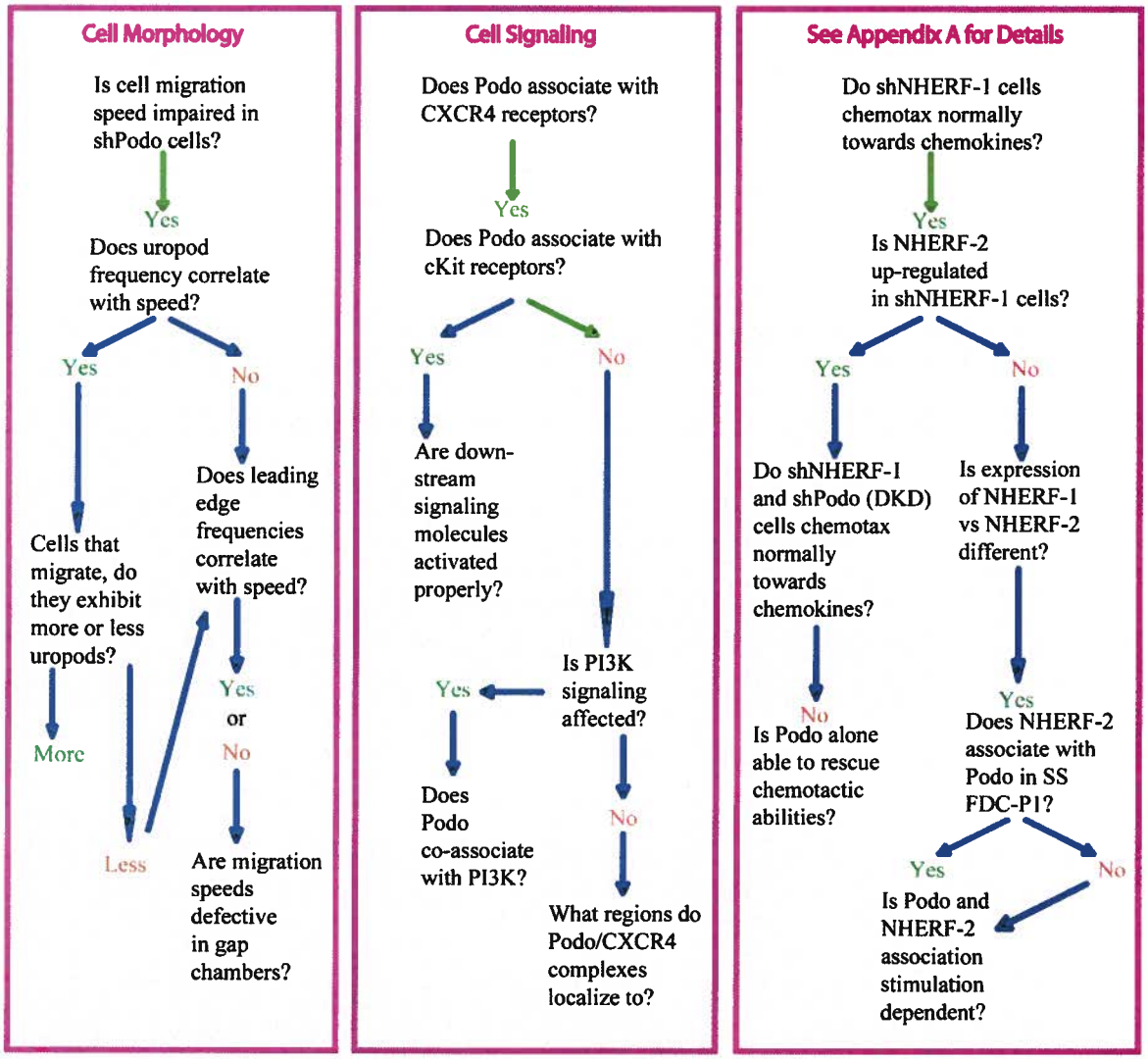

Figure 5.2 Future road map (an excerpt) to further investigate the role of Podocalyxin in cell migration.

This is a future road map which continues from one I generated during my studies (Chapter 1). I have suggested several key questions that would help further address certain results presented in this thesis. Green arrows: decision route I have taken; Blue arrows: suggested future routes for the project.

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Appendix A: Short Story: NHERF-1 Knock-down FDC-P1 cells Show Normal Cell Migration but have Impaired Podocalyxin Surface Expression[1].

Preliminary Results

Concurrent with our studies in Chapter 3, we examined shNHERF-1 cells. These cells were generated according to the material and methods specified in Chapter 3 and the lack of NHERF-1 expression were verified with the same techniques as shPodo cells.

To investigate the role of Podocalyxin and NHERF-1, we decided to use FDC-P1 as our cell-based model. As discussed in Chapter 3 and 4, Podocalyxin plays a role in cell migration. However, the role of NHERF-1 in these cells remains unanswered. NHERF-1 is a cytoplasmic binding ligand for Podocalyxin and their association is IL-3 stimulation dependent. By using three different shRNA oligos, we were able to knock-down NHERF-1 proteins in these cells. We confirmed these results by flowcytometry and by immunobloting techniques (Figure A.1). We proceeded to utilize shNHERF-1A for all of our following studies.

[1]A version of this Appendix is in preparation for publication. Tan PC., Bains J., McNagny MK. Podocalyxin Surface Expression is NHERF-1 Dependent. Brief Communication in preparation for submission July 2008.

A.1.1 NHERF-1 is Successfully Knocked-down in FDC-P1 Cells.

A

Sequences	----------SENSE---------- ---- LOOP ------ ---------- ANTISENSE ----------
shNHERF-1A	5'-TGCATCTTGGGTTCATTTGATTCAAGAGATCAAATGAACCCAAGATGCTTTTTTC-3'
shNHERF-1B	5'-TGCAATGGCCTCATCCTTAATTCAAGAGATTAAGGATGAGGCCATTGCTTTTTTC-3'
shNHERF-1C	5'-TGGAAATGCCTTCAGAAATTTTCAAGAGAAATTTCTGAAGGCATTTCCTTTTTTC-3'

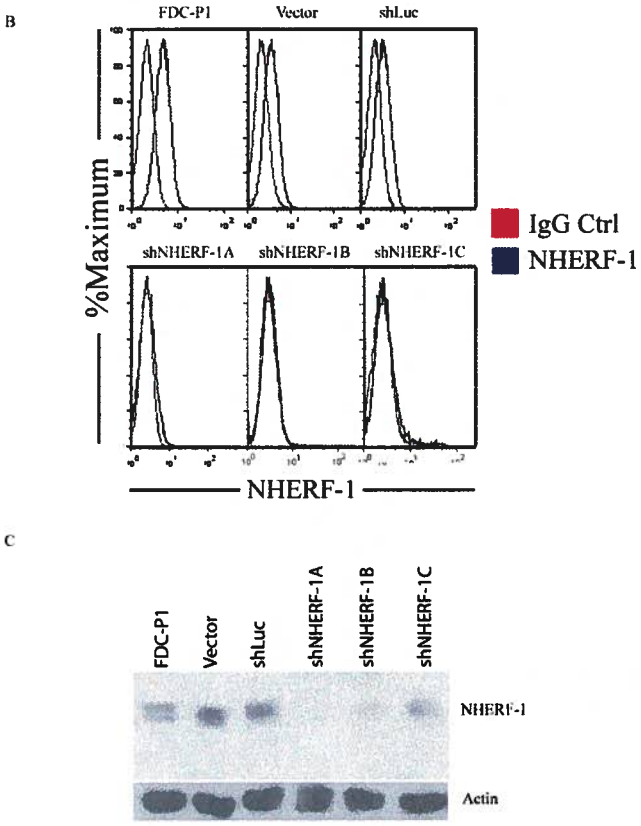

Figure A.1 Schematic of shNHERF-1 sequences for stably silencing NHERF-1 proteins in FDC-P1.

A) Sequences for three shNHERF-1 oligos used for silencing NHERF-1 proteins in FDC-P1 via lentiviral based system. B) FACS analysis for NHERF-1 in FDC-P1 shows successful knock-down with three clones. B) Further confirmation by immunobloting for shNHERF-1 in FDC-P1 cells indicate a >95% knock down in shNHERF-1A and B and only >80% in shNHERF-1C.

A.1.2 Normal Chemotaxis of shNHERF-1 Cells

Similar to findings in Chapter 3, we were curious whether knocking-down NHERF-1 expression in these cells would have the same migration defects. We found no significant difference in cell migration between shNHERF-1 and shLuc cells (Figure A.2). This result suggests that since Podocalyxin continues to be expressed by shNHERF-1 cells cytoplasmically, it is possible that cell migration still occurs. We hypothesize that, although Podocalyxin is required for efficient signaling via CXCR4, the signaling process may not require stable expression of Podocalyxin at the cell surface. We can further speculate that because Podocalyxin is present cytoplasmically, that this is sufficient for stabilizing CXCR4 and thus, activating the signaling pathway.

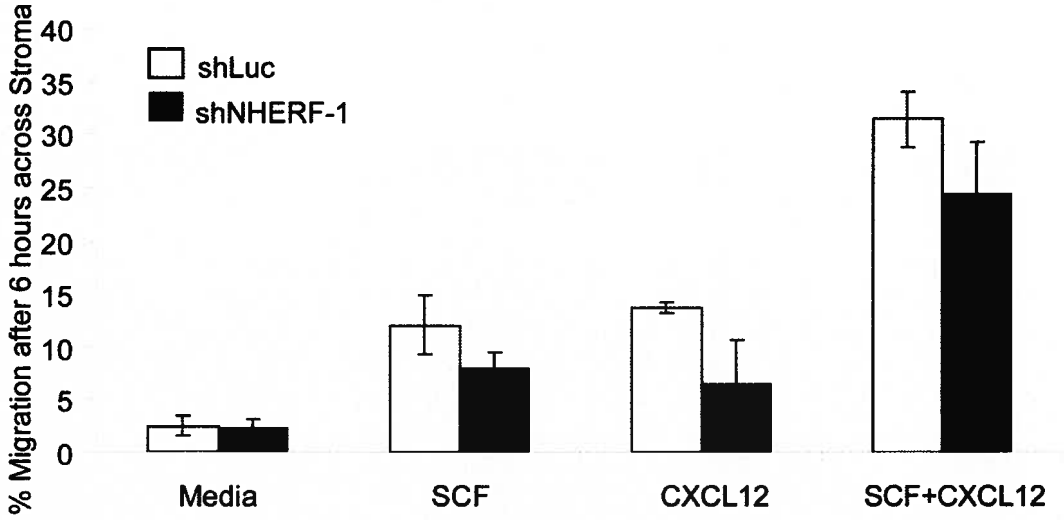

Figure A.2 shNHERF-1 cells chemotaxis normally towards CXCL12 and SCF.

Cells were starved and subsequently placed into transwells and allowed to migrate for 6 hours. There is no statistical significance between shLuc and shNHERF-1 cells. Student's t-test; $p>0.05$, $n = 4$.

A.1.3 Normal levels of pAKT in NHERF-1 knock down cells

Since there is no statistically significant differences with cell migration in shNHERF-1 cells (student's t-test, $p>0.05$), we speculate that signaling downstream of CXCR4 would remain the same. As shown in Figure A.3, shNHERF-1 cells have normal levels of pAKT after stimulation with CXCL12 and SCF and that over a stimulation time course, pAKT enhancement is comparable to shLuc. Therefore, we would expect that shNHERF-1 cells would not have a migration defect.

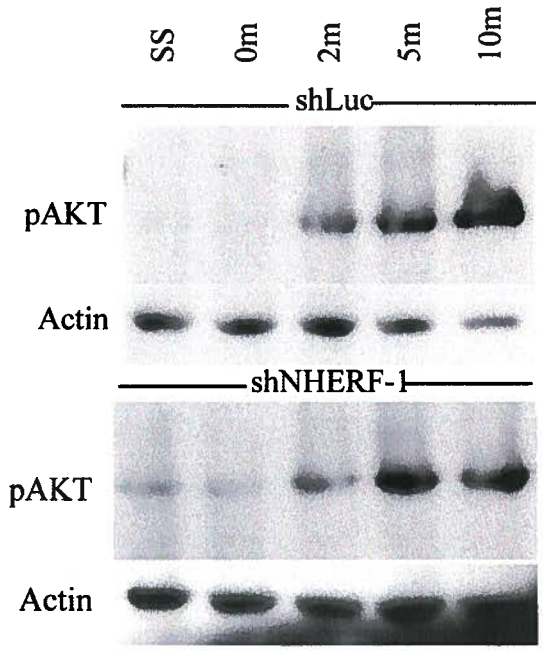

Figure A.3 shNHERF-1 cells express normal levels of pAKT after CXCL12 and SCF stimulation.

shNHERF-1 cells stimulated with CXCL12 and SCF over a time course show normal levels of pAKT and is comparable to shLuc cells. SS: Steady State.

A.1.4 Lack of Cell Surface Podocalyxin Expression in NHERF-1 Knock-out cells

Upon silencing NHERF-1 proteins in FDC-P1 cells, we observed a dramatic affect on the distribution of Podocalyxin at the cell surface. We were surprised to observe that by knocking-down NHERF-1, Podocalyxin is no longer expressed on the surface of cells. Furthermore, the lack of Podocalyxin expression in shNHERF-1 cells is equivalent to that of shPodocalyxin (Figure A.4).

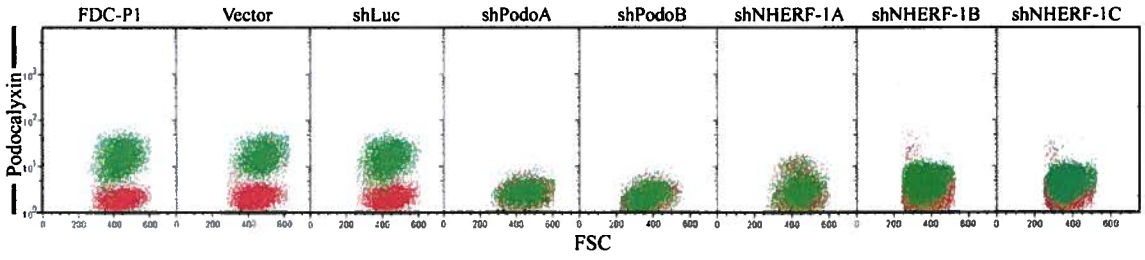

Figure A.4 Lack of Podocalyxin surface expression on shNHERF-1 cells.

FACS analysis of shPodocalyxin and shNHERF-1 cells shows a lack of Podocalyxin expression on the surface of cells. Cells were live-stained to look for surface proteins. Green: Podocalyxin staining; Red: Isotype control.

A.1.5 Punctate Staining of Podocalyxin in shNHERF-1 cells

To further investigate the lack of expression of Podocalyxin in shNHERF-1 cells, we stained these cells for Podocalyxin and proceeded to analyze by confocal imaging. It is essential to fix and permeabilize shNHERF-1 cells prior to staining for Podocalyxin, indicating that the protein is present, however it is retained cytoplasmically. We observed three different phenotypes of Podocalyxin staining pattern: a) global expression, b) punctate and c) polarized or "capped". There is significant difference in the number of punctate stained cells between shNHERF-1A and shLuc cells (Figure A.5). Upon knocking-down NHERF-1, Podocalyxin appear to be synthesized in the cell, however, its expression on the surface is impeded. The NHERF-1 dependent mechanism behind Podocalyxin expression at the cell surface remains to be determined.

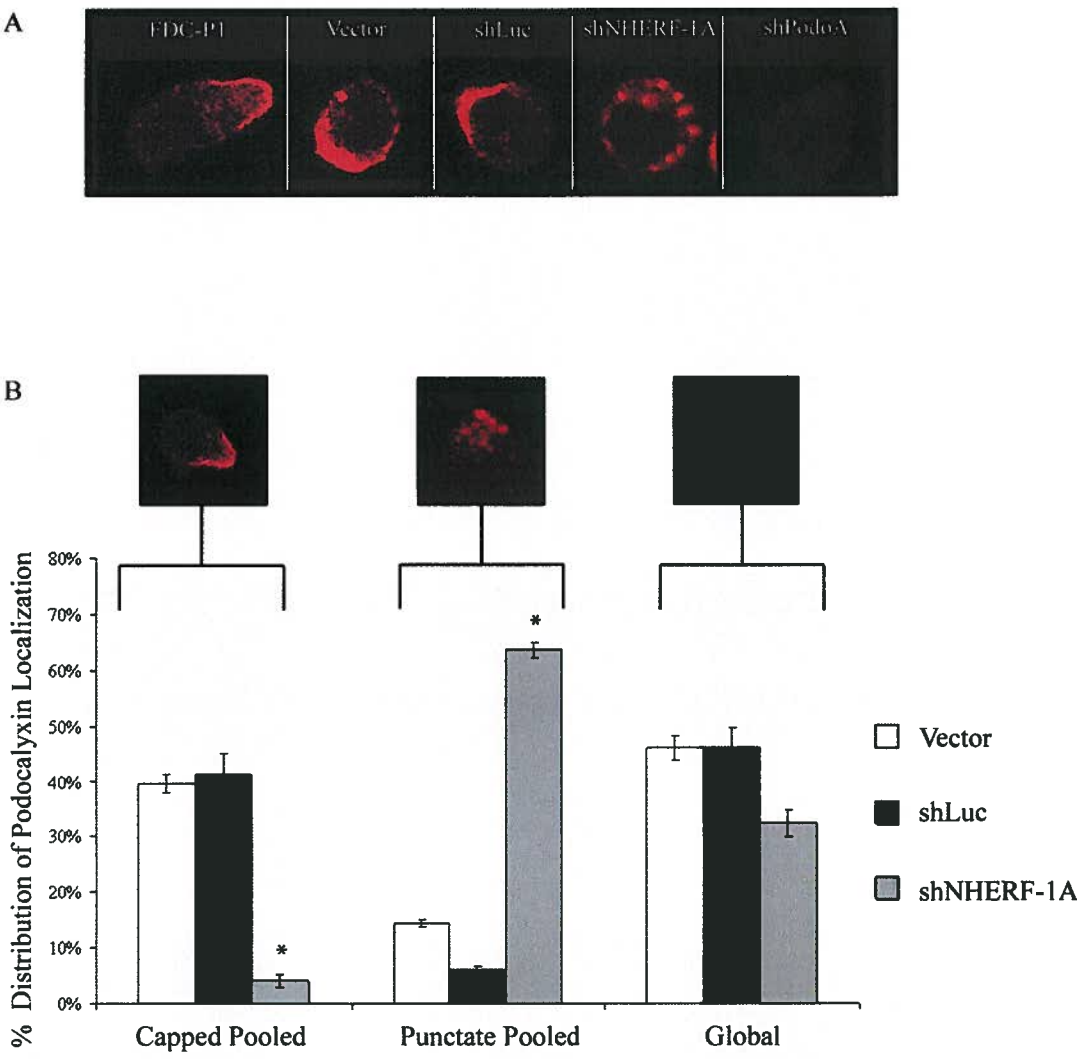

Figure A.5 Podocalyxin is polarized in shNHERF-1 cells but is retained intracellularly. There are significantly more cells with punctuate Podocalyxin staining in shNHERF-1 cells compared to control.

A) Confocal X-Y slices of cells with Podocalyxin staining in shNHERF-1A cells show polarization of Podocalyxin proteins but with a punctuate staining pattern, B) this punctuate staining is significantly higher in shNHERF-1A than shLuc cells. "Capped pool" include cells that show polarized staining, "Punctate pooled" includes cells that display punctuate or polarized punctuate staining pattern and "Global" includes all cells stained with uniform global expression of Podocalyxin on the surface. Inset pictures indicate types of cells counted for each group. Vector: pLL3.7 and * indicates $p<0.05$.

A.1 Brief Discussion

NHERF-1 has long been implicated as a regulatory molecule which helps maintain fine salt balances during the filtration process in the kidney (Weinman et al. 2000; Weinman et al. 2000; Weinman et al. 2001; Weinman et al. 2003). There have been many studies to indicate that NHERF-1 may also play pertinent roles in other cell types (Voltz et al. 2001). In this study, we show that NHERF-1 may play a role in protein expression on the cell surface. There have been other studies to demonstrate that NHERF-1 plays a role in protein localization: molecules such as RAMP-3 (a receptor-activity modifying protein-3) (Bomberger et al. 2005) and CFTR (cystic fibrosis transductance receptor) (Moyer et al. 1999; Moyer et al. 2000).

We have preliminary results to indicate that in FDC-P1 cells, proper expression of Podocalyxin on the cell surface may be dependent on NHERF-1. The *polarization* of Podocalyxin in these cells may not be dependent on NHERF-1 as shown in Figure A.3. Podocalyxin proteins continue to exhibit a "polarize" staining pattern within the cell. In contrast, following C-terminal leucine mutation in CFTR, Moyer and colleagues observed that NHERF-1 binding was abolished and CFTR molecules were not properly localized (Moyer et al. 1999; Moyer et al. 2000).

As demonstrated in Chapter 3, Podocalyxin expression is necessary for efficient chemotaxis and thus, we were curious whether cell migration of shNHERF-1 cells towards both CXCL12 and SCF were also impeded. We found that cell migration was

not impeded, despite the lack of Podocalyxin on the surface. This led us to our next hypothesis that since Podocalyxin proteins retain the ability to "polarize" within the cell, it is still capable of forming a complex with CXCR4 cytoplasmically and thereby, enabling pertinent signaling events (e.g. pAKT) for cell migration to take place. To address our hypothesis, we stimulated shNHERF-1 with both CXCL12 and SCF and found that the levels of phospho-AKT in shNHERF-1 cells are maintained and that over time, pAKT activity is comparable to shLuc cells. Therefore, it is interesting that even with a lack of Podocalyxin expression on the cell surface, cell migration and CXCR4 signaling is normal.

We are currently conducting various preliminary experiments to further investigate NHERF-1's role in protein expression and its subsequent function in FDC-P1 cells. Our next step is to determine what types of cellular structures Podocalyxin is associated with during protein trafficking to the surface. NHERF-1 has a role in trafficking CFTR to the membrane possibly via endosomes (Broere et al. 2007). Similarly, we would like to determine if the lack of Podocalyxin expression on the cell surface of shNHERF-1 cells are due to endosomal compartmentalization within the cell. It is possible that NHERF-1 deficient cells are unable to properly express Podocalyxin on the surface because protein trafficking via endosomal pathway is NHERF-1 dependent.

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Appendix B: Brief Overview of shRNA Silencing.

B.2 Brief Introduction

RNAi, sometimes also referred to as shRNA and sRNA, are a class of 20-25 nucleotide long double stranded RNA molecules. RNA interference (RNAi) is an evolutionary conserved process of post-transcriptional gene silencing. RNAi were first discovered by David Baulcombe's group in plants. Shortly after, synthetic shRNAs were then shown to be able to induce RNAi in mammalian cells by Thomas Tuschl.

Most shRNA interference are produced by generating synthetic short RNA sequences to the proteins of interest. These short pieces of RNA are usually introduced into cells placing it into a viral plasmid which subsequently can be used to infect host cells. To infect mammalian cells, short hairpin RNA (shRNA) are usually used because they are more stable and are expressed long term in the cell. shRNA is characterized by an extended loop at one end of an shRNA oligo duplex.

Once the shRNA is introduced into the cells it triggers the RNAi process. First, it is processed by the RNAse III enzyme, Dicer in an ATP-dependent fashion. In the mouse Dicer is expressed at all stages of development and is consistent with RNAi being an innate cellular defense mechanism. The subsequent shRNA becomes shRNA and it associates with an RNA-inducing silencing complex (RISC) that cleaves and discards the sense strand of the shRNA duplex and further activating RISC. The remaining antisense strand guides RISC to its homologous mRNA, resulting in endonucleolytic cleavage of the target mRNA. As a result of mRNA destruction, the protein of interest is not generated.

B.3 Protocol for Generating shRNA-Lentiviral Plasmids

Annealing shRNA oligos:

Materials:

1 µl Sense oligo (Note: all oligos are resuspended in water at 60pmol/µl)

1 µl Antisense oligo

48 µl Annealing Buffer (100mM K-acetate, 30mM HEPES-KOH pH 7.4, 2mM Magnesium-acetate)

Restriction enzymes (XhoI, HpaI, Xba1, NotI)

Qiagen QIAquick gel extraction kit

Shrimp alkaline phosphatase

Ligase

Stbl-2 competent cells

Method:

Incubate at the shRNA mixture in a PCR machine at 95°C for 4 minutes and followed by 70°C for 10 minutes. Decrease temperature down to 4°C at a rate of 0.1°C/minute and continue to incubate the sample at 4°C for an additional 10 minutes.

Digestion of pLentiLox 3.7-2.0kb:

Digest 1-2µg of pLentiLox3.7-2.0kb with XhoI and HpaI restriction enzymes following manufacturer's recommendation. Following digestion, treat the linearized plasmid with shrimp alkaline phosphatase for 20 minutes at 37°C. Purify the larger linearized fragment

using the commercially available Qiagen QIAquick gel extraction kit. Discard the 2.0kb spacer after digestion. Determine the concentration of plasmid obtained after purification by using a spectrophotometer.

Ligation

Ligate linearized product and annealed oligos at equimolar concentration. I typically use 60 femtomoles of each component in a final concentration of 10μL.

Transformation:

I had success with Invitrogen's Max Efficiency Stbl-2 cells competent cells. These cells are efficient for cloning unstable inserts such as lentiviruses. In addition, Stbl-2 cells have a much lower frequency to undergo recombination during the cloning process.

Testing clones:

We test successful cloning by restriction digest and DNA sequencing.

Preparation of shRNA lentiviral plasmids:

Qiagen Endo-Free Maxiprep Kits were used to purify all plasmids used in transfection of 293T cell lines.

Appendix C: Transwell Migration Assay: Cell Lines and Primary Cells

C.1 Transwell Migration Assay: A Brief Introduction

It has become increasingly common to use permeable supports with microporous membranes to measure chemotaxis and cell migration. Transwell membrane supports are available with different options but for my assays I have always used polycarbonate membranes with 5μm pore sizes. These supports are suitable for growing cell monolayers to aid in with cell migration. Commonly the transwell is referred to as the upper chamber and the well it is placed in is called the lower chamber. These terms will be used to describe the procedure below.

C.2 Protocol for Transwell Migration Assay

Materials:

Cells (FDC-P1 or Ter119 depleted E15 fetal liver cells or M210B4; stromal cells)

0.25% Trypsin in Citrate Solution

Costar 5um 24-well plate transwell chambers

100ng/ml SCF resuspended in 1X PBS

100nM CXCL12 resuspend in 1X PBS

1X conditioned media WEHI-3B (containing IL-3)

100μg/ml Fibronectin

5% BSA solution in PBS

Sterile 1X PBS

Complete RPMI tissue culture media (10% FBS, 4mM L-Glutamine, 1X Penicillin/Streptomycin cocktail)

Starvation media (RPMI plus 10% FBS)

Hemacytometer

Coating Transwells with:

Stromal Cells:

Obtain M210B4 cells from the incubator and wash twice with sterile 1X PBS. Aspirate the last wash and carefully drop enough trypsin to coat the cells in the plate and continue to trypsinize for 5 minutes at 37°C. Tap the sides of the plate to dislodge cells and add 3X the amount of complete media to the cells. Pellet the cells and discard supernatant. Count cells on hemacytometer and apply $1x10^5$ cells per chamber. Replace into the incubator and let the cells adhere overnight. When the chambers are ready to use, carefully aspirate off the media in the upper chamber and replace with media and cells of interest.

Fibronectin Coating:

To coat with fibronectin instead, dilute fibronectin stock to 100µg/ml. Coat each transwell with 100µl of fibronectin overnight or two hours at 37°C and subsequently wash off excess fibronectin 3X with sterile 1XPBS. In parallel, coat extra transwells with 5% BSA as controls. Following coating, block these wells with 5% BSA in PBS for 2 hours at 37°C prior to commencing migration assay.

FDC-P1 Starvation and Primary Cell Preparation:

Pellet FDC-P1 in a centrifuge, aspirate and discard media, followed by washing the cell pellet twice with sterile PBS. Upon last wash resuspend the cell pellet in starvation media and replace the cells into a 37°C incubator and starve the cells for three hours.

For primary cells such as fetal liver cells, carefully obtain cells from E15 embryos, resuspend these cells in PBS and perform red blood cell lysis. Wash the cell pellet twice with sterile PBS and perform a Ter119 depletion using MACS columns and following manufacturer's instructions. Ter119 depleted fetal liver cells are resuspended in complete RPMI supplied with 10% of 1X conditioned WEHI-3B media.

Migration Assay:

Wash off blocking buffer from the transwell with 1X PBS and allow the well to dry for 2-3 minutes. While waiting for the well to dry, prepare the lower chamber by dropping appropriate amounts of CXCL12 and SCF into 500µl of complete media. Prepare cells by resuspending them to the correct concentration and carefully drop a volume of 100µl of cells into the upper chamber. Then, carefully with forceps lift the transwell containing the cells and place it into the 500µl of media at a slight angle to avoid air bubbles from forming at the membrane-liquid interface. If a bubble does occur, carefully lift the transwell straight up and again lower it at an angle. Allow the transwell migration to occur for at least 6 hours.

Quantification of Migrated Cells:

After the migration period, carefully remove the transwell from the lower chamber. Thoroughly resuspend the cells in the lower chamber for counting. Aliquot 10µl of cells into a hemacytometer for counting and determine total number of cells in the lower chamber.